DETECTION OF ANTIMICROBIAL RESISTANCE IN *ESCHERICHIA COLI* AND *SALMONELLA* SPP. ORIGINATED FROM CULTIVATED OYSTERS AND ESTUARINE WATERS

Miss Mullika Kuldee

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By	Miss Mullika Kuldee
Field of Study	Veterinary Public Health
Thesis Advisor	Professor RUNGTIP CHUANCHUEN, D.V.M., M.Sc., Ph.D

Accepted by the FACULTY OF VETERINARY SCIENCE, Chulalongkorn University in Partial Fulfillment of the Requirement for the Master of Science

..... Dean of the FACULTY OF VETERINARY SCIENCE

(Professor SANIPA SURADHAT, D.V.M., Ph.D)

THESIS COMMITTEE

..... Chairman

(Professor ALONGKORN AMONSIN, D.V.M., M. Sc., Ph.D)

..... Thesis Advisor

(Professor RUNGTIP CHUANCHUEN, D.V.M., M.Sc., Ph.D)

..... Examiner

(Assistant Professor TARADON LUANGTONGKUM, D.V.M., M.Sc., Ph.D)

..... Examiner

(Assistant Professor SAHARUETAI JEAMSRIPONG, D.V.M., M.V.P.M., Ph.D)

..... External Examiner

(Associate Professor Sunpeth Angkittitrakul, D.V.M., M.Sc., Ph.D)

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การเพิ่มขึ้นและการแพร่กระจายของแบคทีเรียดื้อยาต้านจุลชีพในคน สัตว์ และ สิ่งแวดล้อม เป็นประเด็นสำคัญทางสาธารณสุข การใช้ยาต้านจุลชีพที่ไม่เหมาะสมช่วยส่งเสริมให้เกิดแบคทีเรียดื้อยา ทำให้เชื้อดื้อยาเหล่านี้กระจายไปในสิ่งแวดล้อมได้ การปนเปื้อนเชื้อดื้อยาให้สิ่งแวดล้อมส่งผลให้เพิ่มความเสี่ยงในการแพร่กระจายไปยังคน สัตว์ และสิ่งแวดล้อม การตรวจติดตามและการเฝ้าระวังเชื้อดื้อยาในด้านสิ่งแวดล้อม ยังมีอยู่อย่างจำกัดเมื่อเทียบกับคนและสัตว์ วัตถุประสงค์ของการศึกษานี้คือ ตรวจหาลักษณะปรากฏและลักษณะทางพันธุกรรมของการดื้อยาต้านจุลชีพและการสร้างเอนไซม์อีเอสบีแอล ใน *Escherichia coli* และ *Salmonella* ที่แยกได้จากหอยนางรมและน้ำกร่อย serovar ของ *Salmonella* และยีนก่อโรคใน *E. coli* และ *Salmonella* จากหอยนางรมและน้ำกร่อย *E. coli* (n=409) และ *Salmonella* (n=126) ในหอยนางรม 144 ตัวอย่าง และน้ำกร่อย 96 ตัวอย่าง จากจังหวัดพังงาในประเทศไทย serovar ของ *Salmonella* ที่พบมากที่สุด คือ Paratyphi B (13.50%) รองลงมาคือ Eastbourne (12.70%) และ II (15.87%) พบการดื้อยาต้านจุลชีพอย่างน้อยหนึ่งชนิด 94.13% ใน *E. coli* และ 96.82% ใน *Salmonella* พบเชื้อดื้อยาหลายกลุ่มใน *E. coli* (42.60%) และ *Salmonella* (23.02%) bla_{TEM} (31.55%) *tet(A)* (25.44%) และ *strA* (14.92%) เป็นยีนดื้อยาที่พบมากที่สุดใน *E. coli* ขณะที่ *sul3* (14.29%) bla_{TEM} (11.91%) และ *cmlA* (11.91%) พบมากใน *Salmonella* ลักษณะที่แสดงออกของการสร้างเอนไซม์ ESBL พบใน *E. coli* จากน้ำกร่อย จำนวน 8 isolates และ *Salmonella* จากหอยนางรม จำนวน 2 isolates *E. coli* 1 isolate และ *Salmonella* 1 isolate ตรวจพบยีน bla_{TEM-1} ซึ่งแสดงถึง broad-spectrum beta-lactamase อย่างไรก็ตาม *E. coli* จำนวน 4 isolates พบยีน bla_{CTX-M} ส่วนยีนก่อโรคที่พบมากใน *E. coli* ได้แก่ *stx1* (17.85%) และ *lt* (11.74%) *Salmonella* มีความชุกของยีนก่อโรคสูงทั้ง *invA* (76.98%) *stn* (76.98%) และ *fimA* (69.05%) *E. coli* ที่ดื้อต่อ ampicillin มีแนวโน้มจะดื้อต่อ chloramphenicol และ trimethoprim (p<0.0001) นอกจากนั้น *E. coli* ที่มียีน *stx1* และ *stx2* มีโอกาสที่จะดื้อต่อ chloramphenicol (p<0.0001) ส่วน *Salmonella* ที่มียีน *invA* และ *fimA* มีโอกาสพบการดื้อยาด้านจุลชีพหลายชนิด โดยสรุปหอยนางรมและน้ำกร่อย เป็นหนึ่งในแหล่งสำคัญของเชื้อดื้อยาในสิ่งแวดล้อม ดังนั้นควรส่งเสริมการตรวจติดตามและเฝ้าระวังการปนเปื้อนของเชื้อดื้อยาในสิ่งแวดล้อม

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The raising and spreading antimicrobial resistance (AMR) bacteria in humans, animals, and environment is a serious global public health issue. Improper use of antimicrobial agents can promote resistant bacteria, and as a result, they can circulate in the environment. Contamination of AMR in the environment can increase the risk of AMR distribution in humans, animals, and the environment. Monitoring and surveillance of AMR in the environmental sector are limited compared to animal and human sectors. The objectives of this study were to determine phenotype and genotype of AMR and extended-spectrum beta-lactamase (ESBL) production in *Escherichia coli* and *Salmonella* isolated from cultivated oysters and estuarine waters, to examine serovars of *Salmonella* isolates, and to detect virulence genes in *E. coli* and *Salmonella* isolated from cultivated oysters (n=144) and estuarine water (n=96). *E. coli* (n=409) and *Salmonella* (n=126) isolates were obtained from cultivated oysters and estuarine waters from Phang Nga, Thailand. The predominant serovars of *Salmonella* were Paratyphi B (13.50%), followed by Eastbourne (12.70%), and II (15.87%). The resistance to at least one antimicrobial agent was found in *E. coli* (94.13%) and *Salmonella* (96.82%). The multidrug resistance *E. coli* (42.60%) and *Salmonella* (23.02%) were observed. The bla_{TEM} (31.55%), *tet(A)* (25.44%), and *strA* (14.92%) were the most prevalent resistance genes found in *E. coli* isolates, while sul*3* (14.29%), bla_{TEM} (11.91%), and *cmlA* (11.91%) were commonly found in resistance *Salmonella*. Phenotypic ESBL production was detected in eight *E. coli* isolates from estuarine waters, and two *Salmonella* isolates from oysters. One of *E. coli* and *Salmonella* isolates that harbored bla_{TEM-1} corresponded to broad-spectrum beta-lactamase. However, four *E. coli* isolates harbored bla_{CTX-M} genes. The most common virulence genes of *E. coli* isolates were *istx1* (17.85%) and *lt* (11.74%). For *Salmonella* isolates, high prevalence of *invA* (76.98%), *stn* (76.98%), and *fimA* (69.05%) were observed. The *E. coli* isolates that resistant to ampicillin were resistant to chloramphenicol and trimethoprim (p<0.0001). Furthermore, the *E. coli* isolates harboring *stx1* and *stx2* were more likely to resistant to chloramphenicol than those did not contain virulence genes (p<0.0001). The *Salmonella* isolates that consisted of *invA* and *fimA* were more likely to be resistant to various antibiotics, including ampicillin, chloramphenicol, tetracycline, and trimethoprim. In conclusion, oysters and estuarine water are one of the potential AMR hotspots in the environment. Therefore, continuing monitoring and surveillance of AMR should be implemented in the environment.

Field of Study:	Veterinary Public Health	Student's Signature .....
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LIST OF ABBREVIATIONS

AMR	antimicrobial resistance
bp	base pair
DNA	deoxyribonucleic acid
DW	distilled water
E. coli	*Escherichia coli*
EDTA	ethylenediaminetetraacetic acid
et al.	at alii, and others
MDR	multidrug resistance
MIC	minimum inhibitory concentration
ml	milliliter
OR	Odds ratio
PCR	polymerase chain reaction
TAE	tris-acetate-EDTA
tris	tris-hydroxymethyl-aminoethane
µg	microgram
µl	microliter
µm	micrometer

CHAPTER I INTRODUCTION

Antimicrobial resistance (AMR) has been recognized as a significant global issue affecting human and animal health. AMR bacteria are spreading rapidly and have become one of the greatest challenges to global public health. Resistant bacteria are naturally developing and can be found in humans, animals, and the environment. Inappropriate use of antimicrobials in humans and animals, including excessive, subtherapeutic doses and misuse of antibiotics, induces bacteria to become resistant to antimicrobial agents. This leads to the difficult treatment of bacterial infections due to a lack of effective antibiotics. In human medicine, antibiotics are used for the treatment of bacterial infections, but in animals, antimicrobials have been used for several purposes, e.g., to treat bacterial infections, promote the growth of animals, and improve animal productivity. For aquatic animals, antibiotics have been used to maintain health status by preventing bacterial infection and reducing morbidity and mortality rates (Watts et al., 2017).

The excessive and widespread use of antimicrobial agents in agriculture is a major selective pressure for AMR, endangering human and animal health. Bacteria can develop resistance to antimicrobial agents, and AMR may possibly spread to inter- and intra-species of bacteria. The occurrence of AMR in human, animal, and environmental sectors has been considered a One Health issue. Many pathways that resistance materials can be transferred include transduction, transformation, and conjugation, to the same bacterial species or other bacterial species.

Salmonella spp. are pathogenic bacteria that are a major cause of foodborne disease, while *Escherichia coli* is recognized as an indicator bacterium of fecal contamination in the environment. Moreover, *E. coli* has been used as a primary indicator of AMR in humans and food-producing animals (ECDC et al., 2017;

EFSA, 2018). The transmission of resistant *E. coli* and *Salmonella* spp. and their virulence genes from animals and the environment to humans can occur by a fecal-oral route through the consumption of contaminated raw or undercooked seafood and exposure to contaminated water. Seafood consumption continues to increase worldwide since seafood is a good source of high-quality protein and is rich in essential amino acids, fatty acids (e.g., DHA and EPA), vitamins (A, D, and B), and minerals (e.g., calcium, iodine, zinc, etc.). However, many foodborne outbreaks have been identified in seafood. Shellfish (e.g., oysters, clams, and mussels) are one significant source of bacterial contamination because they are filter-feeding species that can accumulate and concentrate a variety of organisms (Iwamoto et al., 2010).

The contamination of *Salmonella* has been reported in a wide range of seafood products such as shrimp, crab, fish, and shellfish. The consumption of food and water contaminated with *Salmonella* can cause gastrointestinal illness. Sometimes, a host can recover from infection by self-limiting, but sometimes antibiotics are needed. The prevalence of AMR *Salmonella* has been increasing concern. AMR and multidrug resistance (MDR) *Salmonella* has been a complicated treatment of salmonellosis. In Thailand, the resistant *Salmonella* spp. in seafood was reported with the highest percentage compared with other pathogens, such as *Aeromonas* spp. and *Vibrio* spp. (Woodring et al., 2012).

Extended-spectrum β-lactamases (ESBL) are enzymes produced by bacteria in the family *Enterobacteriaceae* that can inhibit the effect of penicillins as broad-spectrum cephalosporins and monobactams (Rupp and Fey, 2003). The ESBL-producing bacteria are normally found in *E. coli* and *Salmonella* spp. Furthermore, genetic elements of ESBL-producing bacteria have been widely disseminated in humans, animals, and the aquatic environment (Maravić et al., 2015; Conte et al.,

2017). Thus, ESBL-producing bacteria are one of the major AMR issues, especially in a healthcare setting. Treatment of ESBL-producing bacterial infections is more complicated due to the limit of effective of available antibiotics. The infection of ESBL bacteria usually occurs in health care providers, hospitalized patients, or urinary tract infections in healthy people. Additionally, ESBL bacteria can spread through the contaminated surface, food, and environment (CDC, 2019). Previous studies have reported that MDR and ESBL producing strains were detected in fresh seafood (Singh et al., 2020; Mwanza et al., 2021). Another study found AMR *E. coli* that were contaminated in environmental water were consistent with those from human strains (Anastasi et al., 2012). This indicated the circulation of ESBL and MDR in the marine environment had been concerned as public health significance due to possibly contaminate to humans.

The pathogenicity of bacterial was expressed by virulence factors. Most of the virulence genes were mainly carried by plasmids (Li et al., 2017). Virulence plasmids had a variety of mechanisms to cause an illness to a susceptible host by invading into host cells, producing toxins to damage host cells, and against host immune systems (Clark et al., 2019). However, plasmids also confer resistance to many antibiotics, including beta-lactams, chloramphenicol, aminoglycosides, and tetracyclines (Rozwandowicz et al., 2018). The infection with pathogens carrying resistance genes may be more severe and more difficult, in particular, pathogenic bacteria carrying both virulence and resistance genes (Yandag et al., 2017).

AMR monitoring and surveillance is one of the important strategies to tackle the AMR problem. The emergence and spread of AMR in humans and animals may come from different sources, including the environment. The study of the magnitude of AMR, virulence genes, and ESBL production in estuarine environment is needed to fulfill the lack of AMR data in the environment. Therefore, this study

aims to provide background information on *Salmonella* serotypes identification, AMR, virulence genes, and ESBL producing *E. coli* and *Salmonella* isolated from oysters and estuarine waters.

Questions of the study

1. What are the serovars of *Salmonella* isolated from cultivated oysters and estuarine waters?
2. What is the occurrence phenotype and genotype of AMR and ESBL detection in *E. coli* and *Salmonella* originated from cultivated oysters and estuarine waters?
3. What is the prevalence of virulence genes in *E. coli and Salmonella* originated from cultivated oysters and estuarine waters?
4. What is the association between phenotypic and genotypic of AMR, ESBL and virulence genes in *E. coli* and *Salmonella* from cultivated oysters and estuarine waters?

Objectives of the study

1. To detect the serovars of *Salmonella* isolated from cultivated oysters and estuarine waters.
2. To determine the occurrence phenotype and genotype of AMR and ESBL production in *E. coli and Salmonella* isolated from cultivated oysters and estuarine waters.
3. To detect virulence genes in *E. coli* and *Salmonella* isolated from cultivated oysters and estuarine waters.
4. To examine the association between phenotypic and genotypic of AMR, ESBL, and virulence genes in *E. coli* and *Salmonella* from cultivated oysters and estuarine waters

CHAPTER II LITERATURE REVIEW

General characterization and pathogenesis of *E. coli*

E. coli belongs to the family *Enterobacteriaceae,* Gram-negative rod shape with 1-2 μm length, and a facultative anaerobe bacterium. The optimum temperature to grow is 35-37°C. On the biochemical test, this bacterium can ferment lactose and glucose, be positive to catalase and negative to oxidase, reduce nitrate to nitrite, and be resistant to bile salt. This bacterium can persist in the environment for several weeks to months (Rantsiou et al. , 2012; Ananchaipattana et al., 2016; Elhadi et al., 2016). Some strains of *E. coli* can survive in stream waters for up to 234 days (Duffitt et al., 2011). *E. coli* O157:H7 can survive in adverse conditions such as *E. coli*, which can persist in fumigated soil surrounding livestock farming and agriculture areas.

E. coli is commonly colonized in the intestinal tracts of warm-blooded animals, humans, and birds. Most *E. coli* are non-pathogenic bacteria, whereas some pathogenic strains of *E. coli,* such as Shiga toxin-producing *E. coli* (STEC), especially enterohemorrhagic *E. coli* (EHEC), can cause illness or severe complications such as bloody diarrhea in humans (Karmali, 2009). Pathogenic stains colonize the intestinal tracts of their host and cause illness due to virulence factors (Ahmed et al., 2008). *E. coli* is one of the indicator bacteria for the detection of fecal contamination in food, drinking water, and the environment (Zhang et al., 2016). Selective media including brilliant green agar, violet red bile glucose agar, and MacConkey agar are used to detect *E. coli* (Feng et al., 2002). *E. coli* can spread through feces by contamination in drinking water, cooked foods, animal products, and seafood.

Humans can expose to *E. coli* through the fecal-oral route by consumption of contaminated food, seafood, and water. Humans can accidentally be exposed to these bacteria through direct contact during swimming in contaminated areas such as coastal water or recreational water (Marion et al., 2010). Contaminated water may derive from sewage, discharge, or overflows from households. The symptoms of *E. coli* infection include fever, nausea, vomiting, abdominal pain, and diarrhea. Typically, the clinical signs of *E. coli* infection are self-limiting and disappear within ten days. However, severe complications of pathogenic *E. coli* infection (i.e., *E. coli* O157:H7 and verotoxin producing *E. coli*) are such bloody diarrhea, hemorrhagic colitis, hemolytic uremic syndrome (HUS), hemolytic anemia, thrombocytopenia, acute renal failure, and death (Karmali, 2009; Bhunia, 2018a). The severity of clinical signs depends on host factors and the immune status. The outbreak associated with pathogenic *E. coli*, especially *E. coli* O157:H7 have been occurred worldwide, and most of cases were reported in developing countries. Sources of infection have been described from meat, milk, cheese, and raw produce (Laine ES, 2005; Yakubu et al., 2018; Currie A, 2019; Han et al., 2020). The contamination of *E. coli* O157:H7 from fecal contamination from livestock in the environment also is of public health concern.

General characterization and pathogenesis of *Salmonella* spp.

Salmonella spp. is a member of the *Enterobacteriaceae* family and a Gram-negative, facultative anaerobes and non-spore-forming bacillus. *Salmonella* has been divided into two species there are *S. enterica* and *S. bongori.* The *S. enterica* were classified into six subspecies consisting of Subspecies I (*S. enterica* subsp. *enterica*), Subspecies II (*S. enterica* subsp. *salamae*), Subspecies IIIa, (*S. enterica* subsp. a*rizonae*), Subspecies IIIb (*S. enterica* subsp. *diarizonae*), Subspecies IV (*S. enterica* subsp. *diarizona*) and Subspecies VI (*S. enterica* subsp. *indica*).

This bacterium contains peritrichous flagella that are responsible for motility. For bacterial growth, the optimum temperature is 35-37 °C, and the optimum pH is 6-7. Typical colonies of *Salmonella* can be detected in hecton enteric agar, xylose lysine deoxycholate agar, and bismuth sulfite agar. All *Salmonella* species are intracellular pathogens that can invade macrophages, dendritic cells, and epithelial cells causing invasive infection of gastrointestinal tracts in humans and animals (Bhunia, 2018a). Moreover, the pathogenesis of *Salmonella* is derived from virulence genes such as *invA*, *pefA*, *sipB*, *spvC*, *prgH*, *orgA*, *tolC*, and *rck* genes (Srisanga et al., 2017)

Salmonella spp. is a wide-host range bacterium that can colonize in the intestinal tracts of reptiles, birds, farm animals, and humans. It can survive in the environment for several weeks. The transmission of *Salmonella* in animal-to-human or human-to-human can occur through either consumption of contaminated food such as chicken meat, pork, milk, eggs, dairy products, and seafood or direct contact with animals and contaminated environment (Nguyen et al., 2016a; Afroj et al., 2017; Li et al., 2018; Omar et al., 2018).

Salmonella spp. are classified into two subspecies, which are *S. enterica* and *S. bongori*. *Salmonella* also can be classified serovar by detecting O (lipopolysaccharide) and H (flagella) antigens. More than 2,500 *Salmonella* serovars have been reported (Grimont and Weill, 2007). Each serotype is specific to the host. Many subspecies of *Salmonella* can cause diseases in humans and animals. For example, *S. enterica* serovar Typhi causes typhoid fever and systemic diseases in humans and animals. Two forms of *Salmonella* are typhoid and non-typhoid strains. Non-typhoid *Salmonella* (NTS) such as *S. enterica* serovar Typhimurium and Enteritidis are primarily localized in gastrointestinal tracts. NTS can cause self-limiting gastroenteritis and enterocolitis in humans. However, NTS can also cause

systemic infection in immunocompromised hosts such as claves, piglets, younger people, and the elderly.

Consumption of contaminated chicken meat, eggs, pork, raw produce, and seafood has been implicated with salmonellosis (Tadesse and Gebremedhin, 2015). Sometimes, direct contact with birds, reptiles, and contaminated water is also reported (Demirbilek, 2017). the infection caused by contact to *Salmonella* that naturally commensal colonized on birds or reptiles.

Distribution of *E. coli* and *Salmonella* spp. in oyster and estuarine water

The bivalve mollusks, including mussels, clams, scallops, and oysters, have been described as enriching sources of vitamins and minerals, e.g., vitamin B12, omega-3 fatty acids, choline, iron, selenium, and zinc. Although many mollusks have a high value of nutrients than land-based protein sources, these bivalves can harbor contaminated pathogens (Wright et al., 2018). Consumption of raw or partly cooked shellfish can result in bacterial infection.

The distribution of *Salmonella* and *E. coli* to the marine environment has been associated with ineffective wastewater treatment, contamination in sewage, and inappropriate antimicrobial use in agriculture and aquaculture. *Salmonella* serotype Newport and Typhimurium are major serotypes that have been detected from both wastewater treatment plants and marine environments (Abdellah et al., 2017). Wastewater and waste from agriculture are considered significant sources of bacterial contamination in freshwater and saltwater environment. Bacterial contamination in seawater is potentially associated with the elevation of seafood-borne illnesses in humans (Mok et al., 2016). Additionally, seagulls are one of the crucial vehicles carrying AMR bacteria in its gastrointestinal tract that may harbor resistant organisms from household and coastal environments (Alves et al., 2014).

E. coli in surface waters can cause a health risk to humans if they are consumed while participating in recreational activities or if they are exposed to polluted water sources. *E. coli* is being used as indicator bacteria in the detection of contamination. *Salmonella* contamination of seafood happens mostly as a result of contamination from residential and agricultural runoff water into the surrounding environment. Furthermore, contamination might be linked to the harvesting, transportation, and distribution of seafood. Filter-feeding shellfish can concentrate and accumulate pathogens (Bhunia, 2018b). *Salmonella* has been found in 21% of the raw seafood acquired from fresh markets and supermarkets(Woodring et al., 2012). Because oysters are frequently consumed raw, and the entire animal digestive tract is ingested, pathogenic bacteria, especially Salmonella, could be ingested.

STEC O157: H7 is one of the significant foodborne pathogens that has been reported in a wide spectrum of animal species such as cattle, sheep, goats, dogs, cats, and pigeons. This serotype was also reported in aquatic species like finfish (16%) and raw shellfish (33%) from seafood markets in India (Vijay et al., 2018). In humans, the infection of this pathogen often occurs by consumption of contaminated foods, dairy products, water, and the environment.

In the estuarine environment, the prevalence of *Salmonella* varied from 7% to 10% of oysters and 0% to 30% in estuarine water (Ristori et al., 2007). In Thailand, the prevalence of *E. coli* and *Salmonella* were reported high at 93.1% and 30.6% in fresh oysters (Jeamsripong et al., 2018). Additionally, the prevalence of *E. coli* was observed in seafood was 44.1% (Odumosu et al., 2021). The prevalence of *Salmonella* contamination in oysters has been increasingly reported worldwide. For example, high prevalence of *Salmonella* was observed in seafood (41%) (Nguyen et al., 2016a). The prevalence of *Salmonella* was varied from 16-

36% of raw meats and seafood from open markets and supermarkets (Minami et al., 2010). A previous study reported that oysters had shown the highest prevalence (23.1%) compared to freshwater fish (18.6%), shrimp (13.0%), and saltwater fish (12.2%) (Yang et al., 2015). The persistence of bacterial pathogens in the environment is of major public health concern. This is because they can threaten humans.

The contamination of *E. coli* and *Salmonella* should be frequently monitored, especially in cultivated sites of aquatic animal production. Additionally, increased awareness of fully cooked seafood before consumption is needed to strengthen seafood safety.

Antimicrobial resistance of *E. coli* and *Salmonella* spp.

The dissemination of AMR bacteria into the environment from humans and animals is caused by inappropriate or excessive use of antimicrobials, inefficient wastewater management, and contamination of antimicrobial agents from households (Boss et al., 2016). Spreading of contaminated animal manure, sewage, sludge, and antimicrobial residue can distribute AMR bacteria to the environment through horizontal genetic elements transmission. AMR and MDR bacteria have been reported in various bacterial species, especially in the *Enterobacteriaceae* family, such as *Klebsiella pneumoniae*, *E. coli*, *Enterobacter* spp., *Proteus* spp., *Shigella sonnei*, and *Salmonella* spp. On the Norwegian coast, 90% of *E. coli* were isolated from bivalve mollusks, of which 38% of these isolates were resistant to at least one antimicrobial agent (Grevskott et al., 2017). The occurrence of AMR in *Salmonella* and *E. coli* isolated from water was 12% and 18%, respectively (Dolejská et al., 2009). *E. coli* isolates were observed high resistance to tetracycline (92%) and trimethoprim (80%) (Odumosu et al., 2021). Resistant *E. coli* isolated from shellfish farms were found in tetracycline (30%), streptomycin (26%), and

trimethoprim (14%) (Park et al., 2013). *Salmonella* isolates from seafood were high resistance to sulfonamides (57%), followed by tetracycline (34%) and streptomycin (29%), and 43% of resistant isolates were MDR bacteria (Zhang et al., 2015). Additionally, *Salmonella* isolated from uncooked seafood were also found resistant to several important antimicrobials, including ampicillin (46%), followed by tetracycline (42%), chloramphenicol (31%), trimethoprim-sulfamethoxazole (27%), and streptomycin (19%) (Woodring et al., 2012). *E. coli* and *Salmonella* isolates of mollusks that were resistant to at least one antimicrobial agent were observed 75-80%, and 40% were MDR isolates (Giacometti et al., 2021). AMR of *E. coli* and *Salmonella* were observed at 18% and 12% from surface waters (Dolejská et al., 2009). Recently, the emergence of *E. coli* and *Salmonella* resistance to third-generation cephalosporins has been increasing concern. *E. coli* isolated from bivalve mollusks were resistance to extended-spectrum penicillins (83%) and third-generation cephalosporins (7%). Moreover, horizontal gene transfer was observed from these isolates (Grevskott et al., 2017). Bivalve mollusk has been used as an efficient tool for AMR monitoring in the environment, due to the height level of AMR and MDR were observed in mollusk and water.

In the environment, the AMR bacteria can contaminate through wastewater from many activities, such as sewage from antimicrobial manufacturing plants, hospitals, and urban areas that antibiotics were used. Moreover, antibiotic residues in manure and wastewater from plant production can be potential reservoirs of AMR bacteria in the environment (FAO, 2018). Practical strategies to control and prevention of AMR should be implemented across sectors, including human, animal, and environmental sectors. The emergence of AMR in the environment, especially resistance to many classes of antimicrobials and the occurrence of transferable resistant determinants, is a serious public health threat.

Extended-Spectrum ß-Lactamases (ESBL) in *E. coli* and *Salmonella*

Extended-Spectrum ß-Lactamases (ESBL) is an ability of bacterial resistance to penicillins, first-, second-, and third-generation cephalosporins and aztreonam (except cephamycins and carbapenems) (Rawat and Nair, 2010). ESBL was produced by Gram-negative *Enterobacteriaceae* bacteria, including *E. coli* and *Klebsiella pneumoniae*. The production of plasmid-mediated ESBL is the mechanism that ESBL-producing bacteria can hydrolyze ß-lactam rings and resistance to ß-lactam drugs (Batchoun et al., 2009). Increasing ESBL producing bacteria have been reported worldwide. Infection due to ESBL bacteria becomes a major challenge for hospitalized patients, and this can cause a life-threatening infection (Onyedibe et al., 2018).

Moreover, ESBL-producing bacteria have been observed conferring of co-resistance to various classes of antibiotics, resulting in the limitation of therapeutic options. Inappropriate antimicrobial use in humans and livestock and inefficient wastewater treatment would encourage the distribution of ESBL producing bacteria from the land source into the coastal and marine environment. ESBL bacteria has been increasingly examined in seafood and seawaters. ESBL producing *E. coli* (11.3%) and *Salmonella* (8.4%) was observed from the retail markets (Ye et al., 2018). ESBL producing *E. coli* from surface waters were observed high prevalence (Song et al., 2020) 61% from sewage (Reinthaler et al., 2010). For ESBL producing *Salmonella* from broiler supply chains and shedding water was 17% (Nahar et al., 2019).

MDR *E. coli* isolated from surface water and aquaculture samples contained several AMR genes such as *intI1, sul1, qnrA,* and ESBL (Blahna et al., 2006; Ng et al., 2018; Sivaraman et al., 2021). The occurrence of MDR genes in aquaculture farms was approximately 80% found in quinolones, trimethoprim/sulfamethoxazole, and

aminoglycosides. Moreover, ESBL-producing *Enterobacteriaceae* has been observed from several sources of water such as wastewater, river, and water samples from aquaculture areas (Adelowo et al., 2018). Surface water collected from aquaculture farms in Singapore was observed ESBL producing *E. coli* 27% (Ng et al., 2018). The prevalence of ESBL-producing *E. coli* was 30% in surface water (Müller et al., 2016). Overall, the widespread of ESBL in the environment and aquaculture has been an increasing concern since the treatment of bacterial infection becomes more complicated and may increase morbidity and mortality rates. This is because ineffective treatment with the last line antimicrobials. Approximately 42% of wastewater retrieved from rivers and lakes were detected ESBL-producing *E. coli* (Ben Said et al., 2016). More than 90% resistance to the third-generation cephalosporins, including cefotaxime, ceftazidime, and cefpodoxime, were observed in seafood and fish, and the presence of ESBL-encoding genes was high at 70% in these samples (Sanjit Singh et al., 2017). Mammals and aquatic animals living in the marine environment can also be exposed to ESBL producing bacteria and transfer these bacteria along the food chain (Sellera et al., 2018). People can contact contaminated water and aquaculture products harboring AMR bacteria, contributing to the higher probability of the ingestion of AMR, MDR, and ESBL producing bacteria.

Virulence genes of *E. coli* and *Salmonella*

The bacterial infection uses several mechanisms to cause illnesses by colonizing, invading, and penetrating to disturb host cells. Virulence genes were encoded on chromosomal, plasmid, transposon, or temperate bacteriophage DNA. Virulence is a mechanism to survive and multiply, and some bacteria are growing in the host cell, tissue fluid. For example, *Salmonella* can invade host cells, but they multiply in tissue fluids.

The association between AMR, virulence gene, and ESBL production of *E. coli* was observed from diarrheagenic stool samples collected from hospitalized patients (Eltai et al., 2020). *E. coli* from sewage treatment plants and surrounding environmental water can harbor virulence genes, and they were identical 95% associated with intestinal pathogenic *E. coli* (IPEC) or uropathogenic *E. coli* (UPEC) (Anastasi et al., 2012).

The abundant use of antimicrobial agents in livestock can promote AMR bacteria and may select for pathogenic resistance bacteria. The contamination of virulent microorganisms can potentially cause production loss and affects animal health, and the association between AMR genes and virulence factors has become public health concern (Rosengren et al., 2009). For example, there is an association between virulence genes and AMR *E. coli* in pig production. *E. coli* isolates from a pig with diarrhea and healthy pig in Ontario (Boerlin et al., 2005). It is possible that the co-selection between virulence and resistance may occur (Soto et al., 2006).

The prevalence of *Salmonella enterica* is a serious public health concern globally and the foremost cause of foodborne diseases. Treatment of *Salmonella* is increasing more difficult due to the increase of AMR in *Salmonella.* The finding of cointegrates of virulence and resistance determinants has occurred in soil, wastewater, and ready-to-eat food (Turki et al., 2012; Beshiru et al., 2019) and can indicate infection with resistance pathogenic bacteria might cause more severe and more difficult to treat. The study of the association between virulence and resistance also needs to fulfill the environmental sector in Thailand.

CHAPTER III MATERIALS AND METHODS

This study consists of 4 phases: phase 1) Serological identification of *Salmonella* isolates, phase 2) Phenotypic and genotypic detection of AMR in *E. coli* and *Salmonella* isolated from oyster and estuarine water samples, phase 3) Phenotypic and genotypic detection of ESBL in *E. coli* and *Salmonella* isolated from oysters and estuarine waters, and phase 4) Detection of virulence genes of *E. coli* and *Salmonella* isolated from oysters and estuarine water samples (Figure 1).

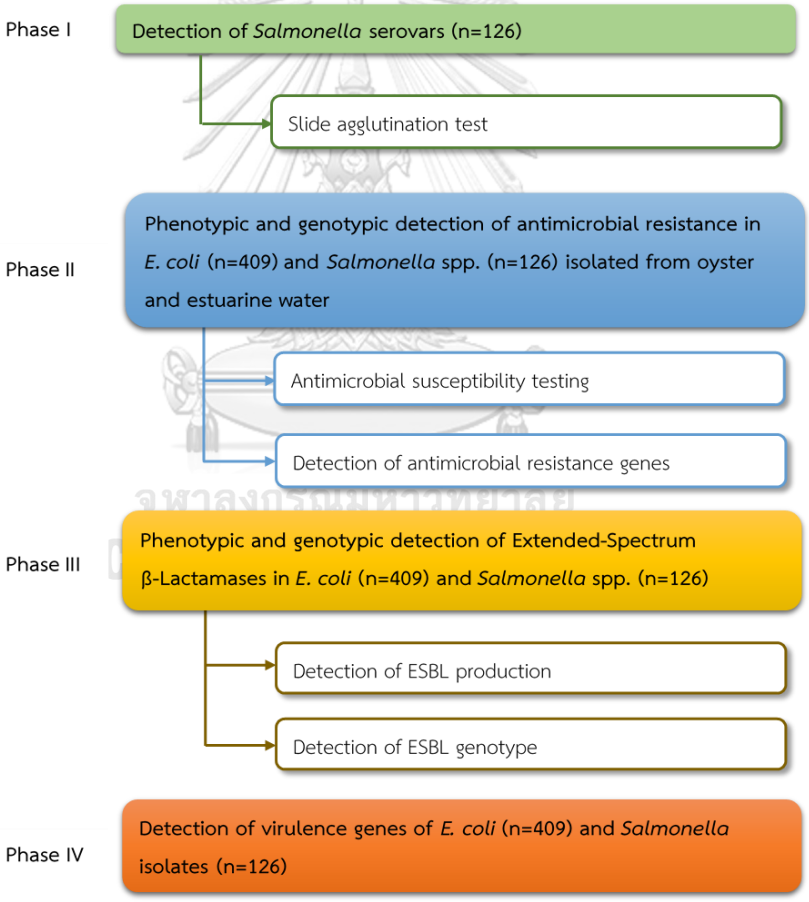

Figure 1 Research design of this experiment

Bacterial isolates

E. coli (n=409) and *Salmonella* spp. (n=126) isolates were collected from the stored collection in the Department of Veterinary Public Health, Faculty of Veterinary Science, Chulalongkorn University (Table 1). The isolates (n=535) were retrieved from 144 samples of oysters and 96 samples of estuarine water samples, which were collected monthly between April 2016 and March 2017 from Phang Nga province in southern Thailand. All bacterial strains were stored in a 20% glycerol stock solution at -80°C freezer.

Table 1 The number of *E. coli* and *Salmonella* isolates from oysters and estuarine waters.

Sample	Number of isolates (n)		Total (n)
	E. coli	*Salmonella*	
Estuarine water	159	3	162
Cultivated oyster	250	123	373
Total	409	126	535

Detection of *Salmonella* serovars

Salmonella isolates (n=126) were tested for serovars by slide agglutination test according to Kauffman-White Scheme (Grimont and Weill, 2007) with an available commercial antiserum (S&A Reagents Lab, Bangkok, Thailand). Each *Salmonella* isolate was cultured in nutrient agar (Difco, Le Pont de Claix, France) at 37 °C for 18-20 hr. The single colony of *Salmonella* was tested for O and H antigens.

Characterization of AMR in *E. coli* and *Salmonella* isolated from oysters and estuarine waters

Antimicrobial susceptibility testing

All bacterial isolates were tested for antimicrobial susceptibility to the antimicrobial agents that are commonly used in human and veterinary medicine (Table 2). Antimicrobial susceptibility test was obtained by determination of minimum inhibitory concentrations (MICs) using agar dilution technique according to the Clinical and Laboratory Standard Institute (CLSI, 2013). The bacterial isolates were cultured in Muller-Hinton agar (MHA) (Difco) at 37 °C for 18-20 hr. The single colonies were selected to adjust the cell density at 0.5 McFarland in 0.85% sodium chloride to estimate cell density at approximately 10^8 CFU/ml and to receive a final dilution of 10^7 CFU/ml before inoculation. The eight antimicrobials and their breakpoints as describe in bracket were ampicillin (32 µg/ml), ciprofloxacin (4 µg/ml), chloramphenicol (32 µg/ml), gentamicin (8 µg/ml), streptomycin (32 µg/ml), sulfamethoxazole (512 µg/ml), tetracycline (16 µg/ml) and trimethoprim (16 µg/ml) (CLSI, 2014). Quality control strains used in this study were *Staphylococcus aureus* ATCC 29213, *E. coli* ATCC 25922, and *Pseudomonas aeruginosa* ATCC 27853.

Detection of AMR genes

DNA templates of all *E. coli* and *Salmonella* isolates were prepared using the boiling method (Dashti et al., 2009). A single colony was grown on LB agar (Difco), suspended into 100 µl of sterile distilled water (DW), heated for 10 min in boiling water, and kept on ice before centrifuging at 12,000 rpm for 5 min. The supernatant was transferred to a sterile microcentrifuge tube and kept in a -20 °C freezer.

All isolates of *E. coli* and *Salmonella* were tested for the presence of AMR genes, including beta-lactams (bla_{TEM}), phenicol (*catA, catB,* and *cmlA*), aminoglycosides (*strA, strB*, *acc* (3)-IV, and *aadA1*), quinolones (*qnrA*, *qnrB*, and *qnrS*), sulfamethoxazole (*sul1, sul2,* and *sul3*), tetracycline (*tet(*A) and *tet(*B)) and trimethoprim (*dfr*A1 and *dfr*A12). All genes and primers tested for resistance genes were presented in (Table 2)

TopTaq PCR Master Mix Kit (Merck, Munich, Germany) was used as manufacturer's instruction. The PCR products were separated by gel electrophoresis using 1.5% agarose gel in 1X Tris-acetate/EDTA (1X TAE). Gels were stained with RedSafe™ Nucleic Acid Staining Solution (iNtRon Biotechnology®, Seongnam, South Korea) and visualized PCR products under UV light using Omega Fluor™ gel documentation system. The DNA templates were used for all PCR.

Table 2 Primer used for AMR genes

Gene	Primer	Primer sequence	Amplicon size (bp)	Reference
Beta-lactams				
bla_{TEM}	blaTEM-F	TTAACTGGCGAACTACTTAC	247	(Khan et al., 2019)
	blaTEM-R	GTCTATTTCGTTCATCCATA		
Phenicol				
catA	catA-F	CCAGACCGTTCAGCTGGATA	454	(Chuanchuen et al., 2008)
	catA-R	CATCAGCACCTTGTCGCCT		
catB	catB-F	CGGATTCAGCCTGACCACC	461	(Chuanchuen et al., 2008)
	catB-R	ATACGCGGTCACCTTCCTG		

Table 2 Primer used for AMR genes (cont.)

Gene	Primer	Primer sequence	Amplicon size (bp)	Reference
cmlA	cmlA-F	TGGACCGCTATCGGACCG	641	(Chuanchuen et al., 2008)
	cmlA-R	CGCAAGACACTTGGGCTGC		
Quinolones				
qnrA	qnrA-F	AGAGGATTTCTCACGCCAGG	580	(Cattoir et al., 2007)
	qnrA-R	TGCCAGGCACAGATCTTGAC		
qnrB	qnrB-F	GGMATHGAAATTCGCCACTG	264	(Cattoir et al., 2007)
	qnrB-R	TTTGCYGYYCGCCAGTCGAAC		
qnrS	qnrS-F	GCAAGTTCATTGAACAGGGT	428	(Cattoir et al., 2007)
	qnrS-R	TCTAAACCGTCGAGTTCGGCG		
Aminoglycosides				
aac(3)IV	aac(3)IV-F	GTGTGCTGCTGGTCCACAGC	627	(Stoll et al., 2012)
	aac(3)IV-R	AGTTGACCCAGGGCTGTCGC		
aadA1	aadA1-F	CTCCGCAGTGGATGGCGG	631	(Chuanchuen et al., 2008)
	aadA1-R	GATCTGCGCGCGAGGCCA		
strA	strA-F	TGGCAGGAGGAACAGGAGG	405	(Chuanchuen et al., 2008)
	strA-R	AGGTCGATCAGACCCGTGC		
strB	strB-F	GGCAGCATCAGCCTTATAATTT	470	(Mala et al., 2016)
	strB-R	GTGGATCCGTCATTCATTGTT		

Table 2 Primer used for AMR genes (cont.)

Gene	Primer	Primer sequence	Amplicon size (bp)	Reference
Tetracycline				
tet(A)	tet(A)-F	GGCGGTCTTCTTCATCATGC	502	(Khan et al., 2019)
	tet(A)-R	CGGCAGGCAGAGCAAGTAGA		
tet(B)	tet(B)-F	CGCCCAGTGCTGTTGTTGTC	615	(Chuanchuen et al., 2008)
	tet(B)-R	CGCGTTGAGAAGCTGAGGTG		
Folate-Pathway inhibitors				
sul1	sul1-F	CGGCGTGGGCTACCTGAACG	433	(Khan et al., 2019)
	sul1-R	GCCGATCGCGTGAAGTTCCG		
sul2	sul2-F	CGGCATCGTCAACATAACCT	721	(Khan et al., 2019)
	sul2-R	TGTGCGGATGAAGTCAGCTC		
sul3	sul3-F	TGTGCGGATGAAGTCAGCTC	244	(Khan et al., 2019)
	sul3-R	GCTGCACCAATTCGCTGAACG		
dfrA1	dfrA1-F	GGAGTGCCAAAGGTGAACAGC	367	(Shahrani et al., 2014)
	dfrA1-R	GAGGCGAAGTCTTGGGTAAAAAC		
dfrA12	dfrA12-F	TTCGCAGACTCACTGAGGG	330	(Chuanchuen et al., 2008)
	dfrA12-R	CGGTTGAGACAAGCTCGAAT		

Phenotypic and genotypic detection of ESBL in *E. coli* and *Salmonella*

ESBL production of bacterial isolates was tested by the disk diffusion method according to CLSI standard method (CLSI, 2013). The detection of ESBL consists of screening and confirmation tests. In the screening test, antimicrobial disks were placed on the plate and incubated at 37 ℃ for 18-20 hr. Ceftazidime (30 µg), cefotaxime (30 µg), and cefpodoxime (10 µg) were used for initial screening. The isolates that showed resistance to at least one of cephalosporins were confirmed for the ESBL production. For confirmation of ESBL production, a combination disk diffusion method using cephalosporins combined with clavulanic acid was used. The positive ESBL producing strains were interpreted by determining the difference of inhibition zone between normal cephalosporin and cephalosporin combined with clavulanic acid. The isolates that observed inhibition zone ≥5 mm is considered as the positive ESBL production.

Positive phenotype ESBL producing of *E. coli* (n=8) and *Salmonella* (n=2) were examined genes corresponding ESBL genes including bla_{TEM}, bla_{SHV}, bla_{CMY-2}, and bla_{CTX-M} using conventional PCR with the specific primer as described in table 3.

Table 3 Primer used for extended-spectrum ß-lactamase genes detection

Gene	Primer	Primer sequence (5'-3')	Amplicon size (bp)	Reference
bla_{TEM}	*bla*TEM-F	TTAACTGGCGAACTACTTAC	247	(Khan et al., 2019)
	*bla*TEM-R	GTCTATTTCGTTCATCCATA		
bla_{SHV}	*bla*SHV-F	AGGATTGACTGCCTTTTTG	393	(Khan et al., 2019)
	*Bla*SHV-R	ATTTGCTGATTTCGCTCG		

Table 3 Primer used for extended-spectrum β-lactamase genes detection (cont.)

Gene	Primer	Primer sequence (5'-3')	Amplicon size (bp)	Reference
bla_{CMY-2}	*bla*CMY-2-F	GACAGCCTCTTTCTCCACA	1000	(Khan et al., 2019)
	*bla*CMY-2-R	GGACACGAAGGCTACGTA		
bla_{CTX-M}	*bla*CTX-M-F	CGATGTGCAGTACCTAA	585	(Batchelor et al., 2005)
	*bla*CTX-M-R	AGTGACCAGAATCAGCGG		

The PCR products were purified using GeneJET PCR Purification Kit (Thermo Scientific, Vilnius, Lithuania) and submitted for DNA sequencing to U2Bio Sequencing Service (Thailand). The sequence result was analyzed using the Blast search tool (https://blast.ncbi.nlm.nih.gov/Blast.cgi.).

Detection of virulence genes of *E. coli* and *Salmonella* isolates

In the *E. coli* isolates, heat-labile toxin (*lt*), heat-stable toxin (*st*), and Shiga-like enterotoxins (*stx1* and *stx2*) and *eae* gene were examined. Virulence genes of *Salmonella,* including fimbriae protein (*fimA*), invasion gene (*invA*), and enterotoxin (*stn*) genes, were also observed in this study. Primer and amplicon size of the virulence genes were described in table 4.

Table 4 Primer used for virulence genes detection in E. coli and Salmonella isolates

Gene	Primer	Primer sequence	Amplicon size (bp)	Reference
E. coli				
lt	lt-F	TCTCTATGCATACGGAG	322	(Hinthong et al., 2017)
	lt-R	CCATACTGATTGCCGCAATT		
st	st-F	TGCTAAACCAGTAGAGTCTTCAAAA	138	(Hinthong et al., 2017)
	st-R	GCAGGCTTACAACACAATTCACAGCAG		
stx1	stx1-F	CAACACTGGATGATCTCAG	349	(Khan et al., 2002)
	stx1-R	CCCCCTCAACTGCTAATA		
stx2	stx2-F	ATCAGTCGTCACTCACTGGT	110	(Khan et al., 2002)
	stx2-R	CTGCTGTCACAGTGACAAA		
eae	eae-F	CCCGAATTCGGCACAAGCATAAGC	881	(Toma et al., 2003)
	eae-R	CCCGGATCCGTCTCGCCAGTATTCG		
Salmonella				
fimA	fimA-F	CCTTTCTCCATCGTCCTGAA	85	(Kumar et al., 2009)
	fimA-R	TGGTGTTATCTGCCTGACCA		
stn	stn-F	CTTTGGTCGTAAAATAAGGCG	260	(Kumar et al., 2009)
	stn-R	TGCCCAAAGCAGAGAGATTC		
invA	invA-F	GTGAAATTATCGCCACGTTCGGGCAA	284	(Kumar et al., 2009)
	invA-R	TCATCGCACCGTCAAAGGAACC		

Statistical analysis

The descriptive statistic was performed on the prevalence of phenotype and genotype AMR, ESBL production, and virulence genes in *E. coli* and *Salmonella* isolates. The logistic regression analyses were performed to examine the association between AMR, ESBL production, and virulence genes in each bacterial strain. The dependent variable was phenotypic AMR in each bacterial species, and the independent variables are other phenotypic AMR, resistance genes, and virulence genes. The analysis with two-side hypothesis testing was performed using Stata version 14.0 (StataCorp, College Station, TX, USA). The statistical significance was considered at a *p-value* ≤ 0.05.

CHAPTER IV RESULTS

In this study, *Salmonella* (n=126) isolates have identified the serovar. *E. coli* (n=409) and *Salmonella* (n=126) has been tested for antimicrobial susceptibility testing and ESBL production. All the isolates were also examined for AMR, ESBL, and virulence genes. The association between AMR, ESBL, and virulence genes was explored.

Serovars of *Salmonella* isolates

From 126 *Salmonella* isolates, 36 serovars were identified from oysters (n=123), and one serovar was reported in estuarine water (n=3). In all isolates, the predominant serovars were II (15.87%), Paratyphi B (13.50%), and Eastbourne (12.70%), respectively. The common serovars of isolates from oysters were II (n=17, 13.83%), Paratyphi B (n=17, 13.83%), and Eastbourne (n=16, 13.01%), whereas Kentucky (n=8, 6.51%), Lomita (n=5, 4.07%) and Newmexico (n=4, 3.26%) also found in lower prevalence. The serovar II (n=3, 100%) was only serovar observed in the estuarine water. The list of serovars has been described in table 5.

Table 5 Salmonella serovars from oysters (n=123) and estuarine waters (n=3)

Serovars	No. of isolates (%)	
	Estuarine water (n=3)	Oyster (n=123)
Aequatoria	-	1 (0.82%)
Agona	-	3 (2.44%)
Alamo	-	1 (0.82%)

Table 5 *Salmonella* serovars from oysters (n=123) and estuarine waters (n=3) (cont.)

Serovars	No. of isolates (%)	
	Estuarine water (n=3)	Oyster (n=123)
Augustenburg	-	3 (2.44%)
Beaudesert	-	1 (0.82%)
Berta	-	1 (0.82%)
Braenderup	-	3 (2.44%)
Chester	-	3 (2.44%)
Croft	-	2 (1.63%)
Eastbourne	-	16 (13.01%)
Emek	-	3 (2.44%)
Florian	-	1 (0.82%)
Hillingdon	-	3 (2.44%)
Hissar	-	2 (1.63%)
II	3 (100%)	17 (13.83%)
II b	-	1 (0.82%)
II-Stern schanze	-	1 (0.82%)
IIIa	-	1 (0.82%)

Table 5 *Salmonella* serovars from oysters (n=123) and estuarine waters (n=3) (cont.)

Serovars	No. of isolates (%)	
	Estuarine water (n=3)	Oyster (n=123)
IIIb	-	4 (3.26%)
Kentucky	-	8 (6.51%)
Larochelle	-	1 (0.82%)
Lomita	-	5 (4.07%)
Mathura	-	3 (2.44%)
Menston	-	2 (1.63%)
Meskin	-	1 (0.82%)
Molade	-	1 (0.82%)
Mono	-	1 (0.82%)
Ndolo	-	2 (1.63%)
Newmexico	-	4 (3.26%)
Othmarschen	-	1 (0.82%)
Paratyphi B	-	17 (13.83%)
Ploufragen	-	2 (1.63%)

Table 5 *Salmonella* serovars from oysters (n=123) and estuarine waters (n=3) (cont.)

Serovars	No. of isolates (%)	
	Estuarine water (n=3)	Oyster (n=123)
Potengi	-	1 (0.82%)
Stockholm	-	1 (0.82%)
Tennessee	-	3 (2.44%)
Westafrica	-	3 (2.44%)
Total	**3 (100%)**	**123 (100%)**

Phenotypic and genotypic detection of AMR in *E. coli* and *Salmonella* isolates

The *E. coli* (n=409) and *Salmonella* spp. (n=126) isolates from oysters and estuarine waters were tested for phenotype and genotype resistance to eight antimicrobial agents.

All 409 *E. coli* isolates were performed for antimicrobial susceptibility testing. Three hundred and eighty-five isolates (94.13%) were resistant to at least one antibiotic, and 24 isolates (5.87%) were susceptible to all tested antibiotics. The common resistance was observed in sulfamethoxazole (77.09%, n=318), followed by ampicillin (55.25%, n=226), tetracycline (40.10%, n=124), and trimethoprim (36.68%, n=150). The prevalence of streptomycin-resistant were 32,28%, while, resistance of chloramphenicol (18.59%, n=76), ciprofloxacin (4.89%, n=20) and gentamicin (4.89%, n=20) was low. Sulfamethoxazole was from oysters (49.88%, n=204) and estuarine waters (27.87%, n=114). The resistance to ampicillin derived from oysters (30.32%, n=124) and estuarine waters (24.94%, n=102), while

tetracycline resistance were from oysters 23.23% (n=95) and estuarine waters 16.87% (n=69) (Figure 2).

In this study, 59 resistance patterns were observed of all *E. coli* isolates. The common patterns were SMX 23.96% (n=98), and AMP-CHL-STR-SMX-TET-TRI 6.85% (n=28). For oyster (n=250), the common resistance patterns were SMX 29.20% (n=73), AMP-SMX 7.60% (n=19) and STR-SMX 5.60% (n=14), while SMX 15.72% (n=25), AMP 10.06% (n=16), and AMP-CHL-STR-SMX-TET-TRI 9.43% (n=15) were found in estuarine waters (n=159) (Table 6). The MDR was observed 42.60% (n=164/385) from oyster (n=86) and estuarine waters (n=78).

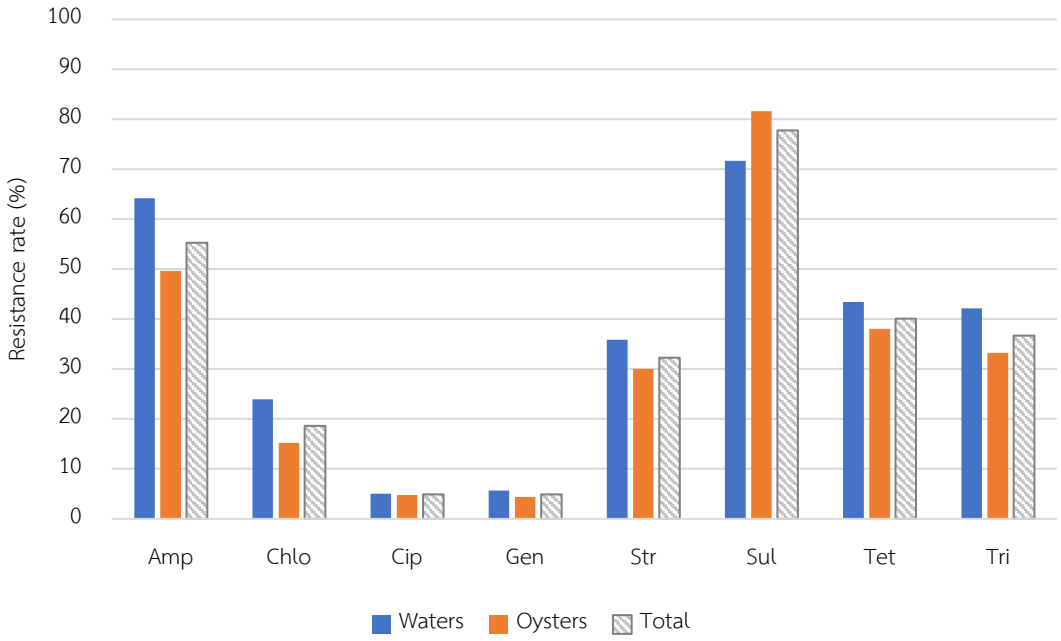

Figure 2 Prevalence of AMR in E. coli isolates (n=409) from oyster (n=250) and estuarine waters (n=159).

Table 6 Resistance pattern of E. coli isolates (n=409) from oysters (n=250) and estuarine waters (n=159)

Resistance pattern	No. of isolates (%)		
	Oysters (n=250)	Waters (n=159)	Total (n=409)
Susceptible	12 (4.80%)	12 (7.55%)	24 (5.87%)
AMP	8 (3.20%)	16 (10.06%)	24 (5.87%)
AMP-CHL-CIP-GEN-STR-SMX-TET-TRI	1 (0.40%)	-	1 (0.24%)
AMP-CHL-CIP-GEN-SMX-TET-TRI	1 (0.40%)	-	1 (0.24%)
AMP-CHL-CIP-STR-SMX-TET-TRI	3 (1.20%)	2 (1.26%)	5 (1.22%)
AMP-CHL-CIP-SMX-TET-TRI	2 (0.80%)	3 (1.89%)	5 (1.22%)
AMP-CHL-GEN-STR-SMX-TET-TRI	1 (0.40%)	4 (2.52%)	5 (1.22%)
AMP-CHL-STR-SMX	1 (0.40%)	-	1 (0.24%)
AMP-CHL-STR-SMX-TET	2 (0.80%)	2 (1.26%)	4 (0.98%)
AMP-CHL-STR-SMX-TET-TRI	13 (5.20%)	15 (9.43%)	28 (6.85%)
AMP-CHL-STR-SMX--TRI	2 (0.80%)	2 (1.26%)	4 (0.98%)
AMP-CHL-STR--TET-TRI	-	1 (0.63%)	1 (0.24%)
AMP-CHL-SMX	1 (0.40%)	-	1 (0.24%)
AMP-CHL-SMX-TET	-	1 (0.63%)	1 (0.24%)
AMP-CHL-SMX-TET-TRI	8 (3.20%)	6 (3.77%)	14 (3.42%)

Table 6 Resistance pattern of *E. coli* isolates (n=409) from oysters (n=250) and estuarine waters (n=159) (cont.)

Resistance pattern	No. of isolates (%)		
	Oysters (n=250)	Waters (n=159)	Total (n=409)
AMP-CHL-SMX-TRI	1 (0.40%)	-	1 (0.24%)
AMP-CHL-TET-TRI	1 (0.40%)	-	1 (0.24%)
AMP-CHL-TRI	-	1 (0.63%)	1 (0.24%)
AMP-CIP-GEN-STR-TET	1 (0.40%)	-	1 (0.24%)
AMP-CIP-STR-SMX-TET	1 (0.40%)	-	1 (0.24%)
AMP-CIP-STR-SMX-TET-TRI	2 (0.80%)	-	2 (0.49%)
AMP-CIP-SMX-TET	-	1 (0.63%)	1 (0.24%)
AMP-CIP-SMX-TET-TRI	-	1 (0.63%)	1 (0.24%)
AMP-CIP-TET	1 (0.40%)	-	1 (0.24%)
AMP-GEN	-	1 (0.63%)	1 (0.24%)
AMP-GEN-STR-SMX-TET	1 (0.40%)	-	1 (0.24%)
AMP-GEN-STR-SMX-TET-TRI	3 (1.20%)	-	3 (0.73%)
AMP-GEN-STR-TET	1 (0.40%)	-	1 (0.24%)
AMP-GEN-SMX-TRI	2 (0.80%)	-	2 (0.49%)
AMP-GEN-TRI	-	3 (1.89%)	3 (0.73%)

Table 6 Resistance pattern of *E. coli* isolates (n=409) from oysters (n=250) and estuarine waters (n=159) (cont.)

Resistance pattern	No. of isolates (%)		
	Oysters (n=250)	Waters (n=159)	Total (n=409)
AMP-STR	1 (0.40%)	1 (0.63%)	2 (0.49%)
AMP-STR-SMX	1 (0.40%)	11 (6.92%)	12 (2.93%)
AMP-STR-SMX-TET	1 (0.40%)	5 (3.14%)	6 (1.47%)
AMP-STR-SMX-TET-TRI	13 (5.20%)	8 (5.03%)	21 (5.13%)
AMP-STR-SMX-TRI	4 (1.60%)	-	4 (0.98%)
AMP-STR-TET	1 (0.40%)	-	1 (0.24%)
AMP-STR-TET-TRI	1 (0.40%)	-	1 (0.24%)
AMP-SMX	19 (7.60%)	3 (1.89%)	22 (5.38%)
AMP-SMX-TET	10 (4.00%)	1 (0.63%)	11 (2.69%)
AMP-SMX-TET-TRI	2 (0.80%)	6 (3.77%)	8 (1.96%)
AMP-SMX-TRI	4 (1.60%)	4 (2.52%)	8 (1.96%)
AMP-TET	4 (1.60%)	4 (2.52%)	8 (1.96%)

Table 6 Resistance pattern of *E. coli* isolates (n=409) from oysters (n=250) and estuarine waters (n=159) (cont.)

Resistance pattern	No. of isolates (%)		
	Oysters (n=250)	Waters (n=159)	Total (n=409)
AMP-TET-TRI	3 (1.20%)	-	3 (0.73%)
AMP-TRI	3 (1.20%)	-	3 (0.73%)
CHL-TET	1 (0.40%)	1 (0.63%)	2 (0.49%)
CIP	-	1 (0.63%)	1 (0.24%)
GEN-SMX-TET	-	1 (0.63%)	1 (0.24%)
STR	2 (0.80%)	-	2 (0.49%)
STR-SMX	14 (5.60%)	-	14 (3.42%)
STR-SMX-TET	-	3 (1.89%)	3 (0.73%)
STR-SMX-TET-TRI	1 (0.40%)	-	1 (0.24%)
STR-SMX-TRI	2 (0.80%)	2 (1.26%)	4 (0.98%)
STR-TET	2 (0.80%)	-	2 (0.49%)
STR-TET-TRI	-	1 (0.63%)	1 (0.24%)
SMX	73 (29.20%)	25 (15.72%)	98 (23.96%)
SMX-TET	7 (2.80%)	3 (1.89%)	10 (2.44%)
SMX-TET-TRI	5 (2.00%)	-	5 (1.22%)

Table 6 Resistance pattern of *E. coli* isolates (n=409) from oysters (n=250) and estuarine waters (n=159) (cont.)

Resistance pattern	No. of isolates (%)		
	Oysters (n=250)	Waters (n=159)	Total (n=409)
SMX--TRI	3 (1.20%)	5 (3.14%)	8 (1.96%)
TET	2 (0.80%)	-	2 (0.49%)
TRI	2 (0.80%)	3 (1.89%)	5 (1.22%)
Total	**250 (100%)**	**159 (100%)**	**409 (100%)**

Abbreviations: AMP: ampicillin; CHL: chloramphenicol; CIP: ciprofloxacin; GEN: gentamicin; STR: streptomycin: SMX: sulfamethoxazole; TET: tetracycline; TRI: trimethoprim

Phenotypic antimicrobial resistance of *Salmonella* isolates

The *Salmonella* isolates (n=126) from oysters (n=123) and estuarine waters (n=3) were examined by the antimicrobial susceptibility test. All isolates of *Salmonella* were susceptible to gentamicin and streptomycin. Approximately 122 isolates (96.82%) were resistant to at least one antimicrobial agent. These isolates were commonly resistance to sulfamethoxazole 95.12% (n=117), followed by trimethoprim 38.21% (n=47), ampicillin 37.40% (n=46), tetracycline 25.20% (n=31), chloramphenicol 9.76% (n=12), and ciprofloxacin 8.73% (n=11), respectively. All three isolates from estuarine water were resistant to only sulfamethoxazole (100%) (Figure 3).

Thirteen resistance patterns were examined in the *Salmonella* isolates, and the most common resistance pattern was SMX 34.92% (n=44), AMP-SMX-TET-TRI 11.11% (n=44), and SMX-TRI 10.32% (n=13) (Table 7). The MDR were identified 23.02% (n=29/122).

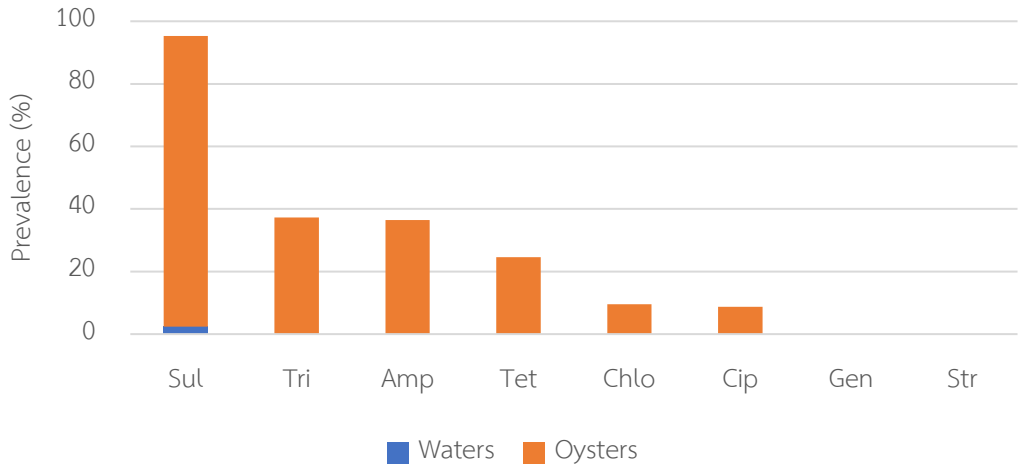

Figure 3 Prevalence of AMR in Salmonella isolates (n=126) from oyster (n=123) and estuarine waters (n=3)

Table 7 Resistance pattern of Salmonella isolates from oyster (n=123) and estuarine water (n=3)

Resistance Pattern	No. of isolates (%)		
	Oyster (n=126)	Estuarine water (n=3)	Total (n=126)
Susceptible	4 (3.17%)	-	4 (3.17%)
AMP-CHL-SMX-TET-TRI	11 (8.73%)	-	11 (8.73%)
AMP-SMX	12 (9.52%)	-	12 (9.52%)
AMP-SMX-TET	4 (3.17%)	-	4 (3.17%)
AMP-SMX-TET-TRI	14 (11.11%)	-	14 (11.11%)
AMP-SMX-TRI	4 (3.17%)	-	4 (3.17%)
AMP-TRI	1 (0.79%)	-	1 (0.79%)
CHL-SMX-TRI	2 (1.59%)	-	2 (1.59%)
CIP-SMX	11 (8.73%)	-	11 (8.73%)
SMX	44 (34.92%)	3 (2.38%)	47 (37.30%)
SMX-TET	1 (0.79%)	-	1 (0.79%)
SMX-TET-TRI	1 (0.79%)	-	1 (0.79%)
SMX-TRI	13 (10.32%)	-	13 (10.32%)
TRI	1 (0.79%)	-	1 (0.79%)
Total	**123 (97.62%)**	**3 (2.38%)**	**126 (100%)**

Abbreviations: AMP: ampicillin; CHL: chloramphenicol; CIP: ciprofloxacin; GEN: gentamicin; STR: streptomycin: SMX: sulfamethoxazole; TET: tetracycline; TRI: trimethoprim.

Genotypic AMR of *E. coli* isolates from oysters (n=250) and estuarine waters (n=159)

In the *E. coli* isolates, bla_{TEM} 31.55% (n=129), *tet(A)* 25.44% (n=104) and *strA* 14.92% (n=61) were predominant resistance genes found in this study. For oyster isolates, the common AMR genes were bla_{TEM} (16.88%, n=69), *tet(A)* (13.21%, n=54), and *strA* (6.85%, n=28), and the water isolates harbored bla_{TEM} (14.67%, n=60), *tet(A)* (12.23%, n=50) and *strA* (8.07%, n=33), respectively (Figure 4).

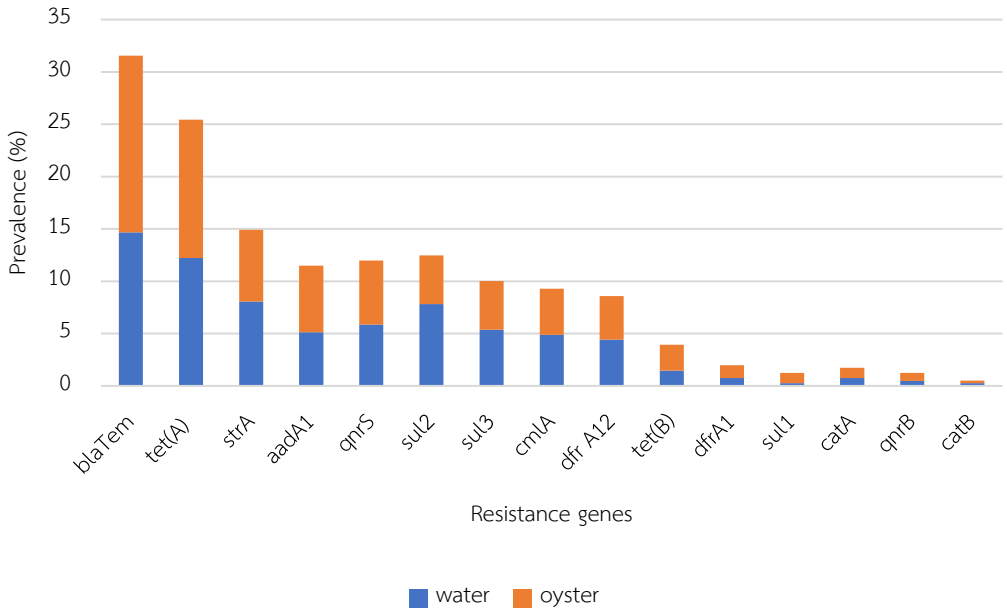

Figure 4 Resistance gene examined in *E. coli* isolates from oyster (n=250) and estuarine waters (n=159)

Genotypic AMR of *Salmonella* isolates from oysters (n=123) and estuarine waters (n=3)

The predominant AMR genes of *Salmonella* isolates were *sul3* 14.29% (n=18), bla_{TEM} 11.90% (n=15) and *cmlA* 11.90% (n=15), respectively. All genes detected were derived from oyster samples, while none of the resistance genes was found in estuarine waters (Figure 5).

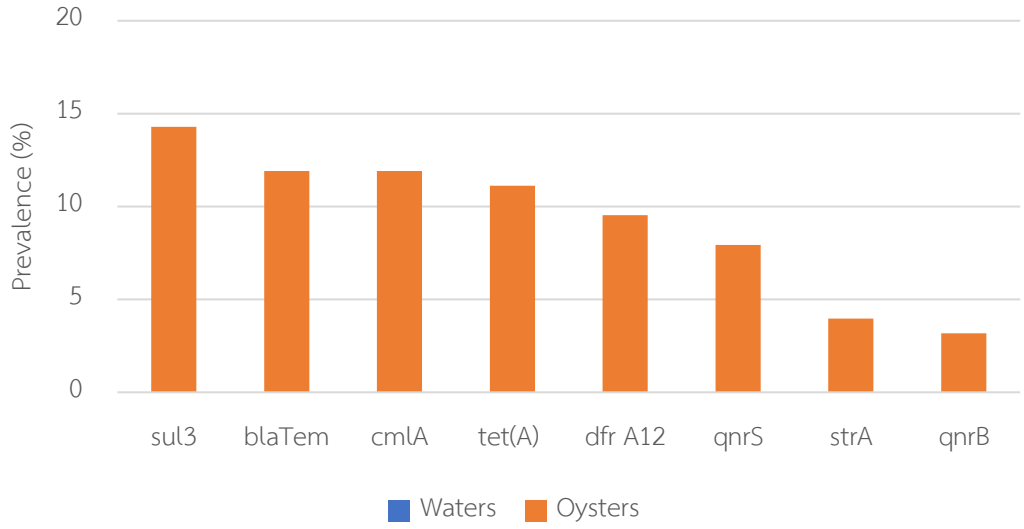

Figure 5 Resistance gene examined in *Salmonella* isolates from oyster (n=123)

Detection of Extended-Spectrum β- Lactamase production in *E. coli* and *Salmonella* isolates from oyster and estuarine water samples

Phenotypic ESBL in *E. coli* and *Salmonella* isolates

Eight isolates of *E. coli* from oysters and while two *Salmonella* isolates, serovar Augustenborg and II from the oysters, were confirmed as ESBL producing isolates.

Genes underlying ESBL production in *E. coli* and *Salmonella* isolates

All positive phenotypic ESBL producing isolates of *E. coli* (n=8) and *Salmonella* (n=2) were tested genes underlying ESBL production. In this study, four *E. coli* isolates from oysters were harbored ESBL genes, consisting of $bla_{\text{CTX-M}}$ (n=4), and one sample harbored broad-spectrum beta-lactamase ($bla_{\text{TEM-1}}$) (n=1). One *Salmonella* isolates from (serovars II) was harbored bla_{TEM}, while the tested genes were not found in Augustenborg.

Virulence genes of *E. coli* and *Salmonella* isolates

For all *E. coli* isolates (n=409), *stx1* and *lt* were predominantly detected from both oysters and estuarine waters. The *stx1* gene (17.85%, n=73) was detected from oysters (10.27%, n=42) and estuarine waters (7.58%, n=31), and *lt* (11.74%, n=48) was reported in oysters (9.54%, n=39), and estuarine waters (2.21%, n=9). The *stx2* and *st* were found only in oyster isolates at 1.23% (n=5/250) and 0.25% (n=1/250), respectively. One isolate from the estuarine water sample harbored the *eae* gene (Figure 6).

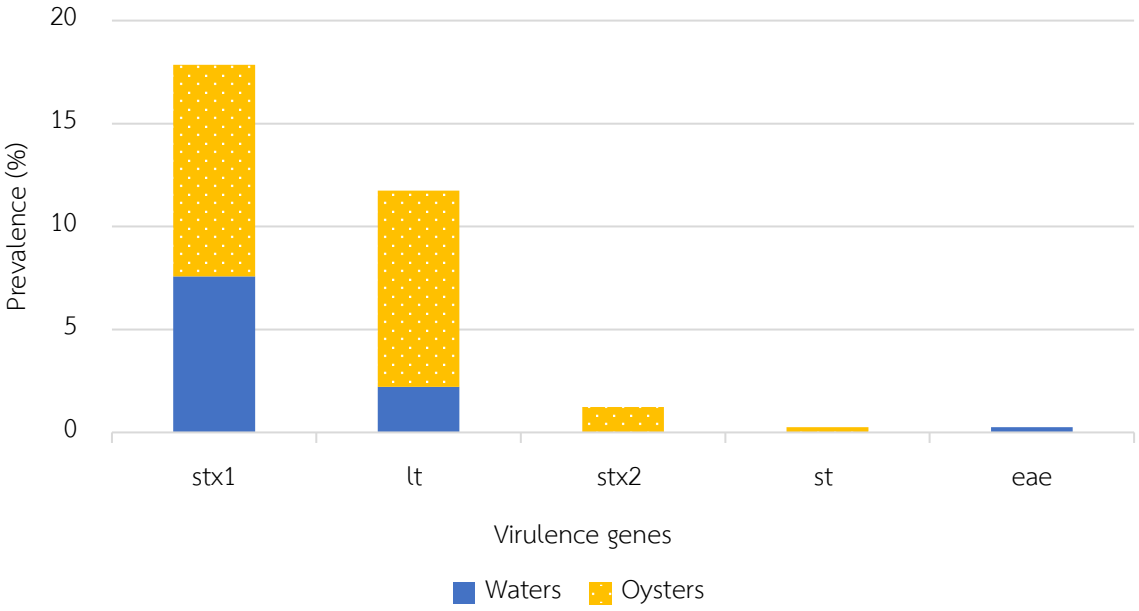

Figure 6 Virulence genes detected in *E. coli* isolates (n=409) from oysters (n=250) and estuarine waters (n=159)

The *Salmonella* isolates from oysters harbored *invA* 76.98% (n=97), *stn* 76.98% (n=97), and *fimA* 69.05% (n=87). While all estuarine waters (n=3) were positive to all tested virulence genes (*invA, stn,* and *fimA*).

Logistic regression models of resistance phenotype and genotype, virulence genes, and ESBL production of *E. coli* and *Salmonella*

The resistance *E. coli* isolates to ampicillin were also resistant to chloramphenicol (OR=21.34) and trimethoprim (OR=5.00) (p<0.0001) (Table 8). However, the isolates that were susceptible to ampicillin were more likely to be resistant to sulfamethoxazole than the isolates that were resistant to ampicillin. The isolates resistant to chloramphenicol were associated with the presence of *cmlA* with OR=18.84 (p<0.0001). The isolates that were resistant to tetracycline were commonly found *tetA* and *tetB* genes than the isolates that susceptible to tetracycline with odds ratio at 34.94 and 13.67 (p<0.0001), respectively. The isolates that phenotypic resistance to chloramphenicol was more harbored the *stx1* gene than those susceptible ones (OR=2.61, p<0.0001), while the resistance to

chloramphenicol isolates had more *stx2* gene than the isolates that were susceptible to chloramphenicol (OR=2.27, $p<0.0001$) (Table 9).

Table 8 Logistic regression analysis of phenotypic resistance in E. coli from oyster (n=250) and estuarine waters (n=159)

Dependent variable	**Independent variable**	**Odds Ratio**	**Robust Std. Err**	**95% C.I.**			***p*>|z|**
AMP	CHL	21.34	1.30	18.93	to	24.05	<0.0001
	SMX	0.50	0.074	0.38	to	0.67	<0.0001
	TRI	5.00	2.36	1.98	to	12.60	0.001
constant		0.94	0.18	0.65	to	1.36	0.747
CHL	*cmlA*	18.84	2.84	14.02	to	25.31	<0.0001
constant		0.14	0.29	0.10	to	0.22	<0.0001
TET	*tetA*	34.94	0.65	33.70	to	36.23	<0.0001
	tetB	13.67	4.22	33.70	to	36.23	<0.0001
constant		0.22	0.01	0.21	to	0.23	<0.0001
CHL	*stx1*	2.61	0.12	2.38	to	2.86	<0.0001
constant		0.19	0.05	0.11	to	0.32	<0.0001
TET	*stx2*	2.27	0.29	1.77	to	2.91	<0.0001
constant		0.66	0.08	0.52	to	0.84	0.001

Abbreviations: AMP: ampicillin; CHL: chloramphenicol; C.I.: confident interval; Std. Err.: standard error; SMX: sulfamethoxazole; TRI: trimethoprim Abbreviations: CHL: chloramphenicol; C.I.: confident interval; Std. Err.: standard error; TET: tetracycline.

Salmonella isolates resistant to ampicillin were commonly harbor bla_{TEM} (OR=15.36) and probably resistance to tetracycline (OR=3.90) than thee isolates that susceptible to ampicillin (p<0.0001). Moreover, these isolates also more likely to harbor *fimA* (OR=4.20) and (p<0.0001) (Table 9), The association between phenotypic resistance *of Salmonella* and virulence genes are phenotypic resistance to chloramphenicol, trimethoprim, and tetracycline isolates were more likely to contain *invA* and *fimA* with statistically significance (p<0.0001). Isolates that resistant to ceftazidime harbored the *invA* gene than those susceptible ones with OR=2.94. The isolates resistant to cefotaxime and cefpodoxime were more likely to present fimA than those susceptible to cefotaxime and cefpodoxime.

Table 9 Logistic regression analysis of phenotypic and resistance genes in Salmonella from oyster (n=123) and estuarine waters (n=3)

Dependent variable	Independent variable	Odds Ratio	Robust Std. Err	95% C.I.			p>|z|
AMP	TET	3.90	0.50	3.80	to	3.99	<0.0001
constant		-1.74	0.08	-1.89	to	-1.58	<0.0001
AMP	bla_{TEM}	15.36	1.18	13.21	to	17.86	<0.0001
constant		0.42	0.03	0.36	to	0.49	<0.0001
CHL	*cmlA*	11.67	0.67	10.43	to	13.05	<0.0001
constant		0.06	0.00	0.05	to	0.06	<0.0001
TET	*tetA*	29.37	1.89ZZ	25.88	to	33.33	<0.0001
constant		0.20	0.13	0.18	to	0.23	<0.0001
AMP	*fimA*	4.20	0.37	3.53	to	5.00	<0.0001
constant		0.44	0.00	0.43	to	0.44	<0.0001
CHL	*invA*	1.25	0.05	1.16	to	1.35	<0.0001
	fimA	7.32	0.23	6.88	to	7.79	<0.0001
constant		0.03	0.00	0.03	to	0.03	<0.0001

Table 9 Logistic regression analysis of phenotypic and resistance genes in *Salmonella* from oyster (n=123) and estuarine waters (n=3) (cont.)

Dependent variable	Independent variable	Odds Ratio	Robust Std. Err	95% C.I.			*p*>|z|
TRI	*invA*	2.40	0.07	2.27	to	2.53	<0.0001
	fimA	2.30	0.22	1.91	to	2.77	<0.0001
constant		1.05	0.01	1.04	To	1.06	<0.0001
TET	*invA*	7.09	0.075	6.95	to	7.24	<0.0001
	fimA	5.23	0.42	4.46	to	6.12	<0.0001
constant		0.08	0.00	0.08	to	0.008	<0.0001
CAZ	*invA*	2.94	0.17	2.62	to	3.29	<0.0001
constant		0.09	0.00	0.09	To	0.09	<0.0001
CTX	*fimA*	1.79	0.10	1.61	to	1.99	<0.0001
constant		0.33	0.00	0.33	to	0.33	<0.0001
CPD	*fimA*	2.90	0.04	2.83	to	2.97	<0.0001
constant		0.08	0.00	0.08	to	0.08	<0.0001
MDR	*fimA*	13.59	0.06	14.48	to	13.71	<0.0001
constant		0.04	0.00	0.04	to	0.04	<0.0001

Abbreviations: AMP: ampicillin; CAZ: ceftazidime; CHL: chloramphenicol; C.I.: confident interval; CPD: cefpodoxime; CTX: cefotaxime; MDR: multidrug-resistant; Std. Err.: standard error; SMX: sulfamethoxazole; TET: tetracycline; TRI: trimethoprim.

CHAPTER V DISCUSSION

AMR is a global problem that has been raised as a public health concern. It is estimated that more than 2.8 million cases and 35,000 people were died due to AMR infection in 2019 (CDC, 2021a). The estimated health care cost due to AMR is more than 4.6 billion USD per year in the U.S. (CDC, 2021b). In Thailand, AMR has been affected by 38,000 deaths, with an economic loss of 1,200 million USD (WHO, 2017; Sumpradit et al., 2021). To better understand the current AMR situation, the One Health approach is recommended by exploring the AMR problem in humans, animals, and the environment. However, studies of AMR in the environment are weak and limit information. This is because the AMR in the environment has been overlooked compared to other sectors under the One Health triad. Moreover, no standardized protocol and contain various environmental niches have been observed. Therefore, this study aimed to fulfill this gap by indicating the possible sources of AMR contamination in the estuarine environment.

E. coli and *Salmonella* have been widely used for AMR monitoring and surveillance in animals and humans. In this study, the resistance rates of *E. coli* and *Salmonella* isolated from oysters and estuarine water were high at 94% and 95%, respectively. Ampicillin, tetracycline, trimethoprim, and sulfamethoxazole were the most prevalent resistance antibiotics in this investigation. This result was consistent with previous studies (Watkinson et al., 2007; Laroche et al., 2009; Silveira et al., 2016; Giacometti et al., 2021). MDR was observed at 43% in *E. coli* and 23% in *Salmonella***.** This finding was similar to the previous study, indicating that the levels of MDR were 38% *E. coli*, but a higher rate of MDR *Salmonella* (44%) was observed in the same study (Giacometti et al., 2021). On the other hand, the lower rate of MDR was reported at 19% in *E. coli* isolated from estuarine water samples (Pereira et al., 2013). Our findings addressed that AMR in *E. coli* and *Salmonella* isolated

from oysters and estuarine water can circulate in the environment and serve as a significant AMR hotspot in the environment. Therefore, the circulation of AMR bacteria in the estuary may possibly transfer from the environment to humans. As a result, it increases the risk of infection in humans.

The resistance to ampicillin, tetracycline, trimethoprim, and sulfamethoxazole was commonly found in our study. In veterinary medicine, sulfonamides have been frequently used in swine and cattle production. Previous studies had indicated that high levels of sulfonamides were examined in livestock manure (Baran et al., 2011; Checcucci et al., 2020). In this study, *E. coli* had a high prevalence of ampicillin and tetracycline resistance, which was consistent with a previous finding in pig farms in Thailand (Lugsomya et al., 2018). However, the prevalence of chloramphenicol and ciprofloxacin resistance was low in comparison with a previous study (Pruksakorn et al., 2016). The resistance to sulfamethoxazole was documented in surface water and soil (Hruska and Fránek, 2012). Greater than 40% of tetracycline-resistant *E. coli* was reported broiler production. Previous studies reported tetracyclines are the most-frequency used in poultry production as prophylaxis, antibacterial therapy, and growth promoter (Kim et al., 2013; Ljubojević et al., 2017; Roth et al., 2019). Similarly, the prevalence of tetracycline resistance was high in pig and vegetable samples (Pu et al., 2018). Due to the low absorption of tetracycline in animals, tetracycline can concentrate and create drug residues in meat, urine, and feces and may eventually distributed into the environment (Mahmoud and Abdel-Mohsein, 2019). In contrast, *Salmonella* isolates were found a lower prevalence of streptomycin-resistant than those in chicken meat (Lertworapreecha et al., 2013). In this study, the prevalence of gentamicin- and ciprofloxacin-resistant of both *Salmonella and E. coli* was low compared to livestock samples (Angkititrakul et al., 2005; Nguyen et al., 2016b). Therefore,

sulfonamide-resistant bacteria found in oyster and estuarine water are predominant environmental pollutants.

The high prevalence of ampicillin, tetracycline, and trimethoprim was observed in seafood, especially in bivalve mollusk (Yang et al., 2015; Silveira et al., 2016; Odumosu et al., 2021). In Thailand, ampicillin and tetracycline were antimicrobials commonly sold in human medicine for treatment of sore throat and common cold. These antibiotics are accounted for 40% of antibiotics sold in pharmacy stores (Pharm et al., 2016). It is estimated that the cost of selling antibiotics in Thailand is 230 million dollars per year (Siltrakool et al., 2021). Even though detected antibiotics in this study were not applied for oyster aquaculture, the contamination of AMR may derive from wastewater and nearby communities. In addition, waste and watersheds from agriculture may be a possible source of AMR in the environment. Based on logistic regression model, the isolates that resistant to ampicillin were more likely to resistance to chloramphenicol and trimethoprim. In addition, a co-selection of resistance genes between ampicillin and chloramphenicol has been revealed (Zhao et al., 2021). The finding agreed with a previous study that combines resistance of both ampicillin and trimethoprim can promote resistance to additional antibiotics, including beta-lactams, aminoglycosides, cephalosporins and ciprofloxacin (Leverstein-van Hall et al., 2003).

Most of the resistance genes detected in this study corresponded to the resistance phenotype. In the *E. coli* isolates, the majority of resistance genes were bla_{TEM}, *tet(A)*, and *strA*, while those in *Salmonella* were *sul3*, bla_{TEM}, *cmlA*, and *tet(A)*. This finding agreed with a previous study that bla_{TEM} was highly prevalent in oysters (Brandão et al., 2017). *Sul3* is most prevalent of sulfonamides class, this result was supported by previous studies of *E. coli* isolated from human, pig, pork and livestock manures (Hammerum et al., 2006; Amador et al., 2019). Moreover,

the distribution of AMR genes in an estuarine environment can be found in different geographical locations (Zhang et al., 2015; Elbashir et al., 2018; Oliveira et al., 2020). This indicated the estuarine environment could be identified as a potential hotspot of resistant organisms that may be transferred resistant bacteria to humans.

In this study, the most common *Salmonella* serovar found were *S*. II (subsp. *salamae*), *S.* Paratyphi B, *S*. Eastbourne, and respectively. *S*. Paratyphi B was the most found in this study. *S.* Paratyphi B is one of the common causes of enteric fever in Asia but the incident still lower than *S.* Paratyphi A (Kim et al., 2019; Grace D. Appiah, 2020). A previous study indicates that *S.* Eastbourne has been detected in various samples, including fish and water samples. Low prevalence of Eastbourne was observed from fish (4.1%) and water samples (4.1%) (Traoré et al., 2015). Although S. subsp. *salamae* is more frequently reported in cold blood animals (Bauwens et al., 2006; Mhongole et al., 2017), such as aquatic turtles (Hidalgo-Vila et al., 2007), Nile perch (*Lates niloticus*) but *S.* subsp. *Salamae* can cause risk to people who are involved with these animals (Casalino et al., 2021), *S.* subsp. *salamae* occasionally be found in raw vegetables due to the contamination from soil and water (Quiroz-Santiago et al., 2009).

Salmonella Eastbourne is not a common serovar that causes salmonellosis in humans. However, this serovar was predominant in a clinically healthy reptile (32%) (Hydeskov et al., 2013). Although rare outbreak of serovar Eastborne was reported, the prevalence of this serovar was prevalent in the estuarine environment. Serovar Agona is one of the global concerns, and it is associated with human foodborne illness (Zhou et al., 2013). In this study, serovar Agona is 2.44% (n=3), which was previously also reported in oysters in the U.S. (Brands et al., 2005). Serovar Augustenburg can be contaminated in several types of samples. In Thailand, this serovar has been previously detected from broiler (Phongaran et al., 2019) and contaminated export fresh produce (Ontoum et al., 2012). Serovar

Braenderup was reported in the environment, including ground water and fertilizers.

Routes of typhoid and paratyphoid infection can occur in various ways, including fecal-oral transmission and contaminated water or food ingests. The emergence of AMR *S.* Typhi and *S.* Paratyphi has been increasing worldwide (Yan et al., 2016). Typhoid and paratyphoid fever are endemic diseases in Thailand and low-income countries. The trends of the disease have variation between 2003-2014. The incidence of typhoid fever declined since 2003, while the trends of paratyphoid were more stable (Techasaensiri et al., 2018). Even though the incidence of paratyphoid is significantly lower than typhoid fever, the large outbreak still occurs of serovars Paratyphi A, B, and C (Butler et al., 1978; Meltzer et al., 2014; Kariuki et al., 2015). The most common enteric fever is Paratyphi A, while Paratyphi B has a more comprehensive host range. Some strains of Paratyphi B cause enteric fever, whereas some of them can cause infection in other animals (Luby, 2014). Shiga toxin is a bacterial exotoxin that relates to highly cytotoxic to host cells. In this study, the *stx1* gene indicating Shiga toxin-producing *E. coli* (STEC) was the most frequently found in this strain collection, while the *eae* gene represents enteropathogenic *E. coli* (EPEC) was only reported in estuarine water at a low rate.

Interestingly, the *stx1* gene was prevalent in this study. Hence, this can raise significant public health concerns of seafood safety. It was speculated that wildlife and aquaculture have served as important sources of STEC spillover from livestock animals (Kim et al., 2019).

The *fimA*, *stn*, and *invA* genes are common virulence genes that play an important role in the pathogenicity of *Salmonella* infection. The *fimA* gene is a structural subunit of type 1 fimbrial protein, while the *stn* gene is heat-labile

Salmonella enterotoxin affecting epithelial cells (Kumar et al., 2009). The *invA* gene is a structural component of *Salmonella* pathogenicity islands, which is related to the invasion of gut epithelial cells in humans and animals (Kumar et al., 2009). This study indicated that 77.0% of *Salmonella* isolates were positive to *invA*, even though this gene has been used for *Salmonella* detection in food animals. However, our finding was supported by previous studies in poultry production (Sharma et al., 2017) and environmental samples (Patel et al., 2020). The absence of invA can occur in some salmonella isolates (Turki et al., 2012).

The variants of ESBL (i.e., SHV, TEM, and CTX-M, and carbapenemase) are common β-lactamases in *Enterobacteriaceae*, particularly in *E. coli* and *K. pneumoniae* (Sanjit Singh et al., 2017). In this study, the prevalence of ESBL-producing of *E. coli* and *Salmonella* was low compared with previous studies. The ESBL in wastewater and the aquatic environment was various from 7-49% (Ben Said et al., 2016). ESBL producing *E. coli* carried $bla_{\text{CTX-M}}$, and those carried $bla_{\text{TEM-1}}$, $bla_{\text{CTX-M-14,}}$ and $bla_{\text{CTX-M-15}}$ were reported in wild fish and marine bivalves, respectively (Sellera et al., 2018).

The association between resistance bacteria, ESBL and virulence genes was examined in this study. This finding showed the complexity of the relationship among phenotype and genotype of resistance and virulence genes of *E. coli* and *Salmonella* isolated from estuarine environment. In this study, *E. coli* resistance to chloramphenicol was positively associated with virulence genes, and S*almonella* resistance to cefpodoxime, cefotaxime, tetracycline, and trimethoprim was positively correlated with *fimA* and *InvA*, but negatively associated with *stn* gene. A quarter of *Salmonella* and most *E. coli* carrying virulence genes were MDR. The relationship between AMR and virulence genes is of public health concern regarding the spread and persistence of these resistance genes in pathogens. As a result, the simultaneous infection of virulence and resistance bacteria may cause

more complicated treatment and could increase morbidity and mortality due to failure treatment of bacterial infection.

The connection of AMR between humans, animals, and the environment is now of particular concern due to human and animal share environment. It is possible that humans can receive AMR from the environment. From our observation, the nearby communities that cultivated oysters were likely to be a potential source of *E. coli* and *Salmonella* contamination in the estuarine environment. Hence, coastal water was considered a potential hotspot of AMR in the environment (Leonard et al., 2015). The interface between humans, animals, and the environment can escalate the spreading of resistant organisms. Consequently, shellfish and estuarine water are carriers of resistant bacteria that can transfer resistance determinants and pose a potent public health risk.

CHAPTER VI CONCLUSION

In summary, the objectives of this study have been archived. The significant findings of this study are as follows:

1. The distribution of AMR, MDR, ESBL, and virulence genes in the estuarine environment has been obtained.
2. Resistant *E. coli* and *Salmonella* isolates were examined in estuarine environment and oysters.
3. The association between AMR and virulence genes was reported in this study.

The result can raise public health concerns of dissemination of AMR in the environment and the infection of *E. coli* and *Salmonella* is more likely to receive resistance genes and virulence genes.

Application

The data obtained from this study can be obtained as:

1. This finding provides the baseline information on AMR in the aquatic environment.
2. The occurrence of AMR in the estuarine environment can raise public health awareness of antimicrobial use in human and veterinary medicine.
3. Improvement of water quality in cultivated area is needed to decrease dissemination of AMR to seafood products and increase seafood safety.
4. The result of this study can be used as part of national AMR monitoring and surveillance in the environment under One Health.

5. The result of this study can be applied for further identification of potential AMR hotspots in the environment. Additionally, this could be guided for the implementation of prevention and control of AMR in the environment.

Recommendations

The findings emphasize the significant role of bacterial contamination in oysters and estuarine waters in AMR transmission between humans, animals, and the environment.

1. The spreading of AMR bacteria from households and agriculture is increasing public health concerns, so comprehensive methods to control and prevent the distribution of AMR are suggested.
2. At the farm level, shellfish should be appropriately harvested to avoid cross-contamination from workers and environment throughout the harvesting process. Decontamination processes such as shellfish purification are needed to reduce seafood contamination.
3. To avoid consuming AMR bacteria from contaminated food, oysters and shellfish should be fully cooked before consumption.

Further investigations

The information of AMR distribution that links humans, animals, and the environment is limited in Thailand, which is essential for the One Health approach. The action that needs to be taken for further investigation are as follows:

1. Monitoring and surveillance of AMR in various geographical distribution are needed to locate the AMR hotspot in the environment.
2. Tracing back sources of AMR contamination in the environment will assist to control and prevent AMR before spreading into the environment.

3. Further studies on resistance genes and other genetic determinants are needed to examine the association between genetic elements under One Health.

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APPENDIX

Appendix A Bacterial growth media and chemical agents

Bacterial growth media

1. Muller Hinton Agar (MHA) (Difco, MD, USA)
    a. Beef extract powder 2.0 g
    b. Acid digest casein 17.5 g
    c. Starch 1.5 g
    d. Agar 17.0 g
2. Luria-Bertani Agar (LB) (Difco, MD, USA)
    a. Tryptone 10.0 g
    b. Yeast Extract 5.0 g
    c. Sodium chloride 5.0 g
    d. Agar 15.0 g
3. Swam agar
    a. Brain Heart Infusion Agar (Difco, MD, USA) 19.0 g
    b. Tryptose 5.0 g
    c. Agar 7.5 g
    d. Distilled water 1,000 ml

Chemical agents

- Sodium chloride
- 50X TAE (Tris-Acetate Buffer)
- Agarose gel (Sigma-Aldrich®, Missouri, USA)
- TE buffer (Tris 10mM and EDTA 1 mM)
- NaOH (0.2M)
- DNA ladder (GeneRuler DNA Ladder, Thermo Scientific)
- Loading Dye (Tritrack DNA Loading Dye, Thermo Scientific)

Appendix B Solvent, concentration range and clinical breakpoint of antimicrobial agents

Antimicrobial agents	Solvent	Concentration range (µg/ml)	Clinical breakpoint (µg/ml)
Ampicillin	Distilled water	0.125-256	32 µg/ml
Chloramphenicol	95% ethanol	0.125-256	32 µg/ml
Ciprofloxacin	0.1 N NaOH	0.00195-64	4 µg/ml
Gentamicin	Distilled water	0.25-128	8 µg/ml
Streptomycin	Distilled water	0.5-256	32 µg/ml
Sulfamethoxazole	1 N NaOH	0.5-2048	512 µg/ml
Tetracycline	70% ethanol	0.0625-256	16 µg/ml
Trimethoprim	Dimethylacetamide	0.25-256	16 µg/ml

Appendix C PCR conditions for AMR detection in E. coli and Salmonella

The AMR genes of *E. coli* and *Salmonella* isolates were examined in this study with the following thermocycler conditions.

The polymerase chain reactions (PCR) condition for bla_{TEM} involved initial denaturation at 94 ℃ for 15 min, followed by 30 cycles of 94 ℃ for 60 sec, 55 ℃ for 60 sec and 72 ℃ for 60 sec. A final extension of 72 ℃ for 10 min was employed (Khan et al., 2019).

A conventional PCR was used to detect *catA* and *catB (*Chuanchuen et al., 2008b). The PCR condition were initial denaturation at 94 ℃ for 5 min, followed by 30 cycles of 95 ℃ for 45 sec, 55 ℃ for 45 sec, and 72 ℃ for 10 min. The final extension of 72 ℃ for 10 min (Chuanchuen et al., 2008b).

The PCR condition for *cmlA* detection was initial denaturation at 94 ℃ for 15 min, followed by 30 cycles of 94 ℃ for 60 sec, 57 ℃ for 3 min and 72 ℃ for 5 min, and followed by final extension of 72 ℃ for 10 min (Chuanchuen et al., 2008a)

Multiplex PCR were performed to examine *qnrA*, *qnrB*, and *qnrS.* The condition was denaturation at 95 ℃ for 10 min, followed by 35 cycles of 95 ℃ for 60 sec, 54 ℃ for 60 sec, and 72 ℃ for 60 sec. A final extension of 72 ℃ for 10 min was employed (Cattoir et al., 2007).

The PCR condition for *aac(3)IV* was initial denaturation at 95 ℃ for 3 min, followed by 35 cycles of 94 ℃ for 30 sec, 55 ℃ for 30 sec, and 72 ℃ for 60 sec. A final extension of 72 ℃ for 10 min was used (Stoll et al., 2012). For *aadA1* and *strA* the conditions were followed to previous study of Chuanchuen et al., 2008c (Chuanchuen et al., 2008b).

The PCR condition for *strB* was initial denaturation at 95 ℃ for 15 min. followed by 35 cycles of 94 ℃ for 30 sec, 57 ℃ for 1.5 min and 72 ℃ for 1.5 min. and followed by a final extension of 72 ℃ for 10 min (Mala et al., 2016).

PCR condition for *tet*(A) was initial denaturation at 95 ℃ for 5 min, followed by 30 cycles of 94 ℃ for 45 sec, 63 ℃ for 60 sec, and 72 ℃ for 10 min. A final extension was 72 ℃ for 10 min (Khan et al., 2019). For *tet*(B) were according to (Chuanchuen et al., 2008b).

The condition of multiplex PCR for *sul1, sul2,* and *sul3* were initial denaturation at 95℃ for 10 min, followed by 30 cycles of 95℃ for 60 sec, 66℃ for 60 sec, and 72℃ for 10 min, and followed by a final extension of 72℃ for 10 min (Khan et al., 2019).

PCR condition for *dfrA1* involved were initial denaturation at 94℃ for 8 min. followed by 32 cycles of 95℃ for 60 sec, 55℃ for 70 sec and 72℃ for 10 min. and followed by final extension of 72℃ for 10 min (Shahrani et al., 2014). and for examine *dfrA12*, PCR condition were set according to (Chuanchuen et al., 2008b).

Appendix D PCR conditions for ESBL detection in E. coli and Salmonella

Multiplex PCR of *bla*$_{TEM}$, *bla*$_{SHV}$, and *bla*$_{CMY-2}$ included initial denaturation at 94℃ for 15 min, followed by 30 cycles of 94 ℃ for 60 sec, 55 ℃ for 60 sec, and 72 ℃ for 60 sec. A final extension of 72 ℃ for 10 min was employed (Khan et al., 2019). For *bla*$_{CTX-M}$, the initial denaturation was at 95℃ for 5 min was performed, followed by 30 cycles of 94 ℃ for 60 sec, 60 ℃ for 60 sec, and 72 ℃ for 60 sec with a final extension of 72 ℃ for 10 min (Batchelor et al., 2005).

Appendix E PCR conditions for virulence genes detection in E. coli and Salmonella

The PCR condition for *lt* and *st* was involved initial denaturation at 95 ℃ for 5 min, followed by 30 cycles of 95 ℃ for 60 sec, 55 ℃ for 60 sec, and 72 ℃ for 60 sec. For final extension, 72 ℃ for 10 min was used (Hinthong et al., 2017). The PCR condition for *stx1* and *stx2* included initial denaturation at 94 ℃ for 5 min, followed by 35 cycles of 94 ℃ for 60 sec, 55 ℃ for 60 sec, and 72 ℃ for 60 sec. A final extension of 72 ℃ for 10 min was employed (Khan et al., 2002).

For *eae* gene detection. The PCR conditions were initial denaturation at 95 ℃ for 5 min, followed by 30 cycles of 95 ℃ for 60 sec, 55 ℃ for 60 sec, and 72 ℃ for 60 sec, and a final extension of 72 ℃ for 10 min (Toma et al., 2003).

To detect the presence of *Salmonella* virulence genes (*fimA*, *stn,* and *invA*). Conventional PCR conditions were involved initial denaturation at 95 ℃ for 2 min, followed by 35 cycles of 95 ℃ for 30 sec, 55 ℃ for 30 sec (the annealing temperature for *invA* was set at 58 ℃) and 72 ℃ for 60 sec followed, by a final extension of 72 ℃ for 5 min (Kumar et al., 2009).

VITA

NAME	Mullika Kuldee
DATE OF BIRTH	19 September 1993
PLACE OF BIRTH	Chiang Rai, Thailand
INSTITUTIONS ATTENDED	The degree of Veterinary Sciences (D.V.M.)