Prevalence and molecular characteristics of antimicrobial resistance of *Aeromonas hydrophila*, *Salmonella* spp., *Vibrio cholerae*, fecal coliform, and *Escherichia coli* in hybrid red tilapia and cultured water

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A Dissertation Submitted in Partial Fulfillment of the Requirements
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ความชุกและลักษณะทางชีวโมเลกุลของการดื้อยาต้านจุลชีพของ *Aeromonas hydrophila Salmonella* spp. *Vibrio cholerae* fecal coliform และ *Escherichia coli* ในปลานิลแดงและน้ำที่ใช้ในการเพาะเลี้ยง

น.ส.วรางคณา เถาธรรมพิทักษ์

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
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Thesis Title	Prevalence and molecular characteristics of antimicrobial resistance of *Aeromonas hydrophila*, *Salmonella* spp., *Vibrio cholerae*, fecal coliform, and *Escherichia coli* in hybrid red tilapia and cultured water
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วรางคณา เถาธรรมพิทักษ์ : ความชุกและลักษณะทางชีวโมเลกุลของการดื้อยาต้านจุลชีพของ*Aeromonas hydrophila Salmonella* spp. *Vibrio cholerae* fecal coliform และ*Escherichia coli* ในปลานิลแดงและน้ำที่ใช้ในการเพาะเลี้ยง. (Prevalence and molecular characteristics of antimicrobial resistance of *Aeromonas hydrophila*, *Salmonella* spp., *Vibrio cholerae*, fecal coliform, and *Escherichia coli* in hybrid red tilapia and cultured water) อ.ที่ปรึกษาหลัก : ผศ. ดร.สหฤทัย เจียมศรีพงษ์

การปนเปื้อนของจุลชีพก่อโรคในอาหารและแบคทีเรียดื้อยาต้านจุลชีพในปลานิลและแหล่งน้ำกลายเป็นปัญหาสำคัญทางสาธารณสุข การศึกษานี้มีวัตถุประสงค์เพื่อตรวจหาความชุกของแบคทีเรียบ่งชี้และแบคทีเรียก่อโรคในปลานิลแดง (*Oreochromis* spp.) และน้ำที่ใช้ในการเลี้ยงปลา และตรวจหาลักษณะปรากฏและลักษณะทางพันธุกรรมของการดื้อยาต้านจุลชีพ ยีนก่อโรคและการสร้างเอนไซม์บีตา-แลคทาเมสชนิดขยายในเอสเชอริเชีย โคไล แอโรโมนาส ไฮโดรฟิลลา ซัลโมเนลลาและวิบริโอ คลอเรลลา จำนวนตัวอย่างปลาทั้งหมด 120 ตัวอย่าง ประกอบด้วยน้ำล้างผิวปลา 120 ตัวอย่าง เนื้อปลา 120 ตัวอย่าง ไส้ปลา 120 ตัวอย่าง ตับและไต 120 ตัวอย่างและน้ำที่ใช้ในการเลี้ยงปลา 120 ตัวอย่าง โดยเก็บจากฟาร์มปลานิลในจังหวัดกาญจนบุรีในประเทศไทย ในเดือนตุลาคม พ.ศ. 2562 ถึงเดือนพฤศจิกายน พ.ศ. 2563 โดยรวมความชุกของฟีคัลโคลิฟอร์มและอี โคไลมีค่าเท่ากับ 74.8% และ 56.7% ตามลำดับ พบปริมาณฟีคัลโคลิฟอร์ม ($2.4 \pm 4.0 \times 10^4$ MPN ต่อกรัม) และอี โคไล ($1.2 \pm 2.9 \times 10^4$ MPN ต่อกรัม) สูงสุดพบในไส้ปลา แอโรโมนาส ไฮโดรฟิลลา ซัลโมเนลลาและวิบริโอ คลอเรลลา มีความชุกเท่ากับ 2.5% 32.0% และ 17.5% ตามลำดับ ตรวจพบแอโรโมนาส ไฮโดรฟิลลาเฉพาะน้ำล้างผิวปลาและน้ำที่ใช้ในการเลี้ยงปลา ในขณะที่ซัลโมเนลลาและวิบริโอ คลอเรลลาส่วนใหญ่ พบในน้ำที่ใช้ในการเลี้ยงปลา การพบซัลโมเนลลาในตัวอย่างเกี่ยวข้องกับการปนเปื้อนของฟีคัลโคลิฟอร์ม อี โคไล วิบริโอ คลอเรลลา ความชื้นสัมพัทธ์และลมกรรโชก ซีโรวาร์ของซัลโมเนลลาที่พบมากที่สุดคือ Saintpaul (18.9%) Neukoelln (15.2%) และ Escanaba (15.2%) เป็ดที่เลี้ยงใกล้ฟาร์มปลานิลอาจเป็นแหล่งของซัลโมเนลลาปนเปื้อนในฟาร์มปลา จากการวิเคราะห์ด้วย rep-PCR อี โคไลทั้งหมดตรวจไม่พบ *stx1* และ *stx2* แอโรโมนาส ไฮโดรฟิลลาทุกไอโซเลตให้ผลบวกกับ *aero* และ *hly* ซัลโมเนลลาทุกไอโซเลตตรวจพบ *invA* วิบริโอ คลอเรลลาทั้งหมดเป็นกลุ่ม non-O1/non-O139 แอโรโมนาส ไฮโดรฟิลลา (100%) ซัลโมเนลลา (100%) และ อี โคไล (79.6%) พบการดื้อยาอย่างน้อย 1 ชนิด ซัลโมเนลลา (72.3%) อี โคไล (53.8%) และแอโรโมนาส ไฮโดรฟิลลา (26.7%) พบการดื้อยาต้านจุลชีพหลายชนิด วิบริโอ คลอเรลลาทั้งหมดมีความไวรับต่อยาต้านจุลชีพทั้งหมดที่ทำการทดสอบ อี โคไลส่วนใหญ่ดื้อยา ampicillin (63.1%) oxytetracycline (58.6%) และ tetracycline (58.0%) ยีนที่พบมากในอี โคไล คือ bla_{TEM} (58.0%) *qnrS* (43.8%) และ *tetA* (29.1%) การศึกษาครั้งนี้พบ bla_{TEM-1} และ $bla_{CTX-M-55}$ แอโรโมนาส ไฮโดรฟิลลาพบการดื้อยา ampicillin (100%) oxytetracycline (26.7%) tetracycline (26.7%) และ trimethoprim (26.7%) ยีนดื้อยาที่พบสูงสุดในแอโรโมนาส ไฮโดรฟิลลา คือ *mcr-3* (20.0%) ตามด้วย *floR qnrS sul1 sul2* และ *dfrA1* ในอัตราการดื้อยาที่เท่ากันคือ 13.3% ซัลโมเนลลาส่วนใหญ่ดื้อต่อ ampicillin (79.3%) oxolinic acid (75.5%) และ oxytetracycline (71.8%) ยีนส่วนใหญ่ที่พบในซัลโมเนลลาคือ *qnrS* (65.4%) *tetA* (64.9%) และ bla_{TEM} (63.8%) วิบริโอ คลอเรลลาส่วนใหญ่พบยีน *sul1* (12.0%) ตามด้วย *catB qnrS tetA tetB strA* และ *dfrA1* ในอัตราการดื้อยาที่เท่ากันคือ 4.0% Class 1 integron ตรวจพบเฉพาะในอี โคไล (19.5%) และแอโรโมนาส ไฮโดรฟิลลา (6.7%) การสร้างเอนไซม์บีตา-แลคทาเมสชนิดขยาย พบเฉพาะในอี โคไล (3.9%) การศึกษาครั้งนี้พบการเกิดขึ้นของยีนดื้อยาโคลิสตินในปลานิลในประเทศไทย ปลานิลและน้ำที่ใช้ในการเลี้ยงปลาเป็นแหล่งกักเก็บของจุลชีพก่อโรคในอาหารและแบคทีเรียดื้อยาต้านจุลชีพที่สำคัญ การทำความสะอาดปลาที่เหมาะสม การมีสุขลักษณะที่ดีและการปรุงปลาให้สุก ทำให้การบริโภคปลานิลมีความปลอดภัย การส่งเสริมแนวคิดสุขภาพหนึ่งเดียวจะช่วยควบคุมและป้องกันการกระจายของเชื้อก่อโรคและเชื้อดื้อยาจากปลานิลไปสู่คนและสิ่งแวดล้อมได้อย่างมีประสิทธิภาพ

สาขาวิชา	สัตวแพทยสาธารณสุข	ลายมือชื่อนิสิต .....
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Varangkana Thaotumpitak : Prevalence and molecular characteristics of antimicrobial resistance of *Aeromonas hydrophila*, *Salmonella* spp., *Vibrio cholerae*, fecal coliform, and *Escherichia coli* in hybrid red tilapia and cultured water. Advisor: Asst. Prof. SAHARUETAI JEAMSRIPONG, D.V.M., M.P.V.M., Ph.D.

Foodborne pathogens and antimicrobial resistant bacteria in tilapia and cultivation water has been emerged as a public health threat. This study aimed to determine the prevalence of indicator and pathogenic bacteria in hybrid red tilapia (*Oreochromis* spp.) and their cultivation water, and to characterize phenotypic and genotypic AMR, virulence genes, and extended-spectrum beta-lactamase (ESBL) production of *Escherichia coli*, *Aeromonas hydrophila*, *Salmonella* spp., and *Vibrio cholerae.* A total of 120 tilapia, which were comprised of carcass rinse (n=120), muscle (n=120), intestine (n=120), liver and kidney (n=120), and cultivation water (n=120) were collected from tilapia farms in Kanchanaburi province, Thailand during October 2019 and November 2020. The overall prevalence of fecal coliforms (74.8%) and *E. coli* (56.7%) were observed. The highest concentration of fecal coliforms ($2.4 \pm 4.0 \times 10^4$ MPN/g) and *E. coli* ($1.2 \pm 2.9 \times 10^4$ MPN/g) were mainly found in fish intestine. The prevalence of *A. hydrophila*, *Salmonella*, and *V. cholerae* were 2.5%, 32.0%, and 17.5%, respectively. *A. hydrophila* was only detected in carcass rinse and cultivation water, while *Salmonella* and *V. cholerae* were most detected in cultivation water. The detection of *Salmonella* was associated with fecal coliforms, *E. coli*, *V. cholerae*, relative humidity, and wind gust. The most common *Salmonella* serovars were Saintpaul (18.9%), Neukoelln (15.2%), and Escanaba (15.2%). Ducks reared nearby in the tilapia farm were postulated that they may be the source of *Salmonella* contamination in tilapia farms based on rep-PCR characterization. All *E. coli* isolates were absent of *stx1* and *stx2*. All *A. hydrophila* isolates were positive for *aero* and *hly*. All *Salmonella* isolates were *invA* positive. All of *V. cholerae* isolates classified as non-O1/non-O139. The *A. hydrophila* (100%), *Salmonella* (100%), and *E. coli* (79.6%) isolates were resistance to at least one antimicrobial. The *Salmonella* (72.3%), *E. coli* (53.8%), and *A. hydrophila* (26.7%) were multidrug resistance. All *V. cholerae* isolates were susceptible to all tested antimicrobials. The predominant resistance in *E. coli* were ampicillin (63.1%), oxytetracycline (58.6%), and tetracycline (58.0%). The bla_{TEM} (58.0%), *qnrS* (43.8%), and *tetA* (29.1%) were the common resistance genes of *E. coli*. The bla_{TEM-1} and $bla_{CTX-M-55}$ were reported in this study. The *A. hydrophila* isolates was resistant to ampicillin (100%), oxytetracycline (26.7%), tetracycline (26.7%), and trimethoprim (26.7%). The *A. hydrophila* isolates were commonly found *mcr-3* (20.0%), followed by *floR*, *qnrS*, *sul1*, *sul2*, and *dfrA1* with the same resistance rates at 13.3%. The *Salmonella* isolates highly resisted to ampicillin (79.3%), oxolinic acid (75.5%), and oxytetracycline (71.8%). The *qnrS* (65.4%), *tetA* (64.9%), and bla_{TEM} (63.8%) were predominant genes found in the *Salmonella* isolates. The *V. cholerae* isolates were mainly carried *sul1* (12.0%), followed by *catB*, *qnrS*, *tetA*, *tetB*, *strA*, and *dfrA1* with the same resistance rate at 4.0%. Class 1 integron was only examined in *E. coli* (19.5%) and *A. hydrophila* (6.7%). For ESBL-producing *E. coli* (3.9%) was detected. This study reported the emerging of colistin resistance gene (*mcr-3*) in tilapia in Thailand. In summary, tilapia and cultivation water are the potential reservoirs of important foodborne pathogens and AMR bacteria. Proper handling, personal hygiene, and fully cooked fish can promote food safety regarding tilapia consumption. To reduce pathogens and resistant bacterial transmission from tilapia to humans and the environment, implementation of One Health should be carried out with effective control and prevention of the dissemination of resistant bacteria.

Field of Study:	Veterinary Public Health	Student's Signature .....
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AIC	Akaike Information Criterion
AMR	antimicrobial resistance
AST	antimicrobial susceptibility test
bp	base pair (s)
°C	degree Celsius
CFU	colony forming unit
CI	confidence interval
cm	centimeter (s)
DNA	deoxyribonucleic acid (s)
DO	dissolved oxygen
ESBL	extended-Spectrum ß-Lactamases
et al.	at alii and others
g	gram (s)
GAP	good agricultural practices
hr	hour (s)
i.e.	id est or that is
L	liter (s)
MAS	motile *Aeromonas* septicemia
MDR	multidrug resistance
MIC	minimum inhibitory concentration
min	minute (s)
ml	milliliter (s)
µM	micromole (s)
MPN	most probable number
PCR	polymerase chain reaction

PFGE	pulsed field gel electrophoresis
pH	potential hydrogen
PMQR	plasmid-mediated quinolone resistance
ppt	part per thousand
RH	relative humidity
m	meter (s)
mm	millimeter (s)
MT	million ton (s)
QRDR	quinolone resistant-determining region
rep-PCR	repetitive sequence-based PCR
s	second (s)
SE	standard error
SD	standard deviation
µg	microgram (s)
µL	microliter (s)
WGS	whole genome sequencing

CHAPTER I

1.1 Importance and Rationale

The demand of protein sources has been growing due to an increase of human population. Aquatic products are one of the main protein sources. Fish is the most significant aquatic products due to high nutritive value, and relatively inexpensive source of animal protein. In 2019, the production of fish was approximately 178 million tons (FAO, 2021). Tilapia (*Oreochromis* spp.) is freshwater fish that widely cultured and consumed globally, because it is easy to culture, fast-growing, and tolerant to low water quality. In Thailand, tilapia production has been over 200,000 tons annually (FAO, 2021), and it has become increasingly due to high domestic tilapia consumption.

Tilapia farming in Thailand has developed in recent decades. The traditional fish production, such as pond culture or small-scale farming has been changed to intensive production system. High stocking density of fish cultivation can generate poor water quality, and deposit of organic matter. Heavy bacterial accumulation in tilapia and environment can resulted in increased risk of bacterial infection in humans. In addition, effluents from household, agriculture, and industry can be the source of bacteria that can potentially go through the natural water resource. Environmental factors, including weather and water parameters are driving factors of increasing bacterial accumulation in the environment.

Pathogenic bacteria contaminated in fish may transmit to human through tilapia consumption or direct contact. The important bacterial pathogens found in farmed tilapia are *Aeromonas hydrophila, Streptococcus* spp*., Edwardsiella tarda, Proteus mirabilis, Klebsiella pneumoniae*, and *Vibrio cholerae* (Halpern and Izhaki, 2017; Lee and Wendy, 2017; Thongkao and Sudjaroen, 2017). Fresh-marketed tilapia,

frozen tilapia, and other tilapia products (such as sashimi, and fillets) have been reported of *Salmonella* spp., *Shigella* spp., and *Staphylococcus aureus* contamination (Elhadi, 2014). Freshwater aquaculture is one of the major sources of pathogenic bacteria accumulation.

Antimicrobial resistance (AMR) is one of the serious threats to global human health. The prolonged use of antimicrobial agents can promote the selection of AMR on bacterial population and develop multidrug resistance (MDR) bacteria. The infection of MDR bacteria have been greatly concerned in public health and contributed to high cost of treatment due to prolong hospital stay and required more potent antimicrobials. A few antimicrobials such as oxolinic acid, oxytetracycline, and sulfonamide compounds have been licensed to use for bacterial infection in fish farming (OIE, 2019). However, improper use of antimicrobial agents is one of the major factors contributing to AMR distribution in aquatic environment. In addition, contaminated effluents discharged to water bodies are a potential source of AMR bacteria distributed in aquaculture. Continuous exposure of indigenous bacteria with high loads of AMR genes can enhance horizontal gene transfer and develop novel AMR bacteria. Thus, fish and aquatic environment can be a hotspot of AMR bacteria. Surveillance and monitoring of AMR in fish and aquatic environment is needed to strengthen multi-collaborative approach to tackle with AMR according to the One Health. Furthermore, the study of bacterial contamination regarding food safety and phenotypic and genotypic distribution of AMR bacteria in fish is limited. Therefore, this study will provide background information of bacterial distribution and AMR bacteria circulation in tilapia and cultivation water.

1.2 Literature Review

1.2.1 Production of tilapia in Thailand

Tilapia is an omnivorous freshwater fish, which is native in Africa. It is introduced to many countries for aquaculture due to rapid growth, palatability, and tolerance with environmental variation. It was estimated that global tilapia production is 4.6 million tons in 2019 (FAO, 2021). China, Indonesia, Egypt, the Philippines, and Thailand are the main producers of tilapia products in the world (FAO, 2021). The production of tilapia in Thailand was over 200,000 tons per year during 2012-2017 (Ferreira et al., 2015) Most of the tilapia produce in Thailand serves for domestic consumption.

The most common methods used to rear tilapia in Thailand are both well-typed fishpond and floating basket in natural water resources. The culturing tilapia in ponds is mostly distributed in the central plain around Bangkok vicinity, such as Pathum Thani, Chainat, and Ayutthaya, while tilapia reared by floating basket is mostly concentrated at the river basin in western Thailand, such as Kanchanaburi, and Suphanburi. The Kwae Noi river in Kanchanaburi is impounded by the dam resulting in water flowing all year round creating the suitable site for tilapia cultivation. This location is considered as one of the hotspots of red tilapia culture using floating baskets. Although these production sites have good water quality, it can be potentially contaminated with bacteria originated from communities, tourist activities, irrigation, and fishing.

1.2.2 Distribution of important bacteria in fish and aquatic environment

Freshwater environment enriches with abundance of bacteria. Regarding the public health concerns, there are two types of bacteria in the freshwater environment classified by bacterial sources. The first group is the bacteria, which naturally habit in the freshwater environment, including *Aeromonas* spp.,

V. cholerae, and *Streptococus agalactiae* (Janda and Abbott, 2010; Senderovich et al., 2010). The second group is exotic bacteria, which are commonly introduced to aquaculture from anthropogenic wastes, such as *Salmonella*, and *Escherichia coli* (Novoslavskij et al., 2016).

Important zoonotic bacteria found in fish and freshwater aquatic systems, such as *A. hydrophila, Salmonella* spp., *S. agalactiae* can pose a serious health risk in humans (Awuor et al., 2011; Lee and Wendy, 2017). Zoonotic bacteria can survive in fish and cultivation water in aquaculture, so bacterial contamination in tilapia and aquatic environment are linked. The association between bacterial communities in fish and the surroundings have been studied. The high prevalence of bacteria can be found in gill and intestine in fish, because fish commonly ingest food from surrounding water (Rocha et al., 2014). The bacteria can also be abundant in fish surface due to continuous exposure with surrounding water. Therefore, many parts of fish can harbor bacteria from the environment. The detection of bacteria in fish meat may originate from post-harvest contamination, such as during fish preparation and handing (Mandal et al., 2009; Rocha et al., 2014).

1.2.3 Environmental parameters associated with distribution of bacteria in fish and production site

A fish spends its entire life in aquatic environment that can lead to high exposure of bacterial contaminants. Environmental parameters become key factors for aquaculture production, since they affected on water quality of cultivation water and fish health status. To better understand of the epidemiology of the bacterial distribution in fish, weather and water parameters should be evaluated.

The basic meteorological components of weather are air temperature, humidity, rainfall, precipitation, heat index, and wind. One of the most important

factors affecting on bacterial distribution in production area is rainfall (Hoa et al., 2011). The rain can drive a massive influx of water that may contain microbial and AMR contaminants from land into water bodies of aquaculture area. Consequently, the fish can harbor many bacteria from cultivation water. It is proved that strong sunlight during high ambient air temperature can inactivate bacteria in the water (Chandran and Mohamed Hatha, 2005). Therefore, ambient air temperature is negatively correlated with presence of bacteria in fish. Other factors such as wind speed and heat index are limited observation on their roles on bacterial distribution in fish.

The common physicochemical properties of water have been used to measure in freshwater fish aquaculture are water temperature, dissolved oxygen (DO), pH, salinity, hardness, and levels of ammonia and nitrite. Water temperature is the main parameter affecting on the survival of bacteria in aquaculture. The water temperature positively correlated with the presence of mesophilic bacteria, such as *Salmonella* and *E. coli* (Gorlach-Lira et al., 2013), while negatively associated with the presence of psychotropic bacteria, such as *A. hydrophila* and *Vibrio* spp. (Ismail et al., 2016). DO is the major parameter that associated with water temperature, turbidity, and the presence of bacteria and plankton (Abdullah et al., 2017). The levels of DO of water is temperature dependent. When water temperature increases, the concentration of DO in water decreases. Therefore, DO has been used to monitor the water quality and bacterial pollution level. High levels of ammonia and nitrite indicate poor water quality, and they are positively correlated with the presence of bacteria in fish (Ismail et al., 2016; Abdullah et al., 2017).

1.2.4 Distribution of bacteria in tilapia aquaculture

Fecal coliform and *E. coli*

Fecal coliform is a Gram-negative, facultative anaerobe, and rod bacterium that is naturally found in intestinal tracts of warm-blooded animals and human. This bacterium is considered as a definitive bacterial indicator for fecal contamination. *E. coli* is a subset of fecal coliform. It is an indicator bacterium that normally lives in gastrointestinal tract of warm blood animals and humans. Transmission of *E. coli* occurs by consumption of contaminated food and water. Majority of *E. coli* are considered as non-pathogenic bacteria, except some strains such as shiga toxin- producing *E. coli* (i.e., STEC O157:H7). People infected with STEC O157:H7 usually show clinical signs within 3-4 days post-infection with clinical presentation of hemorrhagic colitis, hemolytic uremic syndrome, and fatal in immunocompromised persons.

Both of fecal coliform and *E. coli* are not normal flora in fish. Detection of these bacteria in tilapia refers to fecal contamination from cultivation water. High levels of fecal coliforms and *E. coli* in fish can suggest poor water quality in the production area. The contamination of *E. coli* in fish is assumed a higher risk of pathogenic bacteria contamination than those fish did not contamination (Ava et al., 2020). Among different types of tilapia sample, including meat, intestine, and gill, the highest concentration of fecal coliform was found in gill ($3.0 \pm 0.67 \times 10^3$ CFU/g), whereas the highest concentration of *E. coli* was found in intestine ($1.45 \pm 0.19 \times 10^3$ CFU/g) of tilapia area (Mandal et al., 2009). Previous study indicated that gill, meat, and intestine of tilapia sold in the market had higher concentrations of fecal coliform and *E. coli* than those were directly collected from production area (Mandal et al., 2009). This indicates that potential fecal contamination in fish usually occurs during storage and handling.

A. hydrophila

A. hydrophila is a Gram-negative, and facultative anaerobic bacteria belonging to the family of *Aeromonadaceae*. It is ubiquitous in freshwater habitats, ground water, and effluents. This pathogen can cause diseases in wide range of host, such as fish, amphibians, reptiles, and humans. In fish, the infection of *A. hydrophila* causes exophthalmos, fin and tail rot, and epizootic ulcerative syndrome (EUS), and this bacterium is associated with fish disease outbreaks causing high mortality (Rasmussen-Ivey et al., 2016). High stock density, organic material composition, and poor water quality are important predisposing factors for *A. hydrophila* infection in fish (Bebak et al., 2015).

A. hydrophila is a predominant *Aeromonas* species in humans that is associated with foodborne and waterborne illnesses (Zhang et al., 2012). Main sources of *A. hydrophila* are reported in freshwater fish, ready-to-eat fish, water, vegetables, milk, and meat products (Zhang et al., 2012; Abd-El-Malek, 2017). Transmission routes of *A. hydrophila* are consumption of contaminated food and direct contact with contaminated materials or environment. Infection through consumption of contaminated food is the major route of disease transmission causing gastroenteritis, while direct contact with contaminated materials causes extraintestinal infections, including wound infections, cellulitis, and septicemia. Incubation period of *A. hydrophila* infection is 12-48 hr and may be prolonged for days for extraintestinal infections. *A. hydrophila* can harbor various virulence factors encoding adherence proteins, catalyst enzymes, and toxins. An aerolysin (*aero)* gene is highly responsible for *A. hydrophila* colonization and severity of infection. Previous studies showed that the prevalence of *A. hydrophila* in fish from market ranged from 2.7% to 36.0% (Abd-El-Malek, 2017; Ahmed et al., 2018).

Salmonella

Salmonella is a Gram-negative, non-spore forming, and facultative anaerobe bacillus belonging to family *Enterobacteriaceae*. Major routes of disease transmission are fecal-oral route and direct contact with contaminated animals or environment. *Salmonella* can cause serious illnesses in humans, such as acute gastroenteritis, enteric fever, and bacteremia. The incubation period is usually 6-72 hr *Salmonella* spp. consists of two species, including *S. enterica* and *S. bongori. S. enterica* is frequently associated with warm-blooded animals, while *S. bongori* can be found in cold-blooded animals. Virulence genes of *Salmonella* present in pathogenicity islands, chromosome, and plasmid. Virulence genes, such as *invA, tolC, spvC*, and *pefA*, are major virulence genes associated with severity of *Salmonella* infection. More than 2,500 serovars of *Salmonella* can be classified by agglutination with specific antisera to identify the somatic (O) and flagella (H) antigens.

The prevalence of *Salmonella* in tilapia ranged from 30% to 64% (Budiati et al., 2013; Elhadi, 2014; Li et al., 2017), which vary depending on type of sample and sampling location. The highest prevalence of *Salmonella* in tilapia was found in intestine (Li et al., 2017). Even though *Salmonella* can cause illnesses in humans, it does not affect fish health. *S. enterica* serovar Typhimurium, *S.* Agona, *S.* Bovismorbificans, *S.* Covallis, *S.* Enteritidis, *S.* Typhi, *S.* Weltevreden, and *S.* Stanley were commonly isolated from tilapia (Budiati et al., 2013; Li et al., 2017), and these serovars were closely associated with human cases (Hassan et al., 2018). According to the limit of microbiological reference criteria of *Salmonella*, they must not be detected in 25 g of raw freeze fish products (DMSC, 2017).

V. cholerae

V. cholerae is a member of the *Vibrionaceae.* This bacterium is a Gram-negative, facultative anaerobic, non-spore forming, and motile rod. It is naturally inhabitant in freshwater, brackish water, and seawater. *V. cholerae* can be classified by lipopolysaccharide component on the cell wall (O antigen). Among hundreds of O serogroups, only two serogroups of *V. cholerae*, O1 and O139, have been associated with human gastroenteritis and implicated with epidemics of cholera outbreaks.

The major route of *V. cholerae* transmission is fecal-oral route with typical incubation period ranging from 6-72 hr When ingested, this bacterium adheres with epithelium of small intestine and secretes cholera enterotoxin causing massive water and electrolyte secretion into lumen of intestine. Clinical signs are severe watery diarrhea, dehydration, and hypovolemic shock. Life-threatening can be occurred with inappropriate water and electrolyte supplements. Three important virulence genes contribute to pathogenicity of *V. cholerae*, including cholera enterotoxin (*ctx*), toxin-coregulated pilus (*tcpA*), and hemolysin A (*hlyA*). The *ctx* gene controls the production of cholera toxin, *tcpA* gene promotes fimbriae synthesis to attach with intestinal cells of host, and *hlyA* gene regulates hemolysis production causing cell lysis (Hounmanou et al., 2016).

The presence of *V. cholerae* in tilapia and water ranged from 3.8% to 6.0% (Traoré et al., 2014; Li et al., 2019). The occurrence of *V. cholerae* were reported in many aquatic animals, predominantly in freshwater fish rather than marine fish (Senderovich et al., 2010). *V. cholerae* can be detected in copepods (*Crustacea*) or chironomids (*Diptera; Chironomidae*), which are abundant metazoa in aquatic environment (Raz et al., 2010). Majority of fish containing *V. cholerae* are healthy.

A recent hypothesis proposed that fish contained *V. cholerae* through ingestion of these metazoan, and *V. cholerae* live in fish with mutually benefits (Halpern and Izhaki, 2017).

1.2.5 AMU used in aquaculture

Intensification of aquaculture contributes to the widespread of multiple pathogens and decreases of fish immune leading to susceptible to infections (Dong et al., 2015). Various diseases in aquatic animals causes massive mortality and economic loss. To prevent the loss of fish production, the antimicrobial agents have been used for therapeutic and prophylactic purposes.

Of the Office International des Epizooties (OIE) lists for important antimicrobial agents in veterinary medicine, 32 antimicrobial agents have been used in fish (OIE, 2019). Five new antimicrobial agents, including two macrolides (kitasamycin and mirosamycin), one of the second generation of fluoroquinolones (sarafloxacin), and two sulfonamides (sulfamerazine and combined ormetoprim and (sulfadimethoxine) were added to the previous list from OIE. In Thailand, seven single antimicrobial agents, including amoxicillin, enrofloxacin, oxytetracycline, sarafloxacin, oxolinic acid, toltrazuril, sulfamonomethoxine sodium, and five combinations of sulfonamides have been licensed by the Thai Food and Drug Administration (FDA) for aquaculture. In tilapia culture, the most commonly used antimicrobial agents are enrofloxacin and oxytetracycline. These antimicrobials are frequently used to treat fish infection associated with *Aeromonas* spp.

1.2.6 AMR in fish and aquatic environment

AMR problems are emerging crisis to global population. AMR bacteria cause more than 2.8 million cases each year in the United States (CDC, 2019), and the cost of healthcare associated with AMR infection is approximately 20 billion dollars per year (Golkar et al., 2014). In Thailand, infection associated with AMR bacteria was over

80,000 human cases with 38,000 deaths (Pumart et al., 2012). The AMR-infected cases required last-line antimicrobials, prolonged stay, and intensive care. In Thailand, more than three million extra hospital days per year is needed for treatment of AMR-associated infection (Pumart et al., 2012).

AMR bacteria are introduced into natural water from effluents discharged from household and agricultural activities. These AMR bacteria contaminated in fish and aquatic environment can transmit to humans by direct contact and consumption. Consumption of contaminated tilapia poses a potential risk of AMR infection. In Malaysia, *Salmonella* isolated from tilapia (n = 22) resisted to clindamycin (100%), rifampin (86.4%), tetracycline (54.5%), spectinomycin (27.3%), and chloramphenicol (22.7%) (Budiati et al., 2013). The prolonged use and misuse of antimicrobial agents in aquaculture may promote the distribution of new resistance determinants and repeat selected of AMR mutants (Davies and Davies, 2010). This selection can develop MDR bacteria that resist to at least three groups of antimicrobial agents (Nikaido, 2009). Previous studies reported predominant AMR pattern of farmed tilapia in China was sulfametoxazol-trimetoprim-tetracycline (17.6%) (Li et al., 2017). The high prevalence of AMR bacteria was reported in Southeast Asia and Saudi Arabia, and the bacterial isolates were highly resistant to ampicillin, clindamycin, rifampin, and tetracycline (Budiati et al., 2013; Elhadi, 2014).

Extended-Spectrum β-Lactamases (ESBLs) are enzymes produced by Gram-negative bacteria, which can break the chemical structure of β-lactam antibiotics. ESBL-producing bacteria can resist to broad spectrum of β-lactam antibiotics, including first, second, and third generations of cephalosporins. β-lactam antibiotics are commonly used in animal and human therapeutics. Therefore, the emergence of ESBL-producing *Enterobacteriaceae* has been an increasingly

concerned in public health worldwide. Fish can be served as a reservoir of ESBL, AMR, and potentially transmitted to human. Previous study indicated that more than 80% of bacteria isolated from fish harbored ESBL genes, and bla_{CTX-M} was the most frequently found in fish and aquatic environment, and the source of ESBL contamination in aquaculture maybe originated from polluted water (Bollache et al., 2019). Importantly, it is suggested that polluted water in aquatic environment may be an important source of ESBL-producing bacteria contaminated in fish (Bollache et al., 2019).

Among bacteria found in fish and aquatic environment, the highest MDR phenotypes were found in *Salmonella* and *E. coli*. Most *Salmonella* exhibited MDR ranging from 95-100% (Saharan et al., 2020; Ferreira et al., 2021; Dewi et al., 2022). The prevalence of MDR *E. coli* isolated from fish were varied from 34-95% (Saqr et al., 2016; Dewi et al., 2022). Previous study examined 96% of MDR *A. hydrophila* was isolated from fish in Egypt (Ahmed et al., 2018). Half of *V. cholerae* isolates exhibited MDR phenotypes that were observed in the aquatic products in China (Fu et al., 2020).

Many studies reported that *Salmonella* and *E. coli* carried bla_{TEM}, which is a commonly ß-lactamase gene found in both aquatic and food-producing animals (Sellera et al., 2018; Zhao et al., 2021). Quinolone resistance is one of the major concerns in aquaculture due to being commonly used in human medicine. The mutations of *gyrA* and *parC* in Quinolone Resistant-Determining Region (QRDR) resulting in amino acid change are mechanisms that conferred quinolone resistance. The primary target of mutation of *E. coli* was *gyrA* at position 83 (Ser83) or 87 (Asp87) and *parC* at position 80 (Ser80), while only *gyrA* (Ser83) was common for *Salmonella* and *V. cholerae* (Ma et al., 2018; Shaheen et al., 2021). For *A. hydrophila,* the

observed mutations were *gyrA* (Ser83) and *parC* (Ser80) (Chenia, 2016; Yang et al., 2017). For *V. cholerae*, a rare QRDR mutation was documented. Many plasmid-mediated quinolone resistance (PMQR) genes were prevalent in aquatic animals, such as *qnrA*, *qnrB*, and *qnrS*, which were observed in *E. coli* and *Salmonella* (Higuera-Llantén et al., 2018; Sivaraman et al., 2020). However, the *qnrS* was postulated as the major PMQR genes found in *Aeromonads* due to observed high prevalence without the detection of *qnrA* and *qnrB* (Dobiasova et al., 2014; Yang et al., 2017).

Tetracycline resistance genes are common resistance genes found in fish and aquatic environment, because analogs of these antimicrobials were widely applied for treatment in aquatic animals. Oxytetracycline, tetracycline, and doxycycline have been commonly used in fish (OIE, 2019). The common tetracycline resistance genes found in fish and aquatic environment were *tetA*, *tetB*, and *tetD* (Furushita et al., 2016; Ferreira et al., 2021; Odumosu et al., 2021). Besides *tetA* and *tetB,* some uncommon tetracycline resistance genes, such as *tetE* and *tetM* were reported in *A. hydrophila* and *V. cholerae* (Harnisz et al., 2015; Fri et al., 2018; Fauzi et al., 2021). Low prevalence of other AMR gene families, such as *sul*, *str*, *aadA*, and bla_{CTX-M} were reported in fish and aquatic environment (Elhadi, 2016; Fauzi et al., 2021).

Colistin is classified as a polymyxin, which is a last-line antimicrobial used for treatment of MDR bacterial infection. Infection of MDR bacteria is deadly due to unable to find effectively treatment with other antimicrobials. Originally, the resistance of colistin was mediated by chromosome mutation. The first discovered colistin resistance gene (*mcr-1*) was isolated from *E. coli* from meat in China in 2015 (Liu et al., 2016). This raised the global concern, since this gene it can be horizontally transferred. Therefore, the ban of colistin use in livestock was initiated in many countries worldwide (Usui et al., 2021; Lv et al., 2022). The *mcr* genes were mostly

found in Gram-negative bacteria, including *E. coli*. *Acinetobacter baumannii*, *K. pneumoniae*, *Pseudomonas aeruginosa*, and *Aeromonas* spp. (Eichhorn et al., 2018; Alqasim, 2021). The *mcr-2* to *mcr-10* have been continuously discovered worldwide (Sheng and Wang, 2021). Co-harboring of *mcr* and ESBL genes was observed resulting in superbug bacteria (Muktan et al., 2020; Le et al., 2021). In aquaculture, the *mcr-1*, *mcr-3*, and *mcr-4* were detected in fish, and the source of contamination was from discharged water of communities and agriculture (Liu et al., 2020; Kalová et al., 2021). To our knowledge, colistin resistance gene was not reported in fish in Thailand. Only *mcr-1* and *mcr-3* were reported in pig and clinical isolates in Thailand (Eiamphungporn et al., 2018; Pungpian et al., 2021).

Integrons are genetic elements found in plasmids, chromosomes, and transposons. They can express a variety of AMR in horizontal gene transfer. Integrons can be divided into three groups, class 1, 2, and 3 integron. Class 1 integron is dominantly found in fish and aquatic animals that is responsible for the dissemination of different AMR genes in aquatic environment. The prevalence of class 1 integron found in fish was ranging from 41.4%-53.0% (Ryu et al., 2012; Bollache et al., 2019). However, the study of class 2 and 3 integron in bacterial isolated from fish and aquatic environment was limited. For SXT element, it is an integrative and conjugative element, which has a key role in the acquisition and transfer of AMR genes between bacteria and can develop MDR. In Thailand, only SXT element was detected in *V. cholerae* in environmental samples in Thailand (Mala et al., 2017).

AMR becomes an important serious threat to public health. Acquired AMR bacteria in human can lead to treatment failure and life-threatening. However, a few studies have conducted to examine the AMR distribution and their resistant

determinants in fish and aquatic environment. Surveillance and monitoring of AMR in aquaculture under One Health can indicate emerging AMR. Identification of potential source of AMR is needed to reduce the cross-contamination in aquaculture. Rational use of antimicrobial agents, good aquaculture husbandry, and improve biosecurity should be implemented to reduce bacterial contamination and AMR development in aquaculture.

1.3 Research objectives

1. To determine the levels of fecal coliform and *E. coli*, and prevalence of *A. hydrophila, Salmonella* spp., *V. cholerae* in hybrid red tilapia and cultivation water.
2. To detect the serovars of *Salmonella* spp. and serogroups of *V. cholerae* isolated from hybrid red tilapia and cultivation water.
3. To detect the phenotype and genotype of AMR, and genetic determinants, ESBL production, and virulence genes of *A. hydrophila, Salmonella* spp., *V. cholerae*, and *E. coli* isolated from hybrid red tilapia and cultivation water.

1.4 Research outline

This study consisted of 4 phases. The first phase was sample collection and environmental parameter measurement. The second phase was bacterial isolation and confirmation of *A. hydrophila, Salmonella* spp, and *V. cholerae*. In addition, serotyping of *Salmonella* and *V. cholerae* was performed. The third phase and fourth phase were phenotypic and genotypic characterization of AMR, respectively.

Phase 1: Sample collection and environmental data measurement

1. Sample collection

- **Hybrid red tilapia (n = 120)**

  The samples consisted of fish carcass rinse (n = 120), fish meat (n = 120), intestine (n = 120), and liver and kidney (n = 120)

- **Cultivation water (n = 120)**

2. Environmental data measurement

- Onsite weather and water parameter
- Average weather parameters retrieved from the Meteorological Department

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Phase 2: Bacterial isolation and serotyping

1. Bacterial isolation

- Fecal coliforms and *E. coli*: US FDA BAM
- *A. hydrophila*: PHE (2015)
- *Salmonella*: ISO6579-1: 2017
- *V. cholerae*: US FDA BAM

2. Bacterial serotyping

- *Salmonella*: White-Kauffmann classification
- *V. cholerae*: serogroup testing for O1/O139/O141

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Phase 3: Phenotypic characterization of AMR

1. Antimicrobial susceptibility test

- Minimum inhibitory concentration and ESBL production test

⇩

Phase 4: Genotypic characterization of AMR

1. Virulence gene detection

- *A. hydrophila*: *aero*, *hly*
- *Salmonella*: *invA*
- *V. cholerae*: *ctx*, *tcpA*, and *hlyA*

2. AMR genes and other genetic determinants detection

- AMR genes, class 1, 2, 3 integron, and SXT element characterization

3. Sequencing

- Subtyping of bla_{TEM} and bla_{CTX-M}
- Mutations of QRDR

⇩

Goals: 1. To provide baseline information of bacterial contamination and key environmental factors associated with bacterial loads in hybrid red tilapia

2. To examine AMR and resistance determinants in aquaculture.

Figure 1. The research outline of this study

1.5 Advantages of this study

1.5.1 Novel knowledge

1. This study provided background data on distribution of fecal coliform, *E. coli*, *A. hydrophila, Salmonella* spp., and *V. cholerae* in hybrid red tilapia and cultivation water.
2. The key environmental factors impacting on bacterial distribution in hybrid red tilapia and cultivation water were identified.
3. Phenotypic and genotypic characterization of AMR in *E. coli*, *A. hydrophila, Salmonella* spp., and *V. cholerae* isolated from hybrid red tilapia and cultivation water were determined.

1.5.2 Application of knowledges

1. The results of prevalence of pathogenic bacteria and concentrations of *E. coli* can be used for future microbiological standard of tilapia in Thailand.
2. The information on AMR obtained from this study can be used to support the antimicrobial use guideline in aquaculture.
3. The burden of AMR in tilapia highlighted the necessary to integrate the aquaculture being a part of One Health for Thailand's National Strategic Plan on AMR.
4. The phenotypic and genotypic AMR detected in this study can be used to promote antimicrobial stewardship in aquaculture.
5. The results of AMR in this study can be used as a part of the AMR surveillance and monitoring in aquaculture in Thailand.

CHAPTER II

2.1 Materials and methods

2.1.1 Site selection and sample collection

The tilapia farms were located along the Kwae Noi river in Kanchanaburi province. This sampling sites were selected, because they are important sites for tilapia cage farming in Thailand. The hybrid red tilapia cultured in this area were mostly distributed in Bangkok and its vicinity.

In this study, only clinically healthy hybrid red tilapia and marketable-sized (body weight over 600 g) were collected. In total, hybrid red tilapia (*Oreochromis* spp.) (n = 120) and cultivation water (n = 120) were obtained from October 2019 to November 2020. The sampling points occurred every one- to two-month intervals for eight consecutive times. In each sampling, three out of five hybrid red tilapia farms were selected based on available marketable-sized hybrid red tilapia. Each sampling time, a total of 15 fish and 15 cultivation water samples were collected within three farms (5 fish and 5 cultivation water samples/farm). One fish and one cultivation water were sampled from the same cage, and five fish (n = 5), and five cultivation water samples (n = 5) were from different cages were collected.

The hybrid red tilapia were caught by a hand-net and kept individually in a double sterile plastic bag. Two hundred ml of cultivation water from the identical cage of harvested fish was collected at a depth of 45-60 cm below the surface. The water samples were kept in a sterile propylene bottle, and transported in refrigerated boxes at 4 °C and processed within 24 hr after collection at the Department of Veterinary Public Health, Chulalongkorn University.

2.1.2 Duck feces collection

Fresh duck feces (n = 15) were collected from nearby grazing ducks, which were raised at the proximity of a fish farm during 7th and 8th sampling events. The duck fecal samples were collected using a sterile plastic spoon and stored in a sterile plastic bag. These samples were kept and transported in refrigerated boxes at 4 ℃ during transportation. The bacterial determination was performed within 24 hr after collection.

2.1.3 Sample preparation

All hybrid red tilapia samples were weighed, and their width and length were measured. A total of 120 hybrid red tilapia was partitioned as four samples, including carcass rinse (n = 120), fish meat (n = 120), intestine (n = 120), and liver and kidney (n = 120). In total, 480 sample retrieved from hybrid red tilapia were used for bacterial isolation. Additionally, the cultivation water samples (n = 120) were used for bacterial isolation and confirmation.

For sample preparation, an approximate 5 × 5 cm area of fish skin on fish scale was swabbed with a sterile cotton. The swab samples were used to retrieve *A. hydrophila* from fish skin. After that, the whole fish body was rinsed with 50 ml of buffered peptone water (BPW) (Difco, MD, USA) to elute the bacteria on fish skin. This washed BPW was used as fish carcass rinse sample for detection and confirmation of fecal coliforms, *E. coli*, *Salmonella* spp., *V. cholerae*, *V. vulnificus*, and *S. agalactiae*. The fish's skin was sprayed with 70% ethyl alcohol to decontamination. The fish were aseptically dissected to collect 25 g of muscle, 1 g of kidney and liver, and 1 g of intestine. For cultivation water, a sterile cotton swab was immersed in the cultivation water for *A. hydrophila* isolation, and 25 ml of cultivation water was used for other bacterial isolation.

2.1.4 Enumeration and confirmation of fecal coliforms and *E. coli*

The concentrations of fecal coliforms and *E. coli* were enumerated followed the procedure described in the United States Food and Drug Administration's Bacteriological Analytical Manual (U.S. FDA BAM) (Feng et al., 2002). The 25 g of fish meat and 25 ml of cultivation water were individually mixed with 225 ml of BPW (Difco), and 1 ml of fish carcass rinse, intestine, kidney and liver were mixed with 9 ml of BPW to make 1:10 dilution. After that, one ml of the BPW suspension was transferred to three replicate 9 ml of tubes containing lactose broth (LB) (Difco) to make 10-fold serial dilution. The LB tubes were added with a Durham tube for gas production detection. The dilutions (10^{-1} to 10^{-3}) were used for cultivation water, fish meat, and kidney and liver, while the dilutions (10^{-1} to 10^{-5}) were used for intestine and fish carcass rinses. All LB tubes were incubated at 35 ± 2 ºC for 24 ± 2 hr. The positive LB tubes with gas production were selected. A loopful of positive LB tubes was transferred to 9 ml of EC broth (Difco), and incubated in water bath at 44.5 ºC for 24 ± 2 hr. The observed gas production in the EC tube of each dilution were used to calculate most probable number (MPN) of fecal coliforms concentration as MPN/g (fish meat, intestine, and kidney and liver) or MPN/ml (cultivation water and fish carcass rinse).

For *E. coli* enumeration, a loopful of positive LB tubes was streaked on Levine-Eosin-Methylene Blue (L-EMB) (Difco) agar and incubated at 35 ± 2 ºC for 24 ± 2 hr. The suspected colonies of *E. coli* on L-EMB agar are dark centered, flat, and with or without green metallic sheen. The number of positive plates in each dilution was used to calculate the *E. coli* concentration as MPN/g or MPN/ml. Suspected colonies of *E. coli* were confirmed using biochemical tests, such as indole production and catalase test. For indole production test, a colony of suspected *E. coli* were inoculated in tryptone broth (Difco) and incubated at 35 ± 2 ºC for 24 ± 2 hr. After

that, 200 µl of Kovac's reagent (Sigma-Aldrich, Steinheim, Germany) was added. The *E. coli* isolates are positive for indole production when the color of Kovac's reagent at the top of tryptone broth changes from yellow to cherry red. For catalase test, 3% H_2O_2 was dropped on glass slide and a small amount of bacterial colony was picked and transferred to the H_2O_2 solution. The *E. coli* show positive catalase test by detection of gas bubbles. The confirmed *E. coli* colony was streaked on plate count agar (PCA) (Difco), and incubated at 35 ± 2 ºC for 18-24 hr. Three *E. coli* colony in each sample were selected, kept in 20% glycerol, and stored at -20 ºC.

2.1.5 *A. hydrophila* isolation and confirmation

A. hydrophila isolation was performed using standardized guidelines from the Department of Public Health of England with slight modifications (PHE, 2015; Aboyadak et al., 2017). Briefly, a sterile cotton swab of cultivation water, carcass rinse, fish meat, intestine, and kidney and liver samples were streaked on Rimler-Shotts (RS) Medium Base (HiMedia Laboratories Ltd., Mumbai, India) supplemented with novobiocin 5 mg/l. The RS medium plates were incubated at 35 ºC ± 2 ºC for 24 hr. Suspected *A. hydrophila* showed yellow colonies on RS Medium plates. These suspected colonies were further biochemically confirmed using TSI slant agar. Suspected colonies of *A. hydrophila* produce purple in slant (alkaline) and yellow in butt (acid) due to glucose fermentation. *A. hydrophila* cannot produce H_2S production, therefore, blackening of TSI agar must not be observed.

All *A. hydrophila* isolates were confirmed by PCR. Two 16s rRNA genes were amplified with genus-specific primers (Aer-F/Aer-R; 5'-CTA CTT TTG CCG GCG AGC GG-'3 and 5'-TGA TTC CCG AAG GCA CTC CC-'3) and species-specific primers (AH-F/AH-R; 5'-GAA AGG TTG ATG CCT AAT ACG TA-'3 and 5'-CGT GCT GGC AAC AAA GGA CAG-'3)

with 35 cycles of the PCR condition as follows: denaturation at 94 ºC for 5 min, annealing 50 ºC for 40 s, and extension at 72 ºC for 50 s (Ahmed et al., 2018).

2.1.6 *Salmonella* isolation, confirmation, and serotyping

The *Salmonella* were detected followed the ISO 6579-1:2017 standard (ISO, 2017). Briefly, 25 g of fish meat and 25 ml of cultivation water were added with 225 ml of BPW (Difco). Intestine (1 g), kidney and liver (1 g), duck feces (1 g) and fish carcass rinse (1 ml) were enriched in a tube containing 9 ml of BPW (Difco). All mixture suspensions were incubated at 35 ± 2 ºC for 18-24 hr. A 0.1 ml of BPW suspension was pipetted on three sites of Modified Semi-solid Rappaport-Vassiliadis (MSRV) (Difco) medium and incubated at 42 ºC ± 0.5 ºC for 18-24 hr. The samples containing presumptive *Salmonella* spp. showed visible greyish swarming zone. After that, a loopful of positive MSRV medium was streaked on Xylose Lysine Deoxycholate (XLD) (Difco) agar and incubated at 35 ± 2 ºC overnight. Positive colonies of *Salmonella* were red with black centers on XLD agar. Three colonies of suspected *Salmonella* per sample were selected and further biochemically confirmed followed the U.S. FDA BAM using Triple Sugar Iron (TSI) (Difco) slant agar (Andrews et al., 2007). A single colony was inoculated in TSI and incubated at 37 ± 1 ºC for 24 ± 3 hr. *Salmonella* colonies are purple to red in slant (alkaline) and yellow butt (acid) due to only glucose fermentation. H_2S production is observed in all positive *Salmonella* isolates. The typical *Salmonella* colonies were streaked on PCA agar and incubated overnight. They were stored in 20% glycerol and stored at -20 ºC.

Finally, PCR was used to confirm *Salmonella* spp. The presumptive *Salmonella* isolates were confirmed by the amplification of the *invA* gene using a pair of primer (*invA*-F/*invA*-R; 5'- GTGAAATTATCGCCACGTTCGGGCAA-'3 and 5'-

TCATCGCACCGTCAAAGGAACC-'3) with product size 284 bp (Kumar et al., 2015). The PCR condition was 35 cycles of denaturation at 95 ºC for 30 s, annealing 58 ºC for 30 s, and extension at 72 ºC for 60 s.

All *Salmonella* isolates were further determined their serotypes by somatic (O) and flagella (H) antigen detection using slide agglutination test according to the Kauffmann-White scheme (Grimont and Weill, 2007) with available commercial antiserum (S&A Reagents Lab, Bangkok, Thailand).

2.1.7 *V. cholerae* isolation and confirmation

V. cholerae were isolated according to the U.S. FDA BAM (Kaysner and DePaola, 2004). In brief, one ml of the mixture BPW suspension from the sample preparation was added into 9 ml of Alkaline Peptone Water (APW) (Difco). The sample solution was incubated at 35 ºC ± 2 ºC for 24 hr. A loopful of suspension was streaked on Thiosulfate-Citrate-Bile Salts-Sucrose (TCBS) (Difco) agar plate and incubated at 35 ºC ± 2 ºC overnight. The suspected *V. cholerae* are round and yellow colonies on TCBS agar. After that, the suspected colonies of *V. cholerae* were picked and streaked on CHROMagar™ *Vibrio* (HiMedia Laboratories) and incubated at 37 ºC for 24 h. The *V. cholerae* show green blue to turquoise blue colonies on CHROMagar™ *Vibrio*. The suspected *V. cholerae* colonies were biochemically confirmed using TSI slant agar containing 2% NaCl. Positive colonies of *V. cholerae* are yellow in slant and butt (acid) without H_2S production. Molecular confirmation of *V. cholerae* was performed by *OmpW* gene (*OmpW*-F/*OmpW*-R; 5'- CACCAAGAAGGTGACTTTATTGTG-'3 and 5'- GAACTTATAACCACCCGCG-'3) with product size 588 bp (Sathiyamurthy et al., 2013). The PCR condition was 30 cycles of denaturation at 94 ºC for 120 s, annealing 50 ºC for 120 s, and extension at 72 ºC for 30 s.

2.1.8 *V. vulnificus* isolation and confirmation

V. vulnificus were isolated using the U.S. FDA BAM method (Kaysner and DePaola, 2004). One ml of BPW suspension was enriched in APW and incubated at 35 ºC ± 2 ºC. After overnight incubation, a loopful of suspension was streaked on TCBS agar, and the plate was incubated at 35 ºC ± 2 ºC for 24 ± 2 h. The positive colonies of *V. vulnificus* are green colonies on TCBS and blue-green colonies on CHROMagar™*Vibrio* (HiMedia Laboratories) agar plates. The presumptive colonies were biochemically confirmed by TSI (Difco) slant agar containing 2% NaCl. The positive *V. vulnificus* isolates show purple to red slant (alkaline) and yellowish butt (acid) without H_2S production.

2.1.9 *S. agalactiae* isolation and confirmation

The isolation of *S. agalactiae* was performed according to the *Streptococcus* Laboratory, Centers for Disease Control and Prevention (CDC, 2018) and the protocol from Laith *et al*., 2017, with a slight modification. Briefly, the swab sample from internal organs were directly streaked onto Brain Heart Infusion (BHI) (Difco) agar supplemented with 6.5% NaCl and incubated at 30 ºC ± 2 ºC overnight. The pinpoint colonies were streaked on CHROMagar™StrepB (HiMedia Laboratories) agar plates, and incubated at 35 ± 2 ºC for 24 h. The appearance of *S. agalactiae* colonies are mauve. These colonies of *S. agalactiae* were further biochemically confirmed by Gram-stain and catalase test. The *S. agalactiae*, which are the Gram-positive bacteria can be stained with purple color of crystal violet, and can produce gas bubbles in H_2O_2 solution.

2.1.10 Measurement of environmental parameters

Water and weather parameters were measured at eight sampling points. For water parameters, average water temperature (ºC), dissolved oxygen (DO) (mg/l), and

water pH were measured by portable water quality meters (SDL-100 and SDL-150, Extech instruments, NH, USA). Salinity of cultivation water (ppt) was measured by a refractometer (Master-S/MillM, Tokyo, Japan).

For weather parameters, onsite weather parameter and accumulative weather data were collected on sampling date. average ambient air temperature (°C), relative humidity (RH) (%), average wind speed (m/s), maximum wind gust (m/s), dew point (°C), and heat index (°C) were recorded onsite for each sampling time using a weather meter (Kestrel 3000, PA, USA). For accumulative weather data, 7-day average for weather parameters included rainfall (mm), wind speed (m/s), maximum wind gust (m/s), RH (%), and ambient air temperature (°C) data were retrieved from Thai meteorological department at the Kanchanaburi station (https://www.tmd.go.th/index.php).

2.1.11 Antimicrobial susceptibility test

One isolate per one positive sample from *E. coli*, *A. hydrophila*, and *V. cholerae* were included, while one isolate per one *Salmonella* serovar were used to performed AST. Twelve antimicrobials that are commonly used in human, livestock, and aquaculture were selected, including ampicillin, chloramphenicol, ciprofloxacin, enrofloxacin, florfenicol, gentamicin, oxytetracycline, oxolinic acid, streptomycin, sulfamethoxazole, tetracycline, and trimethoprim. The AST was performed by determination of minimum inhibitory concentrations (MICs) using agar dilution technique according to the Clinical and Laboratory Standard Institute (CLSI, 2013). The bacteria were grown on Mueller-Hinton agar (MHA) (Difco) and incubated at 37 °C for 18-20 hr. A single bacterial colony was picked and adjusted to the cell density at 0.5 McFarland in 0.9% NaCl solution. This can make the final dilution of 10^7 CFU/ml before inoculation.

The concentration range of twelve antimicrobials were ampicillin (0.25-1,024 µg/ml), ciprofloxacin (0.015-32 µg/ml), chloramphenicol (1-256 µg/ml), enrofloxacin (0.0075-64 µg/ml), florfenicol (0.5-512 µg/ml), gentamicin (0.125-128 µg/ml), oxolinic acid (0.015-128 µg/ ml), oxytetracycline (0.0625-512 µg/ ml), streptomycin (1-512 µg/ ml), sulfamethoxazole (2-2,048 µg/ ml), tetracycline (0.0625-256 µg/ ml), and trimethoprim (0.25-256 µg/ ml) (CLSI, 2014). *Staphylococcus aureus* ATCC 29213, *E. coli* ATCC 25922, and *Pseudomonas aeruginosa* ATCC 27853 were used as quality control strains.

2.1.12 Extended-spectrum ß-lactamase production

The ESBL production of bacterial isolates was examined by disk diffusion method (CLSI, 2013). Three cephalosporin disks of ceftazidime (30 µg), cefotaxime (30 µg), and cefpodoxime (10 µg) (Oxoid, England, UK) were used for screening test. The *E. coli* isolates that resist at least one of cephalosporins was further confirmed using the combination disk diffusion method. Comparison of inhibition zones between ceftazidime (30 µg) and ceftazidime (30 µg)/clavulanic acid (10 µg), and between cefotaxime (30 µg) and cefotaxime (30 µg)/clavulanic acid (10 µg) were determined. The isolates which have the difference of inhibition zone ≥ 5 mm, are considered as ESBL positive isolates.

2.1.13 AMR gene detection

The DNA template of all bacteria was extracted using whole cell boiling method (Lévesque et al., 1995). After overnight incubation at 37 ºC in nutrient agar (NA) (Difco), a single colony of bacterial cells was picked and suspended into 100 µl of sterile rNase free water. This suspension was heated for 10 min in boiling water, and immediately placed on ice before centrifuged at 12,000 rpm for 5 min. The supernatant was transferred to a sterile centrifuge tube and kept in a -20 ºC freezer.

The PCR reaction was performed in final volume of 50 µl containing 25 µL of TopTaq DNA polymerase (Qiagen, Stockach, Germany), 1X PCR buffer, 200 µM of each dNTP, 0.5 µL of each forward and reverse primer of 10 µM concentration, and 5 µL of DNA template.

The presence of AMR genes, which were corresponding to AMR phenotypes was tested as followed: $bla_{TEM,}$ bla_{SHV}, bla_{CTX-M}, and bla_{PSE} encoding β-lactam resistance and ESBL production; bla_{NDM} and bla_{OXA} encoding carbapenem resistance; *catA*, *catB*, *floR*, and *cmlA* encoding phenicol resistance; *ermB* encoding erythromycin resistance; *qnrA*, *qnrB*, *qnrS*, *aac(6')-Ib-cr*, and *qepA* encoding quinolone resistance; *aadA1*, *aadA2*, and *aac(3)IV* encoding gentamicin resistance; *tetA*, *tetB,* and *tetD* encoding tetracycline resistance; *strA* and *strB* encoding streptomycin resistance; *sul1*, *sul2*, and *sul3* encoding sulfonamide resistance; *dfrA1* and *dfrA12* encoding trimethoprim resistance; *mcr-1* to *mcr-5* encoding colistin resistance.

The PCR condition for bla_{TEM}, bla_{CTX-M}, and bla_{SHV} were initial denaturation at 94 °C for 3 min, followed by 25 cycles of 94 °C for 60 s, 50 °C (bla_{TEM} and bla_{SHV}) or 60 °C (bla_{CTX-M}) or for 60 s, and 72 °C for 60 s (Olesen et al., 2004; Batchelor et al., 2005; Hasman et al., 2005). A final extension was done with 72 °C for 10 min.

The simplex PCR was performed to detect bla_{PSE} as follow initial denaturation at 94 °C for 5 min, followed by 30 cycles of 94 °C for 30 s, 55 °C for 30 s, 72 °C for 50 s, and a final extension of 72 °C for 7 min (Li et al., 2013).

For two carbapenemase genes, including bla_{NDM} and bla_{OXA}, the PCR condition was initial denaturation at 95 °C for 15 min, followed by 30 cycles of 94 °C for 30 s, 52 °C for 40 s (bla_{NDM}) or 62 °C for 90 s (bla_{OXA}), 72 °C for 60 s, and a final extension of 72 °C for 10 min (Costa et al., 2006).

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

For quinolone resistance genes, multiplex PCR was performed to examine *qnrA*, *qnrB*, and *qnrS.* The PCR condition was denaturation at 95 ℃ for 10 min, followed by 35 cycles of 95 ℃ for 60 s, 54 ℃ for 60 s, 72 ℃ for 60 s, and a final extension at 72 ℃ for 10 min (Cattoir et al., 2007).

Erythromycin resistance gene (*ermB*) was amplified following the initial denaturation at 95 ℃ for 5 min, followed by 30 cycles of denaturation at 94 ℃ for 1 min, annealing at 60 ℃ for 40 s, extension at 72 ℃ for 40 s, and a final extension at 72 ℃ for 7 min (Raissy et al., 2012).

The cycling conditions for the detection of *dfrA1*and *dfrA12* were followed initial denaturation at 94 ℃ for 8 min, followed by 32 cycles of 95 ℃ for 60 s, 55 ℃ for 70 s, 72 ℃ for 10 min, and a final extension at 72 ℃ for 10 min (Chuanchuen et al., 2008a; Shahrani et al., 2014).

The amplification of *catA*, *catB*, and *floR* was performed as previously described (Chuanchuen and Padungtod, 2009; Ying et al., 2019). The PCR condition was initial denaturation at 94 ℃ for 5 min, followed by 30 cycles of 95 ℃ for 45 s, 55 ℃ for 45 s (*catA* and *catB*) or 58 ℃ for 60 s (*floR*), 72 ℃ for 10 s, and a final extension at 72 ℃ for 10 min.

PCR amplification for *cmlA* was conducted with the following cycling condition, initial denaturation at 94 ℃ for 5 min, followed by 30 cycles of 94 ℃ for 45 s, 57 ℃ for 45 s, and 72 ℃ for 60 s, and followed by final extension at 72 ℃ for 5 min (Chuanchuen and Padungtod, 2009).

Multiplex PCR were performed to examine *strA* and *strB*. The PCR condition was initial denaturation at 95 ℃ for 15 min, followed by 35 cycles of 94 ℃ for 30 s, 57 ℃ for 1.5 min and 72 ℃ for 1.5 min. One cycle of final extension was done at 72 ℃ for 10 min (Chuanchuen and Padungtod, 2009; Mala et al., 2016).

Simplex PCR condition for *tetA* was initial denaturation at 95 ℃ for 5 min, followed by 30 cycles of 94 ℃ for 45 s, 63 ℃ for 60 s, 72 ℃ for 60 s, and a final extension at 72 ℃ for 10 min (Khan et al., 2019).

For *tetB*, *aadA1*, and *aadA2* amplification, the conditions were initial denaturation at 95 ℃ for 5 min, followed by 30 cycles of 95 ℃ for 45 s, 55 ℃ for 45 s, 72 ℃ for 45 s, and a final extension at 72 ℃ for 10 min (Chuanchuen et al., 2008b).

The cycling condition for *tetD* amplification was initiated with 95 ℃ for 5 min, followed by 35 cycles of 98 ℃ for 5 s, 55 ℃ for 15 s, 72 ℃ for 60 s, and a final extension at 72 ℃ for 10 min (Kumai et al., 2005).

The *aac(3) IV* was amplified with the following PCR condition, initial denaturation at 95 ℃ for 3 min, followed by 35 cycles of 94 ℃ for 30 s, 55 ℃ for 30 s, 72 ℃ for 60 s, and a final extension at 72 ℃ for 10 min (Stoll et al., 2012).

PCR condition for *aac(6')-Ib-cr* was initial denaturation at 95 ℃ for 10 min followed by 32 cycles of 94 ℃ for 45 s, 55 ℃ for 45 s, 72 ℃ for 45 s, and a final extension of 72 ℃ for 10 min (Park et al., 2006).

Multiplex PCR for detection of colistin resistance genes (*mcr-1* to *mcr-5*), was initial denaturation at 94 ℃ for 15 min, followed by 25 cycles of 94 ℃ for 30 s, 58 ℃ for 90 s, 72 ℃ for 60 s, and a final extension at 72 ℃ for 10 min (Rebelo et al., 2018).

The PCR products were analyzed using electrophoresis in a 1.5% (w/v) agarose gel in 1X Tris-acetate/EDTA (1X TAE)., stained with Redsafe™ Nucleic Acid

Staining solution (Intron Biotechnology, Seongnam, Republic of Korea), and visualized by Omega Fluor™ gel documentation system. (Aplegen, CA, USA).

2.1.14 Detection of virulence genes

For *E. coli*, the *stx1* and *stx2*, which are shiga-like enterotoxins, were detected with the initial denaturation at 94 ℃ for 5 min, followed by 35 cycles of 94 ℃ for 60 s, 55 ℃ for 60 s, 72 ℃ for 60 s, and a final extension at 72 ℃ for 10 min (Khan et al., 2002).

The PCR amplification for two virulence genes of *A. hydrophila*, aerolysin (*aero*) and hemolysin (*hly*), was carried out with condition as follows initial denaturation at 94 ℃ for 3 min, followed by 30 cycles of 94 ℃ for 30 s, 52 ℃ for 30 s, 72 ℃ for 30 s, and a final extension of 72 ℃ for 10 min (Yousr et al., 2007; Singh et al., 2008).

The detection of *invA*, which is the virulence genes of *Salmonella* spp., the cycling condition was initial denaturation at 95 ℃ for 2 min, followed by 35 cycles of 95 ℃ for 30 s, 58 ℃ for 30 s, 72 ℃ for 60 s, and a final extension of 72 ℃ for 5 min (Kumar et al., 2015).

Three virulence genes of *V. cholerae* were amplified as previously described (Singh et al., 2002; Wong et al., 2012; Imani et al., 2013). Gene encoded toxin-coregulated pilus (*tcpA)* and gene encoded cholera toxin (*ctx*) were amplified with the initial condition 94 ℃ for 2 min, followed by 30 cycles of 94 ℃ for 60 s, 62 ℃ for 60 s, 72 ℃ for 60 s, and a final extension at 72 ℃ for 10 min (Singh et al., 2002; Wong et al., 2012). For detection of hemolysin gene (*hlyA*) in *V. cholerae*, the initial condition 94 ℃ for 5 min, followed by 35 cycles of 94 ℃ for 60 s, 58 ℃ for 60 s,

72 ℃ for 60 s, and a final extension at 72 ℃ for 5 min was carried out (Imani et al., 2013).

2.1.15 Detection of integrons and SXT element

Multiplex PCR was performed to detect integrons (*int1*, *int2,* and *int3*), and SXT element (int_{SXT}) (Kitiyodom et al., 2010). The amplification was an initial denaturation at 94 ℃ for 5 min, followed by 35 cycles of denaturation at 94 ℃ for 30 s, annealing at 60 ℃ for 30 s, extension at 72 ℃ for 60 s, and a final extension at 72 ℃ for 10 min.

CHAPTER III

Bacterial pathogens and factors associated with *Salmonella* contamination in hybrid red tilapia (*Oreochromis* spp.) cultivated in a cage culture system

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Bacterial pathogens and factors associated with *Salmonella* contamination in hybrid red tilapia (*Oreochromis* spp.) cultivated in a cage culture system

3.1 Abstract

Microbial food safety in cultured tilapia remains a challenge to public health worldwide, due to in part to intensive aquaculture leading to poor water quality and high organic matter deposition. This study aimed to determine the prevalence of indicator and potential pathogenic bacteria in hybrid red tilapia (*Oreochromis* spp.) and their cultivation water, and to identify environmental parameters and other bacterial contaminants associated *Salmonella* contamination. A total of 120 fish were sampled, which were partitioned as fish carcasses (n = 120), muscle (n = 120), intestine (n = 120), liver and kidney (n = 120), and cultivation water (n = 120) from three commercial farms in western Thailand from October 2019 to November 2020. The prevalence of fecal coliforms and *Escherichia coli* in these 600 samples was 74.8% and 56.7%, respectively. The prevalence of *Salmonella*, *Vibrio cholerae*, *Aeromonas hydrophila*, and *Vibrio vulnificus* was 23.0%, 17.5%, 2.5% and 1.7%, respectively. None of the samples tested positive for *S. agalactiae*. Cultivation water exhibited a high prevalence for *Salmonella* (58.3%). Among fish samples, *Salmonella* was the highest prevalence at 14.1%, which was mainly from fish intestine. There was a significant association of *Salmonella* with the presence of fecal coliforms, *E. coli*, *V. cholerae*, and *V. vulnificus*. The predominant serovars of *Salmonella* included Saintpaul, Neukoelln, Escanaba, and Papuana. Grazing ducks that were raised in proximity to these cultured tilapias shared the same isolates of *Salmonella* based on the similarity of their rep-PCR DNA fingerprints, suggesting that ducks may function as

either as a biological reservoir for tilapia or at minimum participate in the environmental replication of this strain of *Salmonella*. Taken together, the results suggest that environment used for tilapia aquaculture may be contaminated with pathogenic bacteria and therefore food safety precautions are needed during processing, transportation, cooking, and consumption.

Keywords: *Aeromonas* spp., grazing duck, *Salmonella* spp., tilapia, *Vibrio* spp.

3.2 Introduction

To meet the high demands for global fish consumption, fish production has increased significantly from 76.5 million tons (MT) in 2016 to 82.1 MT in 2018 (FAO, 2020). *Oreochromis* spp., also known as tilapia, is one of the most important farmed freshwater fish. Tilapia production contributed to 8.3% of the total finfish products in 2018 (FAO, 2020), with Thailand and other Southeast Asian countries representing about 23.4% of total production. Due to the increase in global consumption of tilapia, microbial food safety has become an increasing concern for food safety and public health.

The major zoonotic bacteria found in cultured tilapia are *Salmonella*, *Aeromonas hydrophila*, *Vibrio cholerae*, *V. vulnificus*, and *Streptococcus agalactiae* (Chen et al., 2006; Suanyuk et al., 2008). Although *Salmonella* infection in aquatic animals is generally non-pathogenic for the host animals, this bacterium can be transmitted as a foodborne pathogen and then function as a cause of severe gastroenteritis in humans. Previous study has indicated that up to one third of cultured tilapia can be contaminated with *Salmonella* (Awuor et al., 2011). *S. agalactiae*, which belongs to group B *Streptococcus* spp. (GBS), has been a concern for human and animal health and contributes to severe losses in tilapia

production worldwide (Jantrakajorn et al., 2014; Barato et al., 2015). For example, in Thailand, GBS *Streptococcus* serotype Ia and III (Dangwetngam et al., 2016) were reported in both human patients and in tilapia samples (Suanyuk et al., 2008). *A. hydrophila* is ubiquitous in freshwater environments and is a cause of Epizootic Ulcerative Syndrome (EUS) leading to high mortality in fish. Moreover, *A. hydrophila* infection in human induces gastroenteritis and extra-intestinal disease (i.e., meningitis, and endocarditis) (Zhang et al., 2012). Lastly, *V. vulnificus* and *V. cholerae* are pathogenic bacteria commonly reported in tilapia from various countries, including Thailand, China, and Israel (Chen et al., 2006; Senderovich et al., 2010; Dong et al., 2015). Copepods may function as a source of *V. cholerae* contamination in tilapia, but the source of *V. vulnificus* remains to be elucidated (Chen et al., 2006). Previous studies have been mainly focused on bacterial isolation from moribund fish or disease outbreaks; therefore, food safety data is needed on bacterial contamination in healthy fish intended for human consumption in order to protect public health

In Thailand, aquaculture for hybrid red tilapia is primarily based on cage culture systems, which is dependent on natural surface water sources and therefore susceptible to contamination from urban discharges or municipal wastewater. The Kwae Noi river is one of the key locations for cage-based tilapia aquaculture due to its perennial flows and optimal environmental conditions for fish growth. High stocking density can trigger poor water circulation within the fish cages. Combination of fish fecal wastes and appropriate environmental conditions can result in bacterial growth and accumulation. These cultivation factors can promote bacterial infection in tilapia, which then becomes an important vector for foodborne transmission to humans and a threat to public health. Therefore, the objectives of this study were to determine the prevalence of *Salmonella* spp., *A. hydrophila*, *S. agalactiae*, *V. cholerae*, *V. vulnificus*, along with fecal coliforms and *E. coli* in cultured hybrid red

tilapia, and to identify environmental parameters and other bacterial contaminants associated with *Salmonella* contamination in these cultured fish.

3.3 Materials and methods

3.3.1 Sampling location and sample collection

Hybrid red tilapia is a hybrid of *Oreochromis mossambicus* and *O. niloticus*, which is raised in cages along the Kwae Noi river located in Muang district in Kanchanaburi Province. It takes approximately 6 to 7 months to achieve a marketable body weight of at least 600 g for hybrid red tilapia. Tilapia are fed with formulated pellet feeds three times per day, with temporary aeration provided as needed. Antimicrobial drugs, including enrofloxacin and oxytetracycline, are given in the feed when the fish show clinical signs such as swirling swimming, skin hemorrhage or exophthalmia, or when there is a noticeable increase in morbidity and mortality.

A total of 120 tilapia were sampled from which fish carcass rinses (n = 120), fish muscle (n = 120), intestine (n = 120), liver and kidney (n = 120) were obtained, and cultivation water (n = 120) were collected from October 2019 to November 2020. Fresh fecal deposits from nearby grazing ducks (n = 15), which were reared nearby the tilapia aquaculture site, were collected using a sterile plastic spoon and stored in a sterile plastic bag.

At each sampling event, the hybrid red tilapia (n = 15) and cultivation water (n = 15) were collected from three commercial tilapia farms, replicated eight times, with one- to two-month intervals between sampling events. All fish that were sampled appeared clinically healthy (no evident skin hemorrhage, ulcers, or abnormal swimming behavior). The fish were caught by hand-net and individually

collected in a double sterile plastic bag. Two to three hundred mL of cultivation water from the identical cage of harvested fish was collected at a depth of 45-60 cm below the surface. The water samples were kept in a sterile propylene bottle, with samples transported in refrigerated boxes kept at 4 °C and processed within 24 hr after collection at the Department of Veterinary Public Health, Chulalongkorn University.

3.3.2 Sample preparation

All hybrid red tilapia samples were weighed, and their width and length recorded. The average weight ± standard deviation (± sd) of fish samples was 751.2 ± 174.7 g, ranging from 503.0 to 1,413.0 g per fish. The average ± sd of width and length of fish were 14.0 ± 1.5 cm and 29.1 ± 3.0 cm, respectively.

An approximate 5×5 cm area of the surface of each sampled fish was swabbed with sterile cotton for isolation of *A. hydrophila*. Next, for the fish carcass rinse, the entire external surface was rinsed with 50 ml of buffered peptone water (BPW) (Difco, MD, USA) for detection of fecal coliforms, *E. coli, Salmonella* spp., *V. cholerae*, *V. vulnificus* and *S. agalactiae*. The fish's external surface was then sprayed with 70% ethyl alcohol for decontamination, and the fish were aseptically dissected to collect 25 g of muscle, 1 g of kidney and liver, and 1 g of fish intestine. For cultivation water, a sterile cotton swab was immersed in the water for identification of *A. hydrophila*, and 25 ml of water was collected for detection or enumeration of fecal coliforms, *E. coli*, *Salmonella* spp., *V. cholerae, V. vulnificus*, and *S. agalactiae*. In addition, fecal materials from ducks were used for rep-PCR characterization of *Salmonella* spp. The confirmation of *A. hydrophila*, *Salmonella* and *Vibrio* was performed by PCR. Genomic DNA from suspected colonies were extracted using whole cell boiling method (Lévesque et al., 1995). The PCR products

were analyzed using electrophoresis in a 1.5% (w/v) agarose gel, stained with Redsafe™ Nucleic Acid Staining solution (Intron Biotechnology, Seongnam, Republic of Korea) and visualized by Omega Fluor™ gel documentation system. (Aplegen, CA, USA).

3.3.3 Enumeration of fecal coliforms and *E. coli*

The method of fecal coliforms and *E. coli* enumeration followed the procedure described in the United States Food and Drug Administration's Bacteriological Analytical Manual (U.S. FDA BAM) (Feng et al., 2002). To yield 1:10 dilution, 25 g of muscle and 25 ml of sterile water were mixed with 225 ml of BPW (Difco). Fish carcass rinse, intestine, kidney and liver were mixed with 9 ml of BPW. One ml of the suspension was transferred to three replicate test tubes containing 9 ml of lactose broth (LB) (Difco) with a Durham tube. The dilutions (10^{-1} to 10^{-3}) were used for cultivation water, muscle, and kidney and liver, while the dilutions (10^{-1} to 10^{-5}) were used for intestine and fish carcass rinses. All LB tubes were incubated at 37 °C overnight. Positive LB tubes with gas production were determined. A loopful of positive LB tubes was transferred to 9 ml of EC broth (Difco), incubated in water bath at 44.5 °C for 24 to 48 hr with gas production in the EC tube indicative of positive fecal coliforms (MPN/g or MPN/ml).

For *E. coli* enumeration, a loopful of positive LB tubes was streaked on Levine-Eosin-Methylene Blue (L-EMB) (Difco) agar and incubated at 37 °C overnight. The suspected colonies of *E. coli* on L-EMB agar are dark centered, flat, and with or without green metallic sheen. The concentrations of *E. coli* were calculated as MPN/g (fish muscle, intestine, and kidney and liver) or MPN/ml (cultivation water and fish carcass rinse). Suspected colonies of *E. coli* were confirmed using biochemical tests, such as indole production and catalase test.

3.3.4 *Salmonella* isolation and serotyping

Salmonella isolation followed the ISO 6579-1:2017 standard (ISO, 2017). Twenty-five g of blended fish muscle and 25 ml of water were separately mixed with 225 ml of BPW. Intestine (1 g), kidney and liver (1 g), duck feces (1 g) and fish carcass rinse (1 ml) were individually mixed in a tube containing 9 ml of BPW. All mixture suspensions were incubated at 37 ºC for 24 hr. A 100 µl of solution was dropped on Modified Semi-solid Rappaport-Vassiliadis (MSRV) (Difco) medium and incubated at 42 ºC. After overnight incubation, a loopful of incubated medium was streaked on Xylose Lysine Deoxycholate (XLD) (Difco) agar and incubated at 37 ºC for 24 hr. Typical colonies of *Salmonella* were red with or without black centers on XLD agar. For *Salmonella* confirmation, all isolates were screened for the *invA* gene using a pair of primer (*invA*-F/*invA*-R; 5'-GTGAAATTATCGCCACGTTCGGGCAA-'3 and 5'-TCATCGCACCGTCAAAGGAACC-'3) with product size 284 bp (Kumar et al., 2015).

Three to five suspected colonies of *Salmonella* were biochemically confirmed followed the U.S. FDA BAM using Triple Sugar Iron (TSI) (Difco) slant agar (Andrews et al., 2007). *Salmonella* colonies were purple to red in slant and yellow butt with H_2S production. Three *Salmonella* isolates per one positive sample were selected and performed serotyping using slide agglutination test according to the Kauffmann-White scheme (Grimont and Weill, 2007) with available commercial antiserum (S&A Reagents Lab, Bangkok, Thailand).

3.3.5 *A. hydrophila* isolation

The detection of *A. hydrophila* was performed using standardized guidelines from the Department of Public Health of England with slight modifications (PHE, 2015; Aboyadak et al., 2017). Briefly, a sterile cotton swab of cultivation water, fish carcass rinse, muscle, intestine, and kidney and liver samples were streaked on

Rimler-Shotts (RS) Medium Base (HiMedia Laboratories Ltd., Mumbai, India) supplemented with novobiocin 5 mg/l, and the plates were incubated at 35 ºC overnight. Suspected colonies of *A. hydrophila* were round and yellow color in RS medium plate. The suspected colonies were further biochemically confirmed using TSI slant agar. Suspected colonies of *A. hydrophila* produced purple in slant and yellow in butt without H_2S production.

The confirmation of *A. hydrophila* was performed by PCR. Genomic DNA from suspected colonies was extracted by whole cell boiling method (Lévesque et al., 1995) Two 16s rRNA genes were amplified with genus-specific primers (Aer-F/Aer-R; 5'-CTACTTTTGCCGGCGAGCGG-'3 and 5'-TGATTCCCGAAGGCACTCCC-'3) and species-specific primers (AH-F/AH-R; 5'-GAAAGGTTGATGCCTAATACGTA-'3 and 5'-CGTGCTGGCAACAAAGGACAG-'3) with 35 cycles of the PCR condition as follows: denaturation at 94 ºC for 5 min, annealing 50 ºC for 40 s, and extension at 72 ºC for 50 s (Ahmed et al., 2018). The PCR products was analyzed using electrophoresis in a 1.5% (w/v) agarose gel, stained with RedsafeTM Nucleic Acid Staining solution (Intron Biotechnology, Seongnam, Republic of Korea) and visualized by Omega Fluor™ gel documentation system. (Aplegen, CA, USA).

3.3.6 *V. cholerae* isolation

Identification of *V. cholerae* was performed according to the U.S. FDA BAM (Kaysner and DePaola, 2004). In brief, one ml of the mixture BPW suspension from the sample preparation was added into 9 ml of Alkaline Peptone Water (APW) (Difco). The sample solution was incubated at 37 ºC. After overnight incubation, a loopful of suspension was streaked on Thiosulfate-Citrate-Bile Salts-Sucrose (TCBS) (Difco) agar plate and incubated at 37 ºC overnight. The suspected colonies of *V. cholerae* in TCBS agar are generally large with 2-4 mm diameter, round, and yellow.

The suspected colonies of *V. cholerae* were confirmed on CHROMagar™ *Vibrio* (HiMedia Laboratories) and incubated at 37 ºC for 24 hr. The positive colonies of *V. cholerae* are observed green blue to turquoise blue. The suspected colonies were also biochemically confirmed using TSI slant agar containing 2% NaCl. Positive colonies of *V. cholerae* were yellow in slant and butt without H_2S production. Molecular confirmation of *V. cholerae* was performed by *OmpW* gene (*OmpW* -F/ *OmpW* -R; 5'-CACCAAGAAGGTGACTTTATTGTG-'3 and 5'-GAACTTATAACCACCCGCG-'3) with product size 588 bp (Sathiyamurthy et al., 2013).

3.3.7 *V. vulnificus* isolation

V. vulnificus isolation was performed using the U.S. FDA BAM method (Kaysner and DePaola, 2004). The samples were enriched with APW and incubated at 37 ºC. After overnight incubation, a loopful of suspension was streaked on TCBS agar, and the plate was incubated at 37 ºC for 24 hr. The positive colonies of *V. vulnificus* were green colonies. The suspected colonies were confirmed on CHROMagar™*Vibrio* (HiMedia Laboratories). The positive *V. vulnificus* colonies are blue-green colonies. The presumptive colonies were biochemically confirmed by TSI (Difco) slant agar containing 2% NaCl. The positive of *V. vulnificus* showed red slant and yellowish butt without H_2S production.

3.3.8 *S. agalactiae* isolation

The *Streptococcus* isolation was performed according to the *Streptococcus* Laboratory, Centers for Disease Control and Prevention (CDC, 2018) and the protocol from Laith et al., 2017, with a slight modification. In brief, the swab sample from internal organs were directly streaked onto Brain Heart Infusion (BHI) (Difco) agar supplemented with 6.5% NaCl and incubated at 30 ºC overnight. The pinpoint colonies were picked and confirmed on CHROMagar™StrepB (HiMedia Laboratories)

agar plates. The plates were incubated at 37 ºC for 24 hr. Positive colonies of *S. agalactiae* were mauve. The presumptive colonies of *S. agalactiae* were further biochemically confirmed by Gram-stain and catalase test.

3.3.9 Measurement of environmental parameters

Environmental parameters for water and weather were collected at 8 sampling time points. For water parameters, water temperature (ºC), dissolved oxygen (DO) (mg/l), pH, and salinity (ppt) were recorded during sample collection. Portable water quality meters (SDL-100 and SDL-150, Extech instruments, NH, USA) were used for measurement of water temperature, DO, and pH, while a refractometer (Master-S/MillM, Tokyo, Japan) was used to measure water salinity.

Weather data were collected both on-site and online meteorological data. Ambient air temperature (ºC), relative humidity (RH) (%), average wind speed (m/s), maximum wind gust (m/s), dew point (ºC), and heat index (ºC) were recorded using a weather meter (Kestrel 3000, Nielsen-Kellermen, PA, USA) at the sampling sites. The 7-day average for weather parameters included rainfall (mm), wind speed (m/s), maximum wind gust (m/s), RH (%), and ambient air temperature (ºC) data were from Thai meteorological department at the Kanchanaburi station (https://www.tmd.go.th /index.php).

The average ± sd of water temperature (30.8 ± 2.2 ºC), DO (6.8 ± 0.8 mg/l), pH (7.8 ± 0.4), and salinity (1.00 ± 9.4 × 10^{-4} ppt) were presented (Table 1). Based on the online weather data, average 7-day (± sd) of rain fall 1.1 (± 1.0) mm, wind speed 2.1 (± 0.6) m/s, maximum wind gust 9.4 (± 1.5) m/s, RH 65.6 (± 8.7) %, and temperature 29.0 (± 2.6) ºC were presented (Table 1).

Table 1. Average and standard deviation (± sd) for aquaculture cultivation water and weather parameters, stratified by sampling month from October 2019 to November 2020.

Parameter	Monthly average (± sd)								Total average (±sd)
	(10/19)	(12/19)	(1/20)	(3/20)	(5/20)	(7/20)	(9/20)	(11/20)	
Water parameter									
Temperature (°C)	30.7 (±1.5)	27.5 (±0.4)	28.2 (±0.5)	31.7 (±0.5)	33.9 (±1.3)	33.0 (±0.6)	31.8 (±0.2)	29.7 (±0.2)	30.8 (±2.2)
DO (mg/l)	6.8 (±0.7)	7.2 (±0.6)	7.7 (±0.1)	7.1 (±0.1)	5.3 (±0.3)	6.9 (±0.2)	6.2 (±0.06)	7.1 (±0.2)	6.8 (±0.8)
pH	7.5 (±0.1)	7.6 (±0.07)	7.6 (±0.1)	7.9 (±0.3)	7.5 (±0.4)	7.8 (±0.2)	8.6 (±0.2)	7.5 (±0.4)	7.8 (±0.4)
Salinity (ppt)	1.0 (±6.0× 10^{-4})	1.0 (±2.5× 10^{-4})	1.0 (±0)	1.0 (±0)	1.0 (±6.2× 10^{-4})	1.0 (±0)	1.0 (±0)	1.0 (±0)	1.0 (±9.4× 10^{-4})
Weather parameter									
Ambient air temperature (°C)	34.1 (±1.1)	31.1 (±0.3)	34.0 (±0.6)	36.1 (±1.8)	36.2 (±1.7)	32.4 (±1.1)	32.4 (±1.8)	30.9 (±1.1)	33.4 (±2.3)
Relative humidity (%)	60.4 (±2.3)	58.9 (±2.3)	63.7 (±7.8)	57.6 (±4.70)	56.9 (±5.1)	64.9 (±4.9)	73.4 (±5.9)	82.6 (±7.4)	64.8 (±10.0)
Average wind speed (m/s)	1.2 (±0.3)	2.3 (±1.1)	0.7 (±0.5)	0.2 (±0.4)	0.3 (±0.5)	2.4 (±1.7)	1.0 (±0.1)	1.2 (±0.2)	1.2 (±1.2)
Maximum wind gust (m/s)	1.9 (±0.4)	2.9 (±1.1)	2.7 (±0.7)	2.8 (±0.7)	3.0 (±0.8)	10.4 (±3.0)	5.8 (±2.5)	4.9 (±2.7)	4.9 (±3.5)

Table 1. Average and standard deviation (± sd) for aquaculture cultivation water and weather parameters, stratified by sampling month from October 2019 to November 2020. (Continue)

Parameter	Monthly average (± sd)								Total average (± sd)
	(10/19)	(12/19)	(1/20)	(3/20)	(5/20)	(7/20)	(9/20)	(11/20)	
Dew point (°C)	25.9 (±1.6)	21.8 (±0.4)	27.4 (±2.4)	27.5 (±2.2)	29.3 (±4.1)	25.0 (±1.6)	27.8 (±2.8)	27.2 (±1.5)	26.5 (±3.2)
Heat index (°C)	45.8 (±4.6)	35.6 (±1.8)	49.6 (±7.7)	48.7 (±4.4)	48.6 (±4.3)	40.3 (±3.9)	45.4 (±7.4)	42.5 (±5.1)	44.5 (±6.9)
Average 7-day rainfall (mm)	2.2 (±3.0)	0	0	0	0.9 (±1.6)	2.6 (±4.3)	1.0 (±2.2)	2.2 (±5.2)	1.1 (±1.0)
Average 7-day wind speed (m/s)	2.6 (±0.5)	2.0 (±0.5)	1.7 (±0.7)	2.7 (±0.5)	2.1 (±0.6)	2.3 (±0.9)	2.3 (±1.0)	0.7 (±0.5)	2.1 (±0.6)
Average 7-day maximum wind gust (m/s)	9.0 (±1.2)	10 (±1.0)	8.1 (±1.0)	10.6 (±1.18)	10.7 (±2.4)	10.0 (±1.4)	9.9 (±2.0)	6.3 (±1.0)	9.4 (±1.5)
Average 7-day relative humidity (%)	70.4 (±2.5)	59.3 (±4.3)	53.9 (±7.5)	55.3 (±10.2)	64.9 (±6.6)	69.6 (±4.8)	70.1 (±7.2)	81.7 (±6.8)	65.6 (±8.7)
Average 7-day ambient air temperature (°C)	29.9 (±0.8)	26.6 (±2.6)	27.4 (±0.7)	31.6 (±0.9)	31.9 (±1.4)	30.3 (±0.8)	30.1 (±0.8)	27.4 (±0.8)	29.0 (±2.6)

3.3.10 Repetitive sequence-based PCR (rep-PCR) fingerprinting

Thirty-one isolates of *Salmonella* serovars Saintpaul (n = 18), Newport (n = 4), Papuana (n = 2), and Escanaba (n = 7) were selected based on availability of *Salmonella* serovars in tilapia and duck samples to perform rep-PCR DNA fingerprinting. The rep-PCR fingerprint of each bacterium was generated using 2 primer sets, i.e., ERIC (ERIC1; 5′-ATGTAAGCTCCTGGGGATTCAC-3′, ERIC2; 5′-AAGTAAGTGACTGGGGTGAGCG-3′ and $(GTG)_5$; 5′-GTGGTGGTGGTGGTG-3′ (Prasertsee et al., 2019; Santiyanont et al., 2019). In brief, the 25 µl PCR mixture consist of 100 ng bacterial DNA, 1× *Ex Taq* buffer, 0.2mM dNTPs, 0.8 µM of primer, and 0.625 U of *Ex Taq* DNA polymerase (Takara Bio Inc., Shiga, Japan). The PCR conditions were as follows: one cycle of denaturation at 95 ºC for 5 min, 30 cycles of denaturation at 94 ºC for 45 s, annealing at 52 ºC for ERIC and 40 ºC for $(GTG)_5$ for 1 min, extension at 65 ºC for 10 min and final extension at 65 ºC for 20 min. Five microliters of PCR product were separated using 1% agarose gel electrophoresis. GelComparII version 5.10 (Applied Maths BVBA, Kortrijk, Belgium) was used for clustering of ERIC and $(GTG)_5$ fingerprint patterns. The fingerprint dendrogram was calculated using Unweighted Pairgroup Method with Arithmetic Means (UPGMA) based on composite data between ERIC and $(GTG)_5$.

3.3.11 Statistical analyses

One-way analysis of variance (ANOVA) with multiple post-hoc tests were used to test the association between concentrations of fecal coliforms and *E. coli* and type of sample. Similarly, Pearson's chi-square tests of independence were performed to test the association between the presence of *Salmonella*, *A. hydrophila*, *V. cholerae*, and *V. vulnificus* and type of sample. The distribution of predominant *Salmonella* serovars for each sampling event was illustrated using a heatmap Displayr (http://www.displayr.com). The association between the

presence of *Salmonella* in the sample and various risk factors (i.e., presence of bacterial species evaluated in this study other than *Salmonella* , weather and other environmental parameters, water quality parameters) was performed using logistic regression, with a *P*-value ≤ 0.05 based on the likelihood ratio test considered statistically significant and odds ratios calculated for the association between testing positive for *Salmonella* between the referent and comparison categories. Analyses were performed using Stata version 14.0 (StataCorp, College Station, TX, USA).

3.4 Results

3.4.1 Prevalence of fecal coliforms and *E. coli*

The overall prevalence of fecal coliforms and *E. coli* were 70.8% (340/480) and 50.0% (249/480), respectively, for the combined fish samples (carcass rinses, meat, intestines, liver and kidney), and 90.8% (109/120) and 75.8% (91/120) for the cultivation water), respectively (Table 2). Fish samples with the highest prevalence of fecal coliforms and *E. coli* were from fish carcass rinses (90.8%, 109/120) and intestines (84.2%, 101/120), respectively. The mean concentration of fecal coliforms and *E. coli* were highest in fish intestines (2.4×10^4 and 1.2×10^4 MPN/g, respectively). In contrast, fish muscle contained the lowest prevalence of fecal coliforms (45.0%, 54/120) and *E. coli* (14.2%, 17/120). For cultivation water, the prevalence for fecal coliforms and *E. coli* was 90.8% (109/120) and 75.8% (91/120), respectively, and the mean concentration of these indicator bacteria was 1.4×10^2 and 1.9×10^1 MPN/ml, respectively. Mean concentration of fecal coliforms in fish intestine was significantly higher than that of cultivation water, carcass rinses, and muscle (*P*-value < 0.0001); similarly, the mean concentration of *E. coli* in fish intestine was higher than other samples (*P*-value < 0.0001).

Table 2. Prevalence and concentration (± sd) of fecal coliforms and *E. coli* in hybrid red tilapia and aquaculture cultivation water (n = 600).

Type of sample (*n*)	No. of positive (%)		Concentration (± sd) of positive samples (MPN/g or MPN/ml)	
	Fecal coliforms	*E. coli*	Fecal coliforms	*E. coli*
Carcass rinse	109	89	4.1×10^3	2.0×10^3
(n = 120)	(90.8%)	(74.2%)	($\pm 1.8 \times 10^4$)	($\pm 1.1 \times 10^4$)
Muscle	54	17	2.6×10^1	0.2×10^1
(n = 120)	(45.0%)	(14.2%)	($\pm 2.2 \times 10^2$)	($\pm 0.7 \times 10^1$)
Intestine	108	101	2.4×10^4	1.2×10^4
(n = 120)	(90.0%)	(84.2%)	($\pm 4.0 \times 10^4$)	($\pm 2.9 \times 10^4$)
Liver and kidney	69	42	8.9×10^1	1.0×10^1
(n = 120)	(57.5%)	(35.0%)	($\pm 27 \times 10^2$)	($\pm 4.9 \times 10^1$)
Total	340	249	9.0×10^3	1.1×10^4
(n = 480)	(70.8%)	(50.0%)	($\pm 2.7 \times 10^4$)	($\pm 2.3 \times 10^4$)
Cultivation water	109	91	1.4×10^2	1.9×10^1
(n = 120)	(90.8%)	(75.8%)	($\pm 2.8 \times 10^2$)	($\pm 4.8 \times 10^1$)
Grand total	**449**	**340**	**5.6×10^3**	**2.8×10^3**
(n = 600)	**(74.8%)**	**(56.7%)**	**($\pm 1.6 \times 10^4$)**	**($\pm 1.1 \times 10^4$)**

3.4.2 Prevalence of pathogenic bacteria

Salmonella exhibited the highest overall sample prevalence of 23.0% compared to *V. cholerae* (17.5%), *A. hydrophila* (2.5%), and *V. vulnificus* (1.7%); no samples tested positive for *S. agalactiae* (Table 3). The prevalence for *A. hydrophila* in fish carcass rinses was 8.3% (10/120) and 4.3% (5/120) in cultivation water. *V. cholerae* was common in cultivation water (38.3%, 46/120) and fish intestine (20.8%, 25/120). Pearson's chi-square test indicated a significant association between sample type and presence of pathogens, including *Salmonella*, *A. hydrophila*,

V. cholerae, and *V. vulnificus*. Our results demonstrated that *Salmonella* was most abundant in cultivation water (58.3%, 70/120), followed by fish intestine (38.3%, 46/120) and carcass rinses (17.5%, 21/120). No muscle samples tested positive for the presence of *Salmonella* and *V. vulnificus*, and only ~2-3% tested positive for *A. hydrophila* and *V. cholera.*

Table 3. Distribution of *Salmonella* spp., *A. hydrophila*, *V. vulnificus*, *V. cholerae*, and *S. agalactiae* in hybrid red tilapia (n = 480) and cultivation water (n = 120).

Type of sample (n)	**No. of positive (%)**			
	Salmonella	***A. hydrophila***	***V. vulnificus***	***V. cholerae***
Carcass rinse (n = 120)	21 (17.5%)	10 (8.3%)	2 (1.7%)	24 (20.0%)
Muscle (n = 120)	0 (0%)	0 (0%)	0 (0%)	2 (1.7%)
Intestine (n = 120)	46 (38.3%)	0 (0%)	6 (5.0%)	25 (20.8%)
Liver and kidney (n = 120)	1 (0.8%)	0 (0%)	1 (0.8%)	8 (6.7%)
Total (n = 480)	68 (14.1%)	10 (2.0%)	9 (1.9%)	59 (12.3%)
Cultivation water (n = 120)	70 (58.3%)	5 (4.2%)	1 (0.8%)	46 (38.3%)
Grand total (n = 600)	**138 (32.0%)**	**15 (2.5%)**	**10 (1.7%)**	**105 (17.5%)**

As shown in Table 4, the distribution of *Salmonella* serotypes was Saintpaul (18.9%, 74/394), Neukoelln (15.2%, 60/394), Escanaba (15.2%, 60/394), Papuana (15.0%, 59/394), and Virchow (8.6%, 34/394). In the hybrid red tilapia, the most common serotypes were Saintpaul (25.4%, 47/185), followed by Escanaba (23.8%, 44/185), Neukoelln (14.1%, 26/185) and Papuana (13.5%, 25/185). On the other hand, the predominant serotypes for cultivation water were Neukoelln (16.3%, 34/209), Papuana (16.3%, 34/209), and Saintpaul (12.9%, 27/209); for grazing duck feces, the *Salmonella* serotypes were Saintpaul (36.7%, 11/30), Escanaba (10.0%, 3/30), Fillmore (10.0%, 3/30), and Newport (10.0%, 3/30).

Table 4. *Salmonella* serovars isolated from red tilapia (n = 480) and aquaculture cultivation water (n = 120).

Salmonella serotype	Number of isolate† (%)				Total (%) (n = 394)
	Fish Carcass rinse (n = 61)	Intestine (n = 121)	Liver and kidney (n = 3)	Water (n = 209)	
Athinai	0 (0%)	0 (0%)	0 (0%)	2 (1.0%)	2 (0.5%)
Augustenborg	0 (0%)	0 (0%)	0 (0%)	2 (1.0%)	2 (0.5%)
Bradford	0 (0%)	0 (0%)	0 (0%)	1 (0.5%)	1 (0.3%)
Braenderup	0 (0%)	1 (0.8%)	0 (0%)	0 (0%)	1 (0.3%)
Brazzaville	0 (0%)	0 (0%)	0 (0%)	1 (0.5%)	1 (0.3%)
Breukelen	0 (0%)	0 (0%)	0 (0%)	3 (1.4%)	3 (0.8%)
Chartres	0 (0%)	3 (2.5%)	0 (0%)	3 (1.4%)	6 (1.5%)
Chester	0 (0%)	0 (0%)	0 (0%)	3 (1.4%)	3 (0.8%)
Derby	0 (0%)	2 (1.7%)	0 (0%)	0 (0%)	2 (0.5%)
Enteritidis	2 (3.3%)	2 (1.7%)	0 (0%)	0 (0%)	4 (1.0%)
Escanaba	21 (34.4%)	23 (19.0%)	0 (0%)	16 (7.7%)	60 (15.2%)

Table 4. *Salmonella* serovars isolated from red tilapia (n = 480) and aquaculture cultivation water (n = 120). (Continue)

Salmonella serotype	Number of isolate† (%)				
	Fish Carcass rinse (n = 61)	Intestine (n = 121)	Liver and kidney (n = 3)	Water (n = 209)	Total (%) (n = 394)
Galiema	0 (0%)	1 (0.8%)	0 (0%)	0 (0%)	1 (0.3%)
Hiduddify	0 (0%)	0 (0%)	0 (0%)	3 (1.4%)	3 (0.8%)
II	0 (0%)	2 (1.7%)	0 (0%)	4 (1.9%)	6 (1.5%)
Koessen	0 (0%)	0 (0%)	0 (0%)	2 (1.0%)	2 (0.5%)
Larochelle	0 (0%)	0 (0%)	0 (0%)	1 (0.5%)	1 (0.3%)
Menden	0 (0%)	0 (0%)	0 (0%)	1 (0.5%)	1 (0.3%)
Montevideo	0 (0%)	5 (4.1%)	0 (0%)	21 (10.1%)	26 (6.6%)
Neukoelln	15 (24.6%)	8 (6.6%)	3 (100%)	34 (16.3%)	60 (15.2%)
Newport	0 (0%)	2 (1.7%)	0 (0%)	0 (0%)	2 (0.5%)
Othmarschen	0 (0%)	3 (2.5%)	0 (0%)	16 (7.7%)	19 (4.8%)
Papuana	4 (6.6%)	21 (17.4%)	0 (0%)	34 (16.3%)	59 (15.0%)
Paratyphi B	0 (0%)	4 (3.3%)	0 (0%)	1 (0.5%)	5 (1.3%)
Rending	0 (0%)	0 (0%)	0 (0%)	1 (0.5%)	1 (0.3%)
Saintpaul	11 (18.0%)	36 (29.8%)	0 (0%)	27 (12.9%)	74 (18.9%)
Schwabach	0 (0%)	0 (0%)	0 (0%)	2 (1.0%)	2 (0.5%)
Singapore	0 (0%)	0 (0%)	0 (0%)	3 (1.4%)	3 (0.8%)
Stanley	0 (0%)	0 (0%)	0 (0%)	5 (2.4%)	5 (1.3%)
Strathcona	1 (1.6%)	1 (0.8%)	0 (0%)	2 (1.0%)	4 (1.0%)
Typhimurium	0 (0%)	0 (0%)	0 (0%)	1 (0.5%)	1 (0.3%)
Virchow	7 (11.5%)	7 (5.8%)	0 (0%)	20 (9.6%)	34 (8.6%)
Total	61 (100%)	121 (100%)	3 (100%)	209 (100%)	394 (100%)

† Samples from fish muscle were not positive for *Salmonella* spp.

3.4.3 Logistic regression analyses for determination of risk factors associated with *Salmonella*

Logistic regression analyses indicated that the odds of detecting *Salmonella* in the fish and water samples were associated with the co-occurrence of fecal coliforms (OR 3.5, CI: 1.1-11.2), *E. coli* (OR 2.9, CI: 2.3-3.9), *V. cholera* (OR 2.3, CI: 1.2-4.4), and *V. vulnificus* (OR 2.5, CI: 1.7-3.8) (Table 5). In addition, the odds of detecting *Salmonella* were positively associated with the mean 7-day maximum wind gusts (m/s) and negatively associated with the mean 7-day RH (*P*-value < 0.05). Specifically, the odds of detecting *Salmonella* increased 1.08-times (OR 1.08) for each additional m per second increase in maximum wind gust; in contrast, the odds of detecting *Salmonella* decreased 0.97 (OR 0.97) times for each percentage increase in RH.

Table 5. Logistic regression model for the association between the odds of detecting *Salmonella* in the hybrid red tilapia (n = 480) and cultivation water (n = 120), and the various microbiological and environmental parameters.

Parameter	Odds ratio	SE	CI	*P*-value
The presence of fecal coliforms	3.51	2.08	1.10-11.24	0.034
The presence of *E. coli*	2.94	0.41	2.25-3.86	<0.0001
The presence of *V. vulnificus*	2.50	0.53	1.65-3.79	<0.0001
The presence of *V. cholerae*	2.32	0.76	1.22-4.40	<0.0001
Average maximum wind gust (m/s)†	1.08	0.02	1.05-1.12	<0.0001
Average RH†	0.97	0.0092	0.95-0.97	0.001
Constant	0.23	0.22	0.032-1.55	0.131

AIC = 465.52.

† Average over 7 consecutive days.

Note: SE, standard error; CI, confidence interval; AIC, Akaike Information Criterion.

3.4.4 Fingerprinting of *Salmonella* serovars among tilapia, cultivation water, and duck fecal materials

The dendrogram of rep-PCR showed five major clades of *Salmonella*, designated as A, B, C, D, and E (Figure 2), all of which contained both fish and duck feces isolates. The cut-off value of the dendrogram was established at 80%, with the five clades highly segregated by serovar. Clade A, with the largest number of sequence-similar isolates, was comprised of only serovar Saintpaul, which were isolated from grazing duck feces, fish intestines, cultivation water, and fish carcass rinses. Clade B contained an identical genetic profile for four *S.* Newport isolates from duck feces and fish intestines. Clade C contained only two highly related isolates of *S.* Papuana, both from duck feces. Clades D and E were comprised of only *S.* Escanaba with isolates from grazing duck feces, fish intestines, and cultivation water.

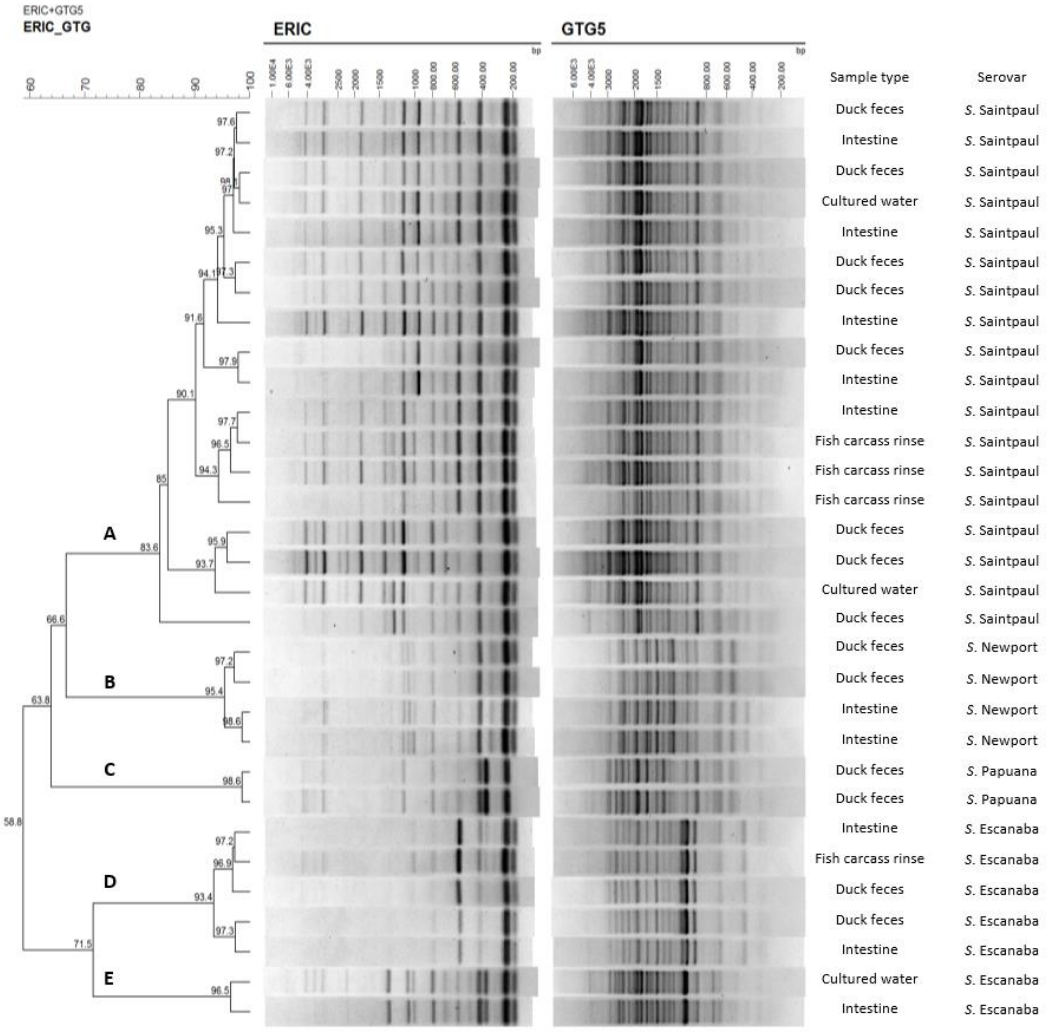

Figure 2. The dendrogram of rep-PCR profiles of *Salmonella* isolates from hybrid red tilapia and nearby duck feces

3.5 Discussion

It is common practice to monitor indicator bacteria to evaluate water quality and aquaculture sanitation supporting fish production. The national standard of microbiological quality criteria for food and food contact containers in Thailand indicates that fish destined for human consumption should not have any detectable *Salmonella* spp. and *V. cholerae*, with the acceptable levels of *Staphylococcus*

aureus and *E. coli* being <100 CFU/g and <10 MPN/g, respectively (BQSF, 2017). The International Commission on Microbiological Specifications for Foods (ICMSF) standard also recommends that the concentrations of fecal coliform and *E. coli* should not exceed 10 MPN/g (ICMSF, 1978). In this study, 62.3% (299/480) of the fish samples met the ICMSF and BQSF standard. Most of the fish muscle (94.2%, 113/120) contained no detectable *E. coli*, while 6% (7/120) of the muscle samples exceeded these *E. coli* standards.

Fish carcass rinses showed the highest prevalence of fecal coliforms. Our findings are consistent with a previous report suggesting that abundant indicator bacteria were found on tilapia skin surfaces due to possibly unhygienic aquaculture conditions (Rocha et al., 2014). Long-term exposure to waterborne microbial contaminants is likely a major cause for the observed high prevalence of indicator bacteria in fish intestines and internal organs. Although fish muscle contained low concentrations of bacteria, it is possible that fish muscle can cross-contaminate tilapia skin or intestinal tissue during fish preparation process. In this study, the levels of bacterial indicators in fish carcass rinses were similar to that of the cultivation water, which is consistent with the speculation that bacterial contamination in tilapia was largely the result of bacteria in cultivation water (Mandal et al., 2009).

The most frequently identified *Salmonella* serovars were Saintpaul, Escanaba, Neukoelln, Papuana, Virchow, and Montevideo. The prevalence of those six serovars in eight sampling events were not uniformly distributed across time (Figure 3). For example, there was an increase in the occurrence of serovar Escanaba at time points IV, V, and VII. *S.* Neukoelln and *S.* Papuana were commonly detected at sampling time points II to IV, whereas the highest prevalence of *S.* Saintpaul was found at sampling time points VI to VIII (Figure 3). Interestingly, *Salmonella* serovars detected

in this study were different from previous work. *Salmonella* serovars Brandenburg, Hadar, Heidelberg, and Saintpaul were reported in farmed fish in Brazil and Vietnam (Nguyen et al., 2016; dos Santos et al., 2019), whereas serovars Albany, Agona, Corvallis, Stanley, Typhimurium, Mikawasima, and Bovis-morbificans were observed in catfish and tilapia in Malaysia (Budiati et al., 2016). Even though *Salmonella* serovar Saintpaul was abundantly found in tilapia in this study, this serovar has been commonly reported in pork in Thailand (Pungpian et al., 2021). An outbreak of *Salmonella* Saintpaul, which was isolated from cucumbers was responsible for 84 illnesses in the U.S. (CDC, 2013).

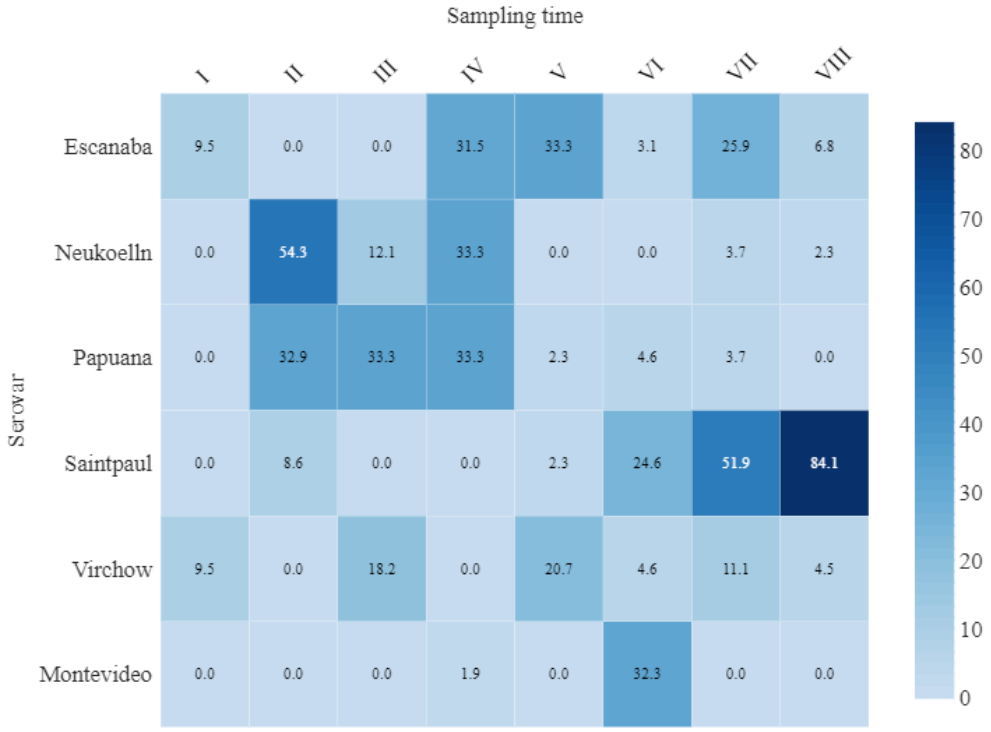

Figure 3. The gradient distribution of the major six *Salmonella* serovars isolated from hybrid red tilapia during eight sampling events (n = 328). The column is the sampling time point from I to VIII, and the row indicates *Salmonella* serovar isolation.

To identifying the source(s) of *Salmonella* contamination in tilapia aquaculture system would be challenging given that *Salmonella* is naturally found in the gastrointestinal tract of a wide variety of vertebrate animals. Grazing ducks raised nearby the tilapia production site were postulated as one potential source of *Salmonella* contamination. Serovars Saintpaul (36.7%) and Escanaba (10.0%) were commonly found in duck feces and were also detected as the major serovars present in the tilapia farms. Previous studies also suggested the presence of Saintpaul and Escanaba have been observed in livestock animals (Negi et al., 2015; Eguale et al., 2018; Awad et al., 2020).

Although previous studies compared molecular typing methods for *Salmonella* (Nath et al., 2010; Fendri et al., 2013; Ferrari et al., 2017), pulsed field gel electrophoresis (PFGE) was set as a standard method for many years for *Salmonella* typing. The discriminatory degree of various typing procedures was compared using 92 strains of *Salmonella* Typhimurium. PFGE, multiple-locus variable number of tandem repeats analysis (MLVA), and ERIC PCR differentiated 72, 53, and 63 types, respectively (Almeida et al., 2015). These findings indicated that the discriminatory efficiency of PFGE was greater than others, however, PFGE can be a time-consuming and labor-intensive method (Winokur, 2003). Despite the moderate discriminatory power, ERIC PCR is rapid and reproducibly distinguishes epidemiological relationships among groups of *Salmonella*. The discriminatory index of ERIC PCR was as high as 0.9981, and 0.983 in typing of 113, and 74 *Salmonella enterica* isolates, respectively (Winokur, 2003; Nath et al., 2010). Therefore, ERIC PCR was utilized in the present study to characterize DNA sequence similarity of serovars *S.* Saintpaul, *S.* Newport, and *S.* Escanaba in fish and with grazing ducks. Based on the DNA similarity of these isolates the results suggested that nearby grazing duck feces may be a source of *Salmonella* that can contaminate the tilapia cultivation site, but such data cannot

definitely prove the original source of contamination, either ducks to fish, fish to ducks, or some third *Salmonella* vertebrate reservoir contaminating both fish and ducks. For example, precipitation and subsequent overland flow could function to erode fecal materials from land-based sources and then runoff into tilapia cultivation sites.

V. vulnificus has been commonly found in estuarine water and shellfish with the prevalence ranging from 13.6% to 15% (Cruz et al., 2016; Baker-Austin and Oliver, 2018; King et al., 2021). However, *V. vulnificus* could also be found in freshwater tilapia raised in Taiwan and Egypt, and the prevalence ranging from 1.7% to 12.5% (Chen et al., 2006; Younes et al., 2016). In the U.S., *V. vulnificus* infection in humans had a high fatality rate compared to other foodborne pathogens, and these bacteria were responsible for more than 95% of seafood-related deaths (Jones and Oliver, 2009). In aquatic animals, the clinical signs of *V. vulnificus* infection include dark coloration, lethargy, and hemorrhage skin lesions. In this study, only a small proportion of the fish intestines and internal organs tested positive for *V. vulnificus* (0.8% to 5%), but none of the fish muscle were positive, indicating that the risk of *V. vulnificus* contamination might be small if wholesale processors and retail consumers of tilapia are careful during cleaning of fish and handling of filets.

In this study, the prevalence of *A. hydrophila* in fish was less than the previous studies (Ahmed et al., 2018; Zaher et al., 2021). The prevalence of *A. hydrophila* found in fish carcass rinses (8.3%) and cultivation water (4.2%). This may contribute from healthy fish were included in our study, while high prevalence of *A. hydrophila* was commonly detected in clinically diseased fish (Salem et al., 2020; Zaher et al., 2021). Even though *S. agalactiae* was not detected during this study, it should be concerned, because *S. agalactiae* sequence type 283 has been

associated with foodborne disease outbreak due to raw tilapia consumption (Barkham et al., 2019). *S. agalactiae* was also frequently reported in diseased tilapia in Thailand (Areechon et al., 2016; Niu et al., 2020).

These production sites were suitable for raising hybrid tilapia in cage-based system. On average, the quality of water, pH (6.8) and DO (7.8 mg/l) in this study were within the required ranges stated in the Thai Aquaculture Standard (TAS) for tilapia cultivation (pH: 6.5-8.0; DO>4 mg/l) (TAS, 2010). However, NH_3-N and alkalinity, which are the important parameters for cultivation quality, were not collected in the study. Further investigations should be performed to explore the quality of water for this tilapia cultivation.

The presence of *Salmonella* in the fish and cultivation water samples was significantly associated with the presence of fecal coliforms, *E. coli*, *V. vulnificus*, and *V. cholerae* (*P*-value < 0.05), with the odds of *Salmonella* detection being 2.3 to 2.5-times higher when these bacteria were also present. Seven-day mean maximum wind gusts and RH were positively and negatively associated with the odds of *Salmonella* in the samples, respectively. The highest wind gusts occurred during the sampling months of July and September; it is possible that high wind gusts could function to transfer *Salmonella* into the tilapia growing area from terrestrial sources, or this environmental parameter is collinear with some other unknown factor(s) causing the increase in bacterial levels. High RH was associated with a lower odds of *Salmonella* detection, which occurred especially during the sampling months of September and November, and the remainder of year having lower RH values (Table 1). Interestingly, a previous study observed that high RH (85%) enhanced the survival of *S*. Typhimurium in a controlled chamber environment (López-Gálvez et al., 2018).

More research would be needed to clarify the mechanism(s) causing these associations between weather parameters and the odds of *Salmonella* in tilapia.

In conclusion, the high levels of fecal coliforms and *E. coli* and the presence of pathogenic bacteria were observed during this study, indicating that food safety precautions are needed regarding human consumption of tilapia. Based on DNA similarity of bacterial isolates, nearby grazing ducks were identified as a potential source of *Salmonella* contamination for tilapia and the cultivation environment, but such speculations based on matching of DNA fingerprints does not prove causality and more data is needed to confirm this speculation. Good animal husbandry, effective farm biosecurity, and where possible, water treatment interventions for tilapia aquaculture may be helpful to reduce environmental levels of bacterial contamination. Furthermore, given the occurrence of bacterial pathogens in various tilapia tissues, it may be prudent to maintain hygienic processing of fish fillets, temperature control during, transport, food preparation and adequate cooking temperature to reduce the risk of bacterial transmission from cultivated tilapia to humans.

CHAPTER IV

Molecular epidemiology of antimicrobial resistance and extended-spectrum β-lactamase production of *Escherichia coli* isolated from farm-raised hybrid red tilapia

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Molecular epidemiology of antimicrobial resistance and extended-spectrum ß-lactamase production of *Escherichia coli* isolated from farm-raised hybrid red tilapia

4.1 Abstract

This study aimed to characterize phenotypic and genotypic of antimicrobial resistance (AMR), virulence genes, and extended-spectrum ß-lactamase (EBSL) production of *Escherichia coli* isolated from hybrid red tilapia and cultivation water. A total of 600 samples retrieved from fish meat (n = 120), intestine (n = 120), carcass rinse (n = 120), liver and kidney (n = 120), and cultivation water (n = 120) was collected from 2019-2020. Of all *E. coli* tested positive isolates, 79.6% (n = 265/333) were resistant to at least one antimicrobial, and 53.8% (n = 179/333) were multidrug resistance. ESBL production of *E. coli* were 3.9% (n = 13/333). The most common *E. coli* isolates were resistant to ampicillin (63.1%), oxytetracycline (58.6%), tetracycline (58.0%), and oxolinic acid (57.4%). AMP-OTC-OXO-TET (9.6%) was the predominant resistance pattern. The most common resistance genes were bla_{TEM} (58.0%), followed by *qnrS* (43.8%), *tetA* (29.1%), and *tetB* (23.7%). Most of AMR genes were detected in intestine. The *int1* gene was detected 19.5%. The bla_{TEM-1} (58.0%) and $bla_{CTX-M-55}$ (2.7%) were predominantly characterized. Therefore, this study indicated that *E. coli* isolated from hybrid red tilapia and cultivation water are potential reservoirs of AMR and their determinants. To mitigate the AMR problem, surveillance of AMR in aquaculture under One Health should be implemented.

Keywords: aquaculture; colistin; *Escherichia coli*; extended-spectrum ß-lactamase; tilapia

4.2 Introduction

Antimicrobial resistance (AMR) is one of the top ten health global threats that required urgent action plans to achieve the sustainable development goals (WHO, 2020). AMR is a challenging issue due to the increase of global emergence of AMR in environment, which an intricated link to human and animals under One Health. It is estimated that almost five million deaths per year occurs due to the infection of AMR bacteria (Murray et al., 2022). This infection can result in prolong hospital stays and increase healthcare costs. In Thailand, total cost of healthcare and treatment of the infection of multidrug resistant (MDR) bacteria are 1.3 billion and 202 million USD, respectively (Thamlikitkul et al., 2015). Estimated annual 45,206 deaths are associated with MDR infections in Thailand (Lim et al., 2016). This reflects that the AMR burden dramatically increases and becomes a significant public health threat.

Improper use and easily affordable antimicrobials are the major drivers contributing to widespread of AMR, and these served as selective pressure of AMR and MDR spreading (Harada and Asai, 2010). Extended-spectrum ß-lactamase (ESBL)-producing bacteria become a major public health concern, because these bacteria can resist to clinically important antibiotics included penicillins, third, fourth, and fifth generation cephalosporins. ESBL bacteria also can co-select with other antimicrobials, such as tetracycline, fluoroquinolone, aminoglycosides, and colistin (Tacão et al., 2014; Zhang et al., 2019). It is increasingly observed that 9,000 deaths caused by ESBL-producing *Enterobacteriaceae* in the United States (CDC, 2019). Hence, last-resort antibiotics such as carbapenem have been required to treat ESBL-producing bacteria.

Quinolones are widely used antimicrobials in humans and animals due to their potential treatment of Gram-positive and Gram-negative bacteria. Quinolone

resistance mainly contributed from chromosomal mutations in the Quinolone Resistance Determining Region (QRDR), which predominantly target at topoisomerase II (*gyrase A*) and IV (*parC*). Other mechanisms mediated quinolone resistance phenotype are overexpression of efflux pump (encoded by *qepA* gene), and the presence of plasmid mediated quinolone resistance (PMQR) genes, including *qnrA*, *qnrB*, *qnrS*, and *aac(6')-Ib-cr*. Resistance to quinolone can co-select with other antimicrobial classes, such as cephalosporins (Ma et al., 2018).

Extensive use of antimicrobials in aquaculture and heavy loads of resistant bacteria from run-off and wastewater can subsequently promote the spreading of AMR in aquatic environment (Adelowo et al., 2018). It is recommended that *E. coli* is an indicator bacterium that generally used for AMR monitoring and surveillance in food-producing animal and aquaculture (EFSA et al., 2019; Noordin et al., 2020). Even though AMR in aquaculture and environment is increasingly concerned, the study of AMR phenotypes and genotypes is still limited. Therefore, the objectives of this study were to determine AMR, virulence genes, and ESBL production of *E. coli* isolated from hybrid red tilapia and cultivation water, and to observe mutations of *gyrA* and *parC* in the QRDR of ciprofloxacin-resistant *E. coli* isolates.

4.3 Materials and methods

4.3.1 Sample collection and preparation

Hybrid red tilapia (*Oreochromis mossambicus* and *O. niloticus*) were raised in cage-cultured method located in Kanchanaburi province, Thailand. Five hybrid red tilapia were collected from one farm and three out of five farms were selected for each sampling event depended on availability of fish. Eight consecutive sampling were performed every 1 to 2 month-period. A total of 600 samples, including fish

carcass rinse (n = 120), fish meat (n = 120), intestine (n = 120), liver and kidney (n = 120), and cultivation water (n = 120) were collected from 2019 to 2020. Healthy fish were caught by hand-net, euthanized by immersion in clove oil (0.1 g/l), and kept in a sterile plastic bag. Cultivation water was collected into a sterile propylene bottle from the same cage as fish collection at depth 40-60 cm from the surface. Enrofloxacin and oxytetracycline are two antimicrobials used as feed medicated in farms. These antimicrobials were applied when observation of the reduction of feed intake and abnormal mortality were detected.

All samples were transported at refrigerated temperature (< 10 ºC) and processed within 24 hr at the Department of Veterinary Public Health, Faculty of Veterinary Science, Chulalongkorn University. Fish surface was thorough washed using buffer peptone water (BPW) (Difco, MD, USA) to received fish carcass rinse. After that, fish surface was decontaminated using 70% ethyl alcohol and aseptically dissected to collect meat, kidney and liver, and intestine for further bacterial determination.

4.3.2 *E. coli* isolation and confirmation

The detection of *E. coli* followed the U.S. Food and Drug Administration's Bacteriological Analytical Manual (U.S. FDA BAM) (Feng et al., 2002). Briefly, 25 g of fish meat, 1 g of intestine, 1 g of liver and kidney, 25 ml of fish carcass rinse, and 25 ml of cultivation water were individually mixed with BPW (Difco) and transfer to lactose broth (Difco). After incubation at 37 ºC for 24 hr, a loopful of LB tubes was transferred to EC broth (Difco) and incubated at 44.5 ºC in water bath. A loopful of positive tubes were streaked on Levine-Eosin-Methylene Blue (L-EMB) (Difco) agar. Suspected colonies of *E. coli* were confirmed using indole and catalase test. One isolate per one positive sample was used for antimicrobial susceptibility testing (AST).

4.3.3 Antimicrobial susceptibility testing

AST was performed using agar dilution method according to standard protocol (CLSI, 2013). *E. coli* isolates (n = 333) were grown on Mueller-Hinton agar (MHA) (Difco) and incubated at 37 ºC for 24 hr. Antimicrobials were selected based on frequently used in human and veterinary medicine, including ampicillin, chloramphenicol, ciprofloxacin, enrofloxacin, florfenicol, gentamicin, oxytetracycline, oxolinic acid, streptomycin, sulfamethoxazole, tetracycline, and trimethoprim. Minimum inhibitory concentration (MIC) was recorded. *Staphylococcus aureus* ATCC 29213, *E. coli* ATCC 25922, and *Pseudomonas aeruginosa* ATCC 27853 were used as quality control strains.

4.3.4 Phenotypic detection of ESBL-producing *E. coli*

The ESBL production of *E. coli* isolates was examined by disk diffusion method (CLSI, 2013). Three antibiotic disks of ceftazidime (30 µg), cefotaxime (30 µg), and cefpodoxime (10 µg) (Oxoid, England, UK) were used for screening test. The *E. coli* isolates that resist at least one of cephalosporins was further confirmed using the combination disk diffusion method. Ceftazidime (30 µg), cefotaxime (30 µg), and these two disks with clavulanic acid were used for EBSL confirmation. The *E. coli* isolates with the difference of inhibition zone of single cephalosporin and cephalosporin containing clavulanic acid ≥ 5 mm is considered as ESBL positive isolates.

4.3.5 Detection AMR genes, virulence genes, integrons, and SXT element

The genomic DNA template was extracted using whole cell boiling method (Lévesque et al., 1995). All primer used were listed (Table 6). Selected AMR genes were corresponding to AMR phenotype as followed: $bla_{TEM,}$ bla_{SHV}, bla_{CTX-M}, and bla_{PSE} encoding β-lactam resistance and ESBL production; bla_{NDM} and bla_{OXA} encoding carbapenem resistance; *catA*, *catB*, *floR*, and *cmlA* encoding phenicol resistance;

ermB encoding erythromycin resistance; *qnrA*, *qnrB*, *qnrS*, *aac(6')-Ib-cr*, and *qepA* encoding quinolone resistance; *aadA1*, *aadA2*, and *aac(3)IV* encoding gentamicin resistance; *tetA*, *tetB,* and *tetD* encoding tetracycline resistance; *strA*, and *strB*, encoding streptomycin resistance; *sul1*, *sul2*, and *sul3* encoding sulfonamide resistance; *dfrA1* and *dfrA12* encoding trimethoprim resistance; *mcr-1* to *mcr-5* encoding colistin resistance. Integrons (*int1*, *int2,* and *int3*), virulence genes (*stx1* and *stx2*), and SXT element (int_{SXT}) were also detected.

PCR reaction contained 25 µl of TopTaq DNA polymerase (Qiagen, Stockach, Germany), 1X PCR buffer, 200 µM of each dNTP, 0.5 µl of each forward and reverse primer of 10 µM concentration, and 5 µl of DNA template to receive a final volume of 50 µl. All amplified PCR products were performed in gel electrophoresis in 1.5% (w/v) agarose gel and stained with Redsafe™ nucleic acid staining solution (Intron Biotechnology, Seongnam, Republic of Korea). The results were visualized by Omega Fluor™ gel documentation system (Aplegen, CA, USA).

4.3.6 Nucleotide sequencing of ESBL and QRDR

The *E. coli* carried bla_{TEM} (n = 193) and bla_{CTX} (n = 9) were used to nucleotide sequencing. The isolates contained the mutation of QRDR associated with fluoroquinolone resistance were selected based on the levels of ciprofloxacin resistance. The resistant *E. coli* isolates (n = 22) to ciprofloxacin were randomly selected and classified as low resistance (1-16 µg/ml) (n = 10), and high resistance (32-128 µg/ml) (n = 12). Eight susceptible ciprofloxacin isolates were served as negative control strains. To observe the mutation of QRDR, all *E. coli* isolates were amplified for *gyrA* and *parC* using primers listed in Table 6.

All PCR products were purified and sequenced (Bionics Co., LTD., Gyeonggi-Do, Republic of Korea). The sequences were analyzed using Molecular Evolutionary

Genetic Analysis (Mega) software version 11 (Tamura et al., 2021). The reference sequences were downloaded in the GenBank database available at the National Centre for Biotechnology Information (NCBI) (https://www.ncbi.nlm.nih.gov).

Table 6. Primers used to detect virulent and AMR genes of *E. coli* isolates (n = 333).

Gene	Primer	Oligonucleotide sequences	Product size (bp)	Reference
Resistance gene				
bla$_{TEM}$	*bla*$_{TEM}$-F	GCGGAACCCCTATTT	964	(Olesen et al., 2004)
	bla$_{TEM}$-R	TCTAAAGTATATATGAGTAAACTT GGTCTGAC		
bla$_{SHV}$	*bla*$_{SHV}$-F	TTCGCCTGTGTATTATCTCCCTG	854	(Hasman et al., 2005)
	bla$_{SHV}$-R	TTAGCGTTGCCAGTGYTG		
bla$_{CTX-M}$	*bla*$_{CTX-M}$-F	CGATGTGCAGTACCAGTAA	585	(Batchelor et al., 2005)
	bla$_{CTX-M}$-R	AGTGACCAGAATCAGCGG		
bla$_{NDM}$	*bla*$_{NDM}$-F	GGTTTGGCGATCTGGTTTTC	621	(Poirel et al., 2011)
	bla$_{NDM}$-R	CGGAATGGCTCATCACGATC		
bla$_{PSE}$	*bla*$_{PSE}$-F	GCTCGTATAGGTGTTTCCGTTT	575	(Li et al., 2013)
	bla$_{PSE}$-R	CGATCCGCCGAHTGTTCCATCC		
bla$_{OXA}$	*bla*$_{OXA}$-F	ACACAATACATATCAACTTCGC	813	(Costa et al., 2006)
	bla$_{OXA}$-R	AGTGTGTGTTTAGAATGGTGATC		
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		
qnrA	*qnrA*-F	AGAGGATTTCTCACGCCAGG	580	(Cattoir et al., 2007)
	qnrA-R	TGCCAGGCACAGATCTTGAC		
qnrB	*qnrB*-F	GGMATHGAAATTCGCCACTG	264	(Cattoir et al., 2007)
	qnrB-R	TTTGCYGYYCGCCAGTCGAAC		

Table 6. Primers used to detect virulent and AMR genes of *E. coli* isolates (n = 333). (Continue)

Gene	Primer	Oligonucleotide sequences	Product size (bp)	Reference
qnrS	*qnrS*-F	GCAAGTTCATTGAACAGGGT	428	(Cattoir et al., 2007)
	qnrS-R	TCTAAACCGTCGAGTTCGGCG		
ermB	*ermB*-F	AGACACCTCGTCTAACCTTCGCTC	640	(Raissy et al., 2012)
	ermB-R	TCCATGTACTACCATGCCACAGG		
dfrA1	*dfrA1*-F	GGAGTGCCAAAGGTGAACAGC	367	(Shahrani et al., 2014)
	dfrA1-R	GAGGCGAAGTCTTGGGTAAAAAC		
dfrA12	*dfrA12*-F	TTCGCAGACTCACTGAGGG	330	(Chuanchuen et al., 2008a)
	dfrA12-R	CGGTTGAGACAAGCTCGAAT		
catA	*catA*-F	CCAGACCGTTCAGCTGGATA	454	(Chuanchuen et al., 2008a)
	catA-R	CATCAGCACCTTGTCGCCT		
catB	*catB*-F	CGGATTCAGCCTGACCACC	461	(Chuanchuen et al., 2008a)
	catB-R	ATACGCGGTCACCTTCCTG		
cmlA	*cmlA*-F	TGGACCGCTATCGGACCG	641	(Chuanchuen et al., 2008a)
	cmlA-R	CGCAAGACACTTGGGCTGC		
strA	*strA*-F	TGGCAGGAGGAACAGGAGG	405	(Chuanchuen et al., 2008a)
	strA-R	AGGTCGATCAGACCCGTGC		
strB	*strB*-F	GGCAGCATCAGCCTTATAATTT	470	(Mala et al., 2016)
	strB-R	GTGGATCCGTCATTCATTGTT		
tetA	*tetA*-F	GGCGGTCTTCTTCATCATGC	502	(Khan et al., 2019)
	tetA-R	CGGCAGGCAGAGCAAGTAGA		
tetB	*tetB*-F	CGCCCAGTGCTGTTGTTGTC	615	(Chuanchuen et al., 2008a)
	tetB-R	CGCGTTGAGAAGCTGAGGTG		
tetD	*tetD*-F	AAACCATTACGGCATTCTGC	787	(Kumai et al., 2005)
	tetD-R	GACCGGATACACCATCCATC		
addA1	*addA1*-F	CTCCGCAGTGGATGGCGG	631	(Chuanchuen et al., 2008a)
	addA1-R	GATCTGCGCGCGAGGCCA		
addA2	*addA2*-F	CATTGAGCGCCATCTGGAAT	500	(Chuanchuen et al., 2008b)
	addA2-R	ACATTTCHCTCATCGCCGGC		

Table 6. Primers used to detect virulent and AMR genes of *E. coli* isolates (n = 333). (Continue)

Gene	Primer	Oligonucleotide sequences	Product size (bp)	Reference
aac(3)IV	*aac(3)IV*-F	GTGTGCTGCTGGTCCACAGC	627	(Stoll et al., 2012)
	aac(3)IV-R	AGTTGACCCAGGGCTGTCGC		
aac(6')-Ib-cr	*aac(6')-Ib-cr*-F	TTGCGATGCTCTATGAGTGGCTA	482	(Park et al., 2006)
	aac(6')-Ib-cr-R	CTCGAATGCCTGGCGTGTTT		
qepA	*qepA*-F	GCAGGTCCAGCAGCGGGTAG	199	(Yamane et al., 2008)
	qepA-R	CTTCCTGCCCGAGTATCGTG		
floR	*floR*-F	ATGGTGATGCTCGGCGTGGGCCA	800	(Ying et al., 2019)
	floR-R	GCGCCGTTGGCGGTAACAGACACC GTGA		
mcr-1	*mcr-1-F*	AGTCCGTTTGTTCTTGTGGC	320	(Rebelo et al., 2018)
	mcr-1-R	AGATCCTTGGTCTCGGCTTG		
mcr-2	*mcr-2-F*	CAAGTGTGTTGGTCGCAGTT	715	(Rebelo et al., 2018)
	mcr-2-R	TCTAGCCCGACAAGCATACC		
mcr-3	*mcr-3-F*	AAATAAAAATTGTTCCGCTTATG	929	(Rebelo et al., 2018)
	mcr-3-R	AATGGAGATCCCCGTTTTT		
mcr-4	*mcr-4-F*	TCACTTTCATCACTGCGTTG	1116	(Rebelo et al., 2018)
	mcr-4-R	TTGGTCCATGACTACCAATG		
mcr-5	*mcr-5-F*	ATGCGGTTGTCTGCATTTATC	1644	(Rebelo et al., 2018)
	mcr-5-R	TCATTGTGGTTGTCCTTTTCTG		
Integrons				
int1	*int1*-F	CCTGCACGGTTCGAATG	497	(Kitiyodom et al., 2010)
	int1-R	TCGTTTGTTCGCCCAGC		
int2	*int2*-F	GGCAGACAGTTGCAAGACAA	247	(Kitiyodom et al., 2010)
	int2-R	AAGCGATTTTCTGCGTGTTT		
int3	*int3*-F	CCGGTTCAGTCTTTCCTCAA	155	(Kitiyodom et al., 2010)
	int3-R	GAGGCGTGTACTTGCCTCAT		
Integrative and conjugative elements				
int_{sxt}	int_{SXT}-F	GCTGGATAGGTTAAGGGCGG	592	(Kitiyodom et al., 2010)
	int_{SXT}-R	CTCTATGGGCACTGTCCACATTG		

Table 6. Primers used to detect virulent and AMR genes of *E. coli* isolates (n = 333). (Continue)

Gene	Primer	Oligonucleotide sequences	Product size (bp)	Reference
Virulence genes				
stx1	*stx-1*-F	CAACACTGGATGATCTCAG	138	(Khan et al., 2002)
	stx-1-R	CCCCCTCAACTGCTAATA		
stx2	*stx-2*-F	ATCAGTCGTCACTCACTGGT	349	(Khan et al., 2002)
	stx-2-R	CTGCTGTCACAGTGACAAA		
QRDR				
gyrA	*gyrA*-F	GCTGAAGAGCTCCTATCTGG	436	(Chuanchuen and
	gyrA-R	GGTCGGCATGACGTCCGG		Padungtod, 2009)
parC	*parC*-F	GTACGTGATCATGGATCGTG	390	(Chuanchuen and
	parC-R	TTCCTGCATGGTGCCGTCG		Padungtod, 2009)

4.3.7 Statistical analysis

Descriptive statistic was used to determine the prevalence of phenotypic and genotypic resistance, virulence genes, integrons, and SXT element. Chi-square tests were used to examine the association between sample type and antimicrobials. Logistic regression models were used to examine the association between AMR and their determinants. All analyses were considered statistical significance with a *P*-value ≤ 0.05 based on the likelihood ratio test. All statistical analysis were performed using Stata version 14.0 (StataCorp, College Station, TX, USA).

4.4 Results

The overall prevalence of *E. coli* isolated from hybrid red tilapia and cultivation water was 55.5% (n = 333). *E. coli* 79.6% (n = 265) were resistant to at least one antimicrobial. MDR bacteria were observed 53.8% (n = 179) with the

highest prevalence of AMR found in the intestine (Table 7). The most common resistance were ampicillin (63.1%), followed by oxytetracycline (58.6%), tetracycline (58.0%), and oxolinic acid (57.4%) (Table 7). The association between sample type and antimicrobials were observed from florfenicol (*P*-value = 0.019) and oxytetracycline (*P*-value = 0.027). All of *E. coli* isolates were resistant to gentamicin at low rate. Sixty-nine AMR patterns were examined (Table 8). The most common resistance patterns were AMP-OTC-OXO-TET (9.6%), AMP-OTC-TET (5.4%), and CIP-ENR-OXO (5.4%). Interestingly, the prevalence of ciprofloxacin and tetracycline resistance increased based on the direction of waterflow from farm 2 to farm 5 (Figure 4). Similarly, the increasing resistance levels along the waterflow were observed in ampicillin, streptomycin, and oxytetracycline.

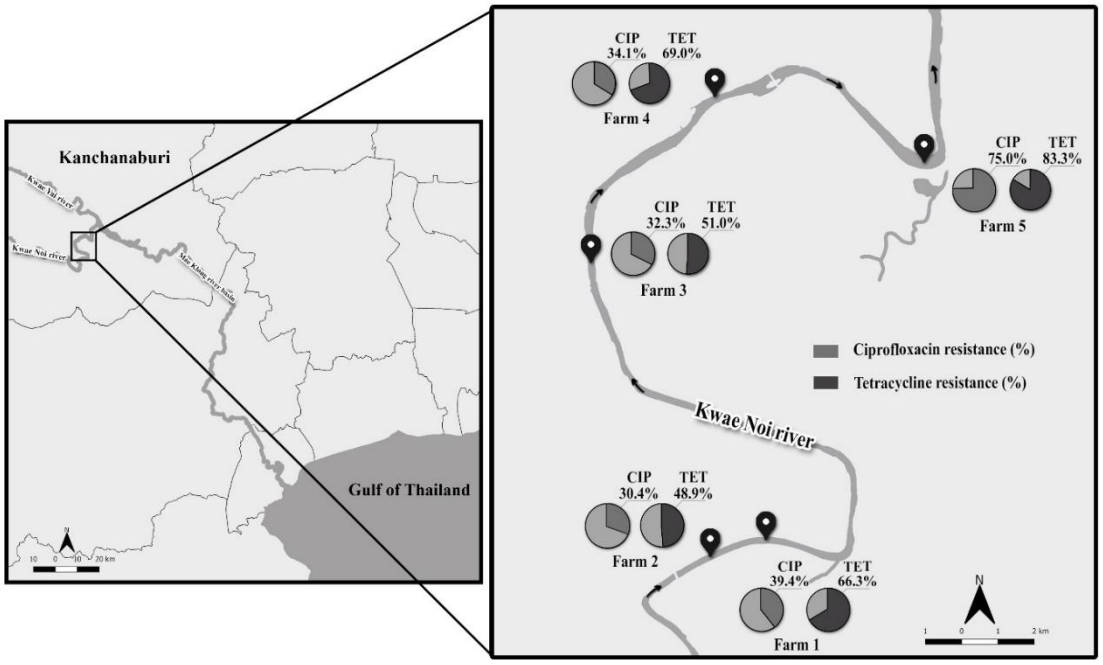

Figure 4. Spatial distribution of ciprofloxacin (CIP) and tetracycline (TET) resistance in *E. coli* isolates (n = 333)

Table 7. Phenotypic resistance of *E. coli* (n = 333) isolated from hybrid red tilapia and cultivation water.

Antimicrobials	Resistance (%)					
	Cultivation water (n = 82)	Carcass rinse (n = 88)	Intestine (n = 99)	Fish meat (n = 22)	Liver and kidney (n = 42)	Grand total (n = 333)
Ampicillin	53 (64.6)	51 (58.0)	71 (71.7)	15 (68.2)	20 (47.6)	210 (63.1)
Chloramphenicol	13 (15.9)	12 (13.6)	27 (27.3)	5 (22.7)	11 (26.2)	68 (20.4)
Ciprofloxacin	25 (30.5)	32 (36.4)	40 (40.4)	6 (27.3)	13 (31.0)	116 (34.8)
Streptomycin	17 (20.7)	15 (17.0)	31 (31.3)	6 (27.3)	9 (21.4)	78 (23.4)
Sulfamethoxazole	24 (29.3)	29 (33.0)	37 (37.4)	8 (36.4)	14 (33.3)	112 (33.6)
Tetracycline	49 (59.8)	49 (55.7)	65 (65.7)	13 (59.1)	17 (40.5)	193 (58.0)
Enrofloxacin	26 (31.7)	31 (35.3)	37 (37.4)	5 (22.7)	13 (31.0)	112 (33.6)
Gentamicin	5 (6.1)	6 (6.8)	10 (10.1)	4 (18.2)	0 (0)	25 (7.5)
Oxytetracycline	49 (59.8)	49 (55.7)	68 (68.7)	13 (59.1)	16 (38.1)	195 (58.6)
Oxolinic acid	41 (50.0)	50 (56.8)	63 (63.6)	13 (59.1)	24 (57.1)	191 (57.4)
Trimethoprim	17 (20.7)	18 (20.5)	34 (34.3)	6 (27.3)	6 (14.3)	81 (24.3)
Florfenicol	15 (18.3)	15 (17.0)	34 (34.3)	7 (31.8)	8 (19.0)	79 (23.7)
MDR	40 (48.8)	45 (51.1)	64 (64.7)	13 (59.1)	17 (40.5)	179 (53.8)

Table 8. Resistance pattern of *E. coli* (n = 333) isolated from hybrid red tilapia and cultivation water.

Resistance pattern	No of isolates (%)					
	Cultivation water (n = 82)	Fish carcass rinse (n = 88)	Intestine (n = 99)	Fish meat (n = 22)	Liver and kidney (n = 42)	Total (n = 333)
Susceptible	20 (24.4)	18 (20.5)	17 (17.2)	6 (27.3)	7 (16.7)	68 (20.4)
AMP	3 (3.7)	1 (1.1)	2 (2.0)	1 (4.5)	1 (2.4)	8 (2.4)
AMP-CHP-CIP-ENR-FFC-GEN-OTC-OXO-SMZ-TET-TMP	0 (0)	0 (0)	1 (1.0)	0 (0)	0 (0)	1 (0.3)
AMP-CHP-CIP-ENR-FFC-GEN-OTC-OXO-STR-SMZ-TET- TMP	4 (4.9)	3 (3.4)	7 (7.1)	1 (4.5)	0 (0)	15 (4.5)
AMP-CHP-CIP-ENR-FFC-GEN-OTC-OXO-TET- TMP	0 (0)	1 (1.1)	0 (0)	0 (0)	0 (0)	1 (0.3)
AMP-CHP-CIP-ENR-FFC-OTC-OXO-SMZ-TET- TMP	1 (1.2)	0 (0)	4 (4.0)	0 (0)	1 (2.4)	6 (1.8)
AMP-CHP-CIP-ENR-FFC-OTC-OXO-STR-SMZ-TET	0 (0)	0 (0)	1 (1.0)	0 (0)	0 (0)	1 (0.3)
AMP-CHP-CIP-ENR-FFC-OTC-OXO-STR-SMZ-TET-TMP	3 (3.7)	0 (0)	0 (0)	0 (0)	1 (2.4)	4 (1.2)
AMP-CHP-CIP-ENR-FFC-OTC-OXO-TET	0 (0)	2 (2.3)	0 (0)	0 (0)	0 (0)	2 (0.6)
AMP-CHP-CIP-ENR-GEN-OTC-OXO-SMZ-TET- TMP	1 (1.2)	2 (2.3)	0 (0)	0 (0)	0 (0)	3 (0.9)
AMP-CHP-CIP-ENR-OTC-OXO-SMZ-TET	1 (1.2)	0 (0)	0 (0)	0 (0)	0 (0)	1 (0.3)
AMP-CHP-CIP-ENR-OTC-OXO-SMZ-TET- TMP	0 (0)	0 (0)	2 (2.0)	0 (0)	1 (2.4)	3 (0.9)
AMP-CHP-CIP-ENR-OTC-OXO-STR-SMZ-TET- TMP	1 (1.2)	0 (0)	2 (2.0)	0 (0)	1 (2.4)	4 (1.2)
AMP-CHP-CIP-FFC-OTC-OXO-STR-SMZ-TET- TMP	0 (0)	0 (0)	2 (2.0)	0 (0)	0 (0)	2 (0.6)

Table 8. Resistance pattern of *E. coli* (n = 333) isolated from hybrid red tilapia and cultivation water (Continue)

Resistance pattern	No of isolates (%)					
	Cultivation water (n = 82)	Fish carcass rinse (n = 88)	Intestine (n = 99)	Fish meat (n = 22)	Liver and kidney (n = 42)	Total (n = 333)
AMP-CHP-CIP-OTC-OXO-SMZ-TET	0 (0)	1 (1.1)	0 (0)	0 (0)	0 (0)	1 (0.3)
AMP-CHP-FFC-OTC-OXO-STR-SMZ-TET-TMP	1 (1.2)	2 (2.3)	0 (0)	1 (4.5)	0 (0)	4 (1.2)
AMP-CHP-FFC-OTC-OXO-TET	0 (0)	0 (0)	1 (1.0)	0 (0)	0 (0)	1 (0.3)
AMP-CHP-FFC-OTC-OXO-TET-TMP	0 (0)	0 (0)	1 (1.0)	0 (0)	0 (0)	1 (0.3)
AMP-CHP-FFC-OTC-STR-SMZ-TET	0 (0)	1 (1.1)	0 (0)	0 (0)	0 (0)	1 (0.3)
AMP-CHP-FFC-OTC-STR-TET	0 (0)	0 (0)	1 (1.0)	0 (0)	0 (0)	1 (0.3)
AMP-CHP-FFC-OXO-STR-SMZ	1 (1.2)	0 (0)	1 (1.0)	1 (4.5)	4 (9.5)	7 (2.1)
AMP-CHP-FFC-OXO-STR-SMZ-TMP	0 (0)	0 (0)	4 (4.0)	0 (0)	0 (0)	4 (1.2)
AMP-CHP-FFC-STR-SMZ-TMP	0 (0)	0 (0)	0 (0)	0 (0)	2 (4.8)	2 (0.6)
AMP-CHP-GEN-OTC-OXO-STR-SMZ-TET-TMP	0 (0)	0 (0)	0 (0)	1 (4.5)	0 (0)	1 (0.3)
AMP-CHP-STR-SMZ-TET	0 (0)	0 (0)	0 (0)	0 (0)	1 (2.4)	1 (0.3)
AMP-CIP-ENR-FFC-OTC-OXO-STR-SMZ-TET- TMP	1 (1.2)	1 (1.1)	0 (0)	0 (0)	0 (0)	2 (0.6)
AMP-CIP-ENR-FFC-OTC-OXO-STR-TET	1 (1.2)	0 (0)	0 (0)	0 (0)	0 (0)	1 (0.3)
AMP-CIP-ENR-FFC-OTC-OXO-TET	1 (1.2)	3 (3.4)	7 (7.1)	3 (13.6)	0 (0)	14 (4.2)
AMP-CIP-ENR-OTC-OXO-SMZ-TET	1 (1.2)	0 (0)	0 (0)	0 (0)	0 (0)	1 (0.3)
AMP-CIP-ENR-OTC-OXO-SMZ-TET-TMP	1 (1.2)	0 (0)	1 (1.0)	0 (0)	0 (0)	2 (0.6)
AMP-CIP-ENR-OTC-OXO-STR-SMZ-TET-TMP	1 (1.2)	2 (2.3)	2 (2.0)	0 (0)	0 (0)	5 (1.5)
AMP-CIP-ENR-OTC-OXO-STR-TET	1 (1.2)	3 (3.4)	3 (3.0)	0 (0)	0 (0)	7 (2.1)
AMP-CIP-ENR-OTC-OXO-TET	4 (4.9)	4 (4.5)	4 (4.0)	0 (0)	0 (0)	12 (3.6)

Table 8. Resistance pattern of *E. coli* (n = 333) isolated from hybrid red tilapia and cultivation water (Continue)

Resistance pattern	No of isolates (%)					
	Cultivation water (n = 82)	Fish carcass rinse (n = 88)	Intestine (n = 99)	Fish meat (n = 22)	Liver and kidney (n = 42)	Total (n = 333)
AMP-CIP-ENR-OTC-OXO-TET-TMP	2 (2.4)	1 (1.1)	1 (1.0)	0 (0)	0 (0)	4 (1.2)
AMP-CIP-GEN-OTC-OXO-STR-SMZ-TET-TMP	0 (0)	0 (0)	1 (1.0)	0 (0)	0 (0)	1 (0.3)
AMP-CIP-OTC-SMZ-TET	0 (0)	0 (0)	0 (0)	1 (4.5)	0 (0)	1 (0.3)
AMP-FFC-OTC-OXO-TET	0 (0)	2 (2.3)	1 (1.0)	0 (0)	0 (0)	3 (0.9)
AMP-FFC-OTC-TET	1 (1.2)	0 (0)	0 (0)	0 (0)	0 (0)	1 (0.3)
AMP-GEN-OTC-OXO-STR-SMZ-TET-TMP	0 (0)	0 (0)	1 (1.0)	2 (9.1)	0 (0)	3 (0.9)
AMP-OTC-OXO-SMZ-TET	0 (0)	0 (0)	0 (0)	0 (0)	1 (2.4)	1 (0.3)
AMP-OTC-OXO-SMZ-TET-TMP	0 (0)	2 (2.3)	1 (1.0)	0 (0)	0 (0)	3 (0.9)
AMP-OTC-OXO-STR-SMZ-TET	1 (1.2)	0 (0)	1 (1.0)	0 (0)	0 (0)	2 (0.6)
AMP-OTC-OXO-TET	9 (11.0)	8 (9.1)	8 (8.1)	2 (9.1)	5 (11.9)	32 (9.6)
AMP-OTC-OXO-TET-TMP	1 (1.2)	0 (0)	0 (0)	0 (0)	0 (0)	1 (0.3)
AMP-OTC-SMZ-TET	0 (0)	4 (4.5)	0 (0)	0 (0)	0 (0)	4 (1.2)
AMP-OTC-SMZ-TET-TMP	0 (0)	0 (0)	1 (1.0)	0 (0)	0 (0)	1 (0.3)
AMP-OTC-STR-SMZ-TET	1 (1.2)	0 (0)	1 (1.0)	0 (0)	0 (0)	2 (0.6)
AMP-OTC-STR-SMZ-TET-TMP	0 (0)	1 (1.1)	1 (1.0)	0 (0)	0 (0)	2 (0.6)
AMP-OTC-STR-TET	0 (0)	1 (1.1)	0 (0)	0 (0)	0 (0)	1 (0.3)
AMP-OTC-TET	8 (9.8)	2 (2.3)	5 (5.1)	1 (4.5)	2 (4.8)	18 (5.4)
AMP-OXO	1 (1.2)	3 (3.4)	1 (1.0)	1 (4.5)	0 (0)	6 (1.8)
AMP-OXO-STR	0 (0)	0 (0)	1 (1.0)	0 (0)	0 (0)	1 (0.3)
AMP-OXO-STR-SMZ-TET-TMP	0 (0)	1 (1.1)	1 (1.1)	0 (0)	0 (0)	2 (0.6)
AMP-SMZ	1 (1.2)	0 (0)	0 (0)	0 (0)	0 (0)	1 (0.3)
AMP-TET	1 (1.2)	0 (0)	0 (0)	0 (0)	0 (0)	1 (0.3)
CHL-CIP-ENR-FFC-OTC-OXO-SMZ-TET-TMP	0 (0)	0 (0)	0 (0)	1 (4.5)	0 (0)	1 (0.3)

Table 8. Resistance pattern of *E. coli* (n = 333) isolated from hybrid red tilapia and cultivation water (Continue)

Resistance pattern	No of isolates (%)					
	Cultivation water (n = 82)	Fish carcass rinse (n = 88)	Intestine (n = 99)	Fish meat (n = 22)	Liver and kidney (n = 42)	Total (n = 333)
CIP-ENR-OTC-OXO-TET	0 (0)	2 (2.3)	1 (1.0)	0 (0)	0 (0)	3 (0.9)
CIP-ENR-OXO	1 (1.2)	7 (8.0)	1 (1.0)	0 (0)	9 (21.4)	18 (5.4)
ENR-FFC-OTC-OXO	1 (1.2)	0 (0)	0 (0)	0 (0)	0 (0)	1 (0.3)
FFC	0 (0)	0 (0)	2 (2.0)	0 (0)	0 (0)	2 (0.6)
FFC-OTC-TET	0 (0)	0 (0)	1 (1.0)	0 (0)	0 (0)	1 (0.3)
OTC	0 (0)	0 (0)	1 (1.0)	0 (0)	0 (0)	1 (0.3)
OTC-OXO	0 (0)	0 (0)	1 (1.0)	0 (0)	1 (2.4)	2 (0.6)
OTC-SMZ	0 (0)	0 (0)	1 (1.0)	0 (0)	0 (0)	1 (0.3)
OTC-TET	2 (2.4)	1 (1.1)	2 (2.0)	0 (0)	3 (7.1)	8 (2.4)
OXO-STR	1 (1.2)	0 (0)	0 (0)	0 (0)	0 (0)	1 (0.3)
SMZ	4 (4.9)	7 (8.0)	0 (0)	0 (0)	1 (2.4)	12 (3.6)
SMZ-TET	0 (0)	0 (0)	0 (0)	0 (0)	1 (2.4)	1 (0.3)
SMZ-TMP	0 (0)	2 (2.3)	0 (0)	0 (0)	0 (0)	2 (0.6)
STR-SMZ-TMP	0 (0)	0 (0)	1 (1.0)	0 (0)	0 (0)	1 (0.3)

AMP: ampicillin; CHP: chloramphenicol; CIP: ciprofloxacin; ENR: enrofloxacin; FFC: florfenicol; GEN: gentamicin; OTC: oxytetracycline; OXO: oxolinic acid; SMZ: sulfamethoxazole; STR: streptomycin; TET: tetracycline; TMP: trimethoprim

The most common AMR genes were bla_{TEM} (58.0%), followed by *qnrS* (43.8%), *tetA* (29.1%), *tetB* (23.7%), and *strA* (16.5%) (Table 9). An association between type of the sample and the presence of *strA* (*P*-value = 0.033) was observed. Abundance of resistance genes were mainly detected in fish intestines. Among transferable quinolone resistance genes, *qnrS* (43.8%) was the most prevalent. The prevalence of *tetA* (29.1%), *tetB* (23.7%), *floR* (16.5%), *sul2* (15.6%), *sul3* (15.6%), *dfrA1* (9.6%) and *dfrA12* (6.3%) were reported. The bla_{SHV}, bla_{PSE}, *strB*, *ermB*, *catB*, *addA1*, aac*(3)IV*, and

carbapenemase genes (bla_{NDM} and bla_{OXA}) were not detected in this study. Some PMQR genes (*qnrA*, aac*(6')-lb*-cr, and *qepA)* and series of colistin resistance genes (*mcr-1* to *mcr-5*), and virulence genes (*stx1* and *stx2*) were tested negative. The presence of *int1* gene was 19.5%, while the *int2*, *int3*, and int_{SXT} were not detected.

Table 9. AMR and virulence genes of *E. coli* isolates (n = 333).

Gene	Prevalence (%)					
	Cultivation water (n = 82)	Carcass rinse (n = 88)	Intestine (n = 99)	Fish meat (n = 22)	Liver and kidney (n = 42)	Grand total (n = 333)
Antimicrobial resistance gene						
bla_{TEM}	50 (61.0)	52 (59.1)	62 (62.6)	12 (54.5)	17 (40.5)	193 (58.0)
bla_{SHV}	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
bla_{CTX-M}	0 (0)	0 (0)	5 (5.1)	4 (18.2)	0 (0)	9 (2.7)
bla_{PSE}	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
bla_{NDM}	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
bla_{OXA}	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
catA	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
catB	0 (0)	0 (0)	4 (4.0)	1 (4.5)	0 (0)	5 (1.5)
cmlA	5 (6.1)	5 (5.7)	7 (7.1)	3 (13.6)	4 (9.5)	24 (7.2)
floR	5 (6.1)	8 (9.1)	19 (19.2)	3 (13.6)	7 (16.7)	42 (12.6)
ermB	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
qnrA	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
qnrB	0 (0)	1 (1.1)	3 (3.0)	0 (0)	1 (2.4)	5 (1.5)
qnrS	34 (41.5)	38 (43.2)	51 (51.5)	11 (50.0)	12 (28.6)	146 (43.8)
aac(6')-lb-cr	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
qepA	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
addA1	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
addA2	9 (11.0)	11 (12.5)	10 (10.1)	2 (9.1)	5 (11.9)	37 (11.1)
aac (3) IV	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
tetA	27 (32.9)	20 (22.7)	31 (31.3)	8 (36.4)	11 (26.2)	97 (29.1)

Table 9. AMR and virulence genes of *E. coli* isolates (n = 333). (Continue)

Gene	Prevalence (%)					
	Cultivation water (n = 82)	Carcass rinse (n = 88)	Intestine (n = 99)	Fish meat (n = 22)	Liver and kidney (n = 42)	Grand total (n = 333)
Antimicrobial resistance gene						
tetB	19 (23.2)	22 (25.0)	25 (25.3)	5 (22.7)	8 (19.0)	79 (23.7)
tetD	1 (1.2)	1 (1.1)	2 (2.0)	0 (0)	0 (0)	4 (1.2)
strA	9 (11.0)	9 (10.2)	24 (24.2)	6 (27.3)	7 (16.7)	55 (16.5)
strB	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
sul1	4 (4.9)	5 (5.7)	5 (5.1)	1 (4.5)	0 (0)	15 (4.5)
sul2	13 (15.9)	9 (10.2)	18 (18.2)	6 (27.3)	6 (14.3)	52 (15.6)
sul3	8 (9.8)	10 (11.4)	18 (18.2)	6 (27.3)	10 (23.8)	52 (15.6)
dfrA1	6 (7.3)	4 (4.5)	9 (9.1)	2 (9.1)	0 (0)	21 (6.3)
dfrA12	6 (7.3)	8 (9.1)	11 (11.1)	3 (13.6)	4 (9.5)	32 (9.6)
mcr-1	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
mcr-2	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
mcr-3	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
mcr-4	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
mcr-5	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Integrons						
int1	14 (17.1)	12 (13.6)	28 (28.3)	5 (22.7)	6 (14.3)	65 (19.5)
int2	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
int3	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Integrative and conjugative elements						
int_{SXT}	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Virulence genes						
stx1	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
stx2	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)

Based on logistic regression modeling, the statistical association between resistant phenotype and genotype were observed among the *E. coli* isolates. The ampicillin resistant isolates can carry multiple resistance phenotype and genotype. For example, among resistance to ampicillin of *E. coli* isolates, the resistance to sulfamethoxazole, tetracycline, and the presence of bla_{TEM}, *sul2,* and *qnrS* genes (OR > 1) were examined (Table 10). The odds of observing *E. coli* resistant isolate to ampicillin was statistical associated with the co-occurrence of bla_{TEM} (OR 61.8), *qnrS* (OR 26.8), tetracycline resistance (OR 25.5), *sul2* (OR 13.6), and sulfamethoxazole resistance (OR 5.6), respectively.

Table 10. Logistic regression model for the association between phenotypic and genotypic AMR (n = 333).

AMP	Odds ratio	SE	*P*-value	95% CI
SMZ	5.64	3.34	0.004	1.73 to 18.34
TET	25.53	5.14	<0.0001	17.20 to 37.88
bla_{TEM}	61.76	29.76	<0.0001	24.02 to 158.81
qnrS	26.83	26.78	0.001	3.80 to 189.72
sul2	13.55	5.61	<0.0001	6.02 to 30.49
constant	0.02	0.01	<0.0001	0.01 to 0.06

SE, Standard Error; CI, Confidence interval; SMZ: sulfamethoxazole; TET: tetracycline

The *E. coli* (3.9%, n = 13/333) isolates were ESBL-producing bacteria (Table 11). Out of 13 isolates, four of each isolate from carcass rinse and meat, three isolates from intestine, and two isolates from cultivation water were identified. Among ß-lactam resistance genes, the predominant genes were bla_{TEM} (58.0%, n = 193) and bla_{CTX-M} (2.7%, n = 9). Interestingly, all ESBL positive isolates were MDR

that harbored *bla*$_{CTX-M}$ (53.9%, n = 7), *sul* (84.6%, n = 11), *qnr* (92.3%, n = 12), and *tet* (84.6%, n = 11). The presence of *bla*$_{CTX-M}$ was associated with type of sample (*P*-value < 0.0001). No emergence of *bla*$_{SHV}$, *bla*$_{PSE}$, *bla*$_{NDM}$, and *bla*$_{OXA}$ genes was reported in this study. The unique sequenced positive-*bla*$_{TEM}$ *E. coli* isolates (n = 193) and *bla*$_{CTX-M}$ (n = 9) were *bla*$_{TEM-1}$ and *bla*$_{CTX-M-55}$, respectively.

Table 11. ESBL positive *E. coli* (n = 13) isolates with their AMR patterns and AMR genotypes.

Resistance phenotype	Sample type (n)	*bla*$_{CTX-M}$	AMR genotype (n)
AMP-CHP-CIP-ENR-FFC-GEN-OTC-OXO-SMZ -STR-TET-TMP	Water (2)	Positive	*bla*$_{TEM}$, *sul2, sul3, qnrS, floR*
		Negative	*bla*$_{TEM}$, *sul3, qnrS, dfrA1, tetA*
	Carcass rinse (1)	Negative	*bla*$_{TEM}$, *sul1, sul2, int1, qnrS, strA, tetD, tetB, floR*
	Intestine (3)	Negative	*bla*$_{TEM}$, *sul1, int1, qnrB, qnrS, dfrA1. strA, tetA*
		Positive	*bla*$_{TEM}$, *sul3, qnrS, dfrA1, tetA. floR*
		Positive	*bla*$_{TEM}$, *sul3, qnrS, catB, strA, floR*
	Meat (1)	Positive	*bla*$_{TEM}$, *sul2, sul3, qnrS, catB, strA, tetA, floR*
AMP-CHP-GEN-OTC-OXO-SMZ-STR-TET-TMP	Meat (1)	Positive	*bla*$_{TEM}$, *sul2, int1, qnrS, strA, tetA*
AMP-GEN-OTC-OXO-SMZ-STR-TET-TMP	Meat (2)	Positive	*bla*$_{TEM}$, *sul1, sul2, int1, qnrS, dfrA1, strA, tetA*
		Positive	*bla*$_{TEM}$, *sul2, int1, qnrS, strA, tetA*
AMP-CIP-ENR-OTC-OXO-STR-TET	Carcass rinse (1)	Negative	*bla*$_{TEM}$, *qnrS, tetB, aadA2*
AMP-OTC-SMZ-TET	Carcass rinse (1)	Negative	*bla*$_{TEM}$, *sul2, tetA*
AMP-OTC-OXO-TET	Carcass rinse (1)	Negative	*bla*$_{TEM}$, *qnrS, tetB*
Total	13		

AMP: ampicillin; CHP: chloramphenicol; CIP: ciprofloxacin; ENR: enrofloxacin; FFC: florfenicol; GEN: gentamicin; OTC: oxytetracycline; OXO: oxolinic acid; SMZ: sulfamethoxazole; STR: streptomycin; TET: tetracycline; TMP: trimethoprim

All selected ciprofloxacin resistant isolates (n = 22) observed the mutation of *gyrA* and *parC* (Table 12). Only two positions of mutation in *gyrA* were reported in Ser83Leu at position 248 from C to T (100%, n = 22/22) and Asp87Asn at position 259 from G to A (86.4%, n = 19/22). The latter isolates at Asp87Asn had Ser83Leu, called double point mutation of *gyrA*. The *E. coli* isolates (40.9%, n = 9/22) were observed *parC* mutation from Ser80Iso at position 239 from G to T with double point mutation of *gyrA*. None of silent nucleotide substitution in either *gyrA* or *parC* was observed in this study. The MIC range of ciprofloxacin resistant isolates with *gyrA* mutation and combined *gyrA* and *parC* mutations were similar, which were from 2-128 µg/ml. Among *E. coli* isolates with *gyrA* mutation, the MIC of ciprofloxacin resistant isolates with and without additional PMQR genes (*qnrB* and *qnrS*) ranged from 2-128 µg/ml and 16-64 µg/ml, respectively. For *E. coli* isolates with double point mutation, the MIC of ciprofloxacin resistant isolates with and without additional PMQR genes (*qnrB* and *qnrS*) ranged from 8-128 µg/ml and 2-32 µg/ml, respectively.

Table 12. MIC concentrations of ciprofloxacin resistant *E. coli* (n = 22) isolates with PMQR genes and amino acid change in *gyrA* and *parC* in the QRDR.

Ciprofloxacin resistant category	No of isolates (%)	PMQR	QRDR		
			gyrA mutation		*parC* mutation
Low resistance (MIC: 2-16 µg/ml)	2 (20.0	*qnrS*	Ser83Leu	-	-
	1 (10.0)	*qnrS*	Ser83Leu	Asp87Asn	
	1 (10.0)	*qnrS*	Ser83Leu	Asp87Asn	Ser80Iso
	1 (10.0)	-	Ser83Leu	-	-
	3 (30.0)	-	Ser83Leu	Asp87Asn	-
	2 (20.0)	-	Ser83Leu	Asp87Asn	Ser80Iso
High resistance (MIC: 32-128 µg/ml)	2 (16.7)	*qnrS*	Ser83Leu	Asp87Asn	-
	3 (25.0)	*qnrS*	Ser83Leu	Asp87Asn	Ser80Iso
	1 (8.3)	*qnrB, qnrS*	Ser83Leu	Asp87Asn	-
	1 (8.3)	*qnrB, qnrS*	Ser83Leu	Asp87Asn	Ser80Iso
	4 (33.3)	-	Ser83Leu	Asp87Asn	-
	1 (8.3)	-	Ser83Leu	Asp87Asn	Ser80Iso
Total	22 (100)				

MIC: Minimum Inhibitory Concentration

4.5 Discussion

This study highlighted the emergence AMR bacteria in hybrid red tilapia and aquatic environment. High resistance to ampicillin, enrofloxacin, oxolinic acid, tetracycline, and oxytetracycline were observed in the *E. coli* isolates. Most of resistant *E. coli* isolates were derived from fish intestine indicating that good personal hygiene and sanitation practice is needed for fish preparation and cooking. A previous study indicated the resistance to ampicillin of *E. coli* was 97.5%, which much higher than this study (Saqr et al., 2016). Even though high resistance to β-lactam antibiotics

have been commonly reported in livestock (LjubojeviĆ et al., 2017; Zhang et al., 2017; Jahantigh et al., 2020; Moennighoff et al., 2020), β-lactam resistant bacteria were increasingly reported in aquatic species, such as shrimp, oyster, and carp with prevalence ranging from 86.4-99.0% (He et al., 2016; Kang et al., 2016; Zdanowicz et al., 2020). This emphasized the widespread of β-lactam resistant bacteria across agriculture and aquaculture that may potentially transmit the resistance to human referring to the important of One Health.

This study exhibited the similar resistance rate of the *E. coli* isolates to enrofloxacin (33.6%) and ciprofloxacin (34.8%), which was lower than a previous study of enrofloxacin in Egypt (54.5%) (Algammal et al., 2022). However, a similar rate of ciprofloxacin resistance was observed from Ghana. However, a similar rate of ciprofloxacin resistance was observed from Ghana (Agoba et al., 2017). Low resistance to ciprofloxacin (16.8%) was previously reported in Malaysia (Dewi et al., 2022). Tetracycline and oxytetracycline are the first generation tetracyclines and broad-spectrum antibiotics. In Thailand, enrofloxacin and oxytetracycline have been approved in aquaculture by Thailand FDA (Baoprasertkul et al., 2012). Oxytetracycline is frequently used in intensive tilapia farming due to inexpensive and highly effective to *Aeromonas* and *Vibrio*. (Julinta et al., 2017; El-Gohary et al., 2020). In this study, the prevalence of tetracycline (58.0%) and oxytetracycline (58.6%) resistance were disagreed with previous studies in Malaysia and Guatemala (García-Pérez et al., 2021; Dewi et al., 2022). Surprisingly, high resistance rate to oxytetracycline (79.7%) was reported in ornamental fish in Thailand (Saengsitthisak et al., 2020). It should be noted that the observed high resistance of quinolones and tetracyclines were corresponded to the frequent use of these antimicrobials in tilapia cage culture of Thailand (Rico et al., 2014). It is evidence that the use of antimicrobials in aquaculture contributed to the development of AMR in aquaculture and possibly

widespread to the environment (Payne et al., 2021). In this study, the *E. coli* isolates from downstream tends to accumulate more bacterial resistome than those from upstream. However, this study failed to identify the source of AMR, which may originate from different sources such as aquaculture, livestock, or human wastes. Further studies of the source of AMR in aquaculture should be conducted.

E. coli isolated from hybrid red tilapia and catfish in Vietnam exhibited high resistance to many antimicrobials such as nalidixic acid (92.9%) and gentamycin (88.3%) (Hon et al., 2016). However, resistance to gentamicin (< 10%) was low in this study, which was supported by a previous study indicated that all isolates were susceptible to gentamicin (Kikomeko, 2016). Importantly, resistant *E. coli* to last-resort antibiotics, including polymyxin B (19.4%), fosfomycin (10.3%), and colistin (18.3%) was reported in aquaculture (Schar et al., 2021; Dewi et al., 2022). Integrons are mobile genetic elements, which carry various gene cassettes that confer to MDR. Class 1 integron with *aadA22* and *dfrA12-aadA2* in gene cassette were the dominant gene pattern found in *Enterobacteriaceae* (Su et al., 2011; Ryu et al., 2012). Only *int1* (19.5%) was detected in this study, which was consistent with a study in South Africa (Chenia and Jacobs, 2017). In this study, more than half of the *E. coli* isolates were MDR bacteria, which agreed to previous findings (Ferreira et al., 2021; Dewi et al., 2022). This addresses the widespread of MDR *E. coli* isolates and important mobile genetic element already existed in aquaculture.

The occurrence of bla_{TEM}, *qnrS*, *tetA*, and *tetB* was commonly reported in this study, which agreed with previous studies (Saqr et al., 2016; Odumosu et al., 2021). It is evidence that the use of oxytetracycline to treat bacterial infection in tilapia increased the occurrence of *tetA* genes in tilapia guts (Payne et al., 2021). On the other hand, none of *tetB* was detected in *E. coli* isolates in Nigeria and India

(Sivaraman et al., 2020; Odumosu et al., 2021). The resistance genotype may not correspond to their phenotype in similar rate (Samanta and Bandyopadhyay, 2020). This agreed with this study that the prevalence of *qnrS*, *tetA*, and *tetB* were higher than their corresponding phenotypes. Other mechanisms such as, efflux pump or mutation involve with resistance (Le Roy et al., 2021).

Colistin is a last-resort antimicrobial for treatment of MDR *Enterobacteriaceae* infection, especially carbapenem resistance. Colistin resistance gene can co-express with other plasmid-mediated resistance genes, such as *qnr*, $bla_{\text{CTX-M}}$, and *tet* (Joshi et al., 2019; Zhang et al., 2019; Cheng et al., 2020). In this study, the absence of series of *mcr* gene was examined, which disagreed with previous studies from prawns in Vietnam and grass carp in China (Lv et al., 2018; Ellis-Iversen et al., 2020). However, none of the colistin genes were previously reported in fish from Thailand. Only *mcr-1* and *mcr-3* were detected in livestock and human clinical isolates (Eiamphungporn et al., 2018; Poolperm et al., 2020; Lay et al., 2021). The presence of additional ESBL-producing *Enterobacteriaceae* in food animals is an important challenge of public health. ESBL-producing bacteria were reported in many aquatic animals, including shellfish and fish (Sanjit Singh et al., 2017; Gawish et al., 2021). This study found ESBL-producing bacteria in carcass rinse and meat. It is postulated that fish carcass rinse and gill continuously expose to water, which may be a source of ESBL bacteria (Hassen et al., 2020). Homogenous sequence of $bla_{\text{TEM-1}}$ and $bla_{\text{CTX-M-55}}$ were detected in this study. The distribution of $bla_{\text{TEM-1}}$ agreed with previous studies (Moremi et al., 2016; Gawish et al., 2021), while some studies mainly observed $bla_{\text{CTX-M-1}}$ and $bla_{\text{CTX-M-15}}$ (Hassen et al., 2020; Sivaraman et al., 2020). The $bla_{\text{CTX-M-55}}$ was reported in pig and wastewater from Thailand (Runcharoen et al., 2017; Lay et al., 2021). Different of CTX-M subtypes may be found regarding to fish species and geographical variation.

Based on logistic regression analysis, ampicillin resistant *E. coli* isolates were significantly associated with resistance to sulfamethoxazole, tetracycline, bla_{TEM}, *sul2,* and *qnrS*. A previous study also observed co-existence among bla_{TEM} and *qnrS* (Azargun et al., 2018). It is documented that the resistance to β-lactam was associated with sulfonamides, tetracyclines, and quinolone (Charfi et al., 2017; Algammal et al., 2022). Major mechanism of β-lactam resistance was encoded by plasmid mediated (Arabi et al., 2015). These results indicated that hybrid red tilapia can function as a significant reservoir of β-lactam, quinolone, and tetracycline resistant determinants.

The mutations of QRDR are major mechanism of quinolone resistance. Ser83Leu and Asp87Asn in *gyrA* were the most prevalent that conferred to high ciprofloxacin resistance, which were compatibility with previous studies in oysters from India and pork from Thailand (Santhosh et al., 2017; Pungpian et al., 2021). The mutation of *parC*, Ser80Iso agreed with a study from Taiwan (Chenia, 2016). The MIC of ciprofloxacin resistant *E. coli* isolates with single *gyrA* mutation did not markedly higher than those isolates with both *gyrA* and *parC* mutation. This finding was consistent with previous study showing similar MIC concentrations among these two mutation groups (Chenia, 2016). In this study, all *parC* variant isolates had double point mutation in *gyrA*, which was supported that *parC* was mutated by the initiation of *gyrA* mutation (Hopkins et al., 2005). To compare between low and high levels of ciprofloxacin resistance, PMQR genes were more prevalent in high ciprofloxacin resistant isolates. The *E. coli* having either *gyrA* or *gyrA* and *parC* mutations with additional PMQR genes showed the higher MIC than the isolates without PMQR genes. Hence, this study was in agreement with a study postulated that additional PMQR genes may increase the MIC of resistance to fluoroquinolones (Rezazadeh et

al., 2016). In this study, the role of *gyrA* and *parC* mutations conferred quinolone resistance in *E. coli* was still inconclusive.

In conclusion, hybrid red tilapia reared in open water system are vulnerable for resistant bacterial contamination. The results of this study indicated that hybrid red tilapia and cultivation water are potential hotspots of resistance and MDR *E. coli*. The high burden of resistance phenotype and genotype was majority found in fish intestine indicating that the process for fish preparation is very significant to reduce the cross-contamination during consumption. The spread of ESBL-producing *E. coli* with MDR in aquaculture alarms public health of effective prevention and control of AMR in other sectors. Continuing monitoring and prompt actions to tackle of AMR in aquaculture are required to limit the development and distribution of resistant bacteria in the environment. A comprehensive One Health should be considered to manage AMR problems efficiently and effectively.

CHAPTER V

Emergence of colistin resistance and antimicrobial resistance characterization of foodborne pathogens isolated from hybrid red tilapia and cultivation water

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Emergence of colistin resistance and antimicrobial resistance characterization of foodborne pathogens isolated from hybrid red tilapia and cultivation water

5.1 Abstract

Colistin resistant bacteria have been increasingly concerned worldwide, since colistin is a last-resort antimicrobial for treatment of Gram-negative bacterial infection. The *mcr-1*, *mcr*-3, and *mcr-4* has been documented in fish and aquatic environment. This study aimed to determine antimicrobial resistance (AMR), virulence genes, and ESBL production of *Aeromonas hydrophila*, *Salmonella* spp., and *Vibrio cholerae* isolated from farmed hybrid red tilapia and cultivation water. A total of 278 isolates consisted of 15 *A. hydrophila*, 188 *Salmonella*, and 75 *V. cholerae* isolates from hybrid red tilapia and cultivation water were used. The results showed that all *A. hydrophila* and *Salmonella* isolates were resistant to at least one antimicrobial. *A. hydrophila* (26.7%) and *Salmonella* (72.3%) exhibited multidrug resistance (MDR). *A. hydrophila* was resistant to ampicillin (100%), followed by oxytetracycline (26.7%), tetracycline (26.7%), trimethoprim (26.7%), and oxolinic acid (20%). The most common resistance genes in *A. hydrophila* were *mcr-3* (20.0%), followed by *floR*, *qnrS*, *sul1*, *sul2*, and *dfrA1* with the same resistance rates at 13.3%. One out of three non-MDR isolates harbored *mcr-3* were also carried *int1*. The *Salmonella* isolates exhibited high resistance to ampicillin (79.3%), oxolinic acid (75.5%), oxytetracycline (71.8%), chloramphenicol (62.8%), and florfenicol (55.3%), respectively. The predominant AMR genes in *Salmonella* were *qnrS* (65.4%), *tetA* (64.9%), bla_{TEM} (63.8%), and *floR* (55.9%). All *V. cholerae* isolates were non-O1/non-O139 serogroups, and all of them were susceptible to tested antimicrobials. The *sul1* (12.0%), followed

by *catB*, *qnrS*, *tetA*, *tetB*, *strA*, and *dfrA1* with the same resistance rate (4.0%) were detected in *V. cholerae*. One isolate of *A. hydrophila* was *int1* positive. None of integrons or SXT element were detected in *Salmonella* and *V. cholerae*. This study addressed the emerging of colistin resistance in hybrid red tilapia of Thailand. The spreading of colistin resistant isolates alarmed a serious public health concern. Therefore, hybrid red tilapia was one of potential reservoirs of AMR and genetic determinants of foodborne pathogens.

KEYWORDS: *Aeromonas hydrophila*, antimicrobial resistance, *mcr*, *Salmonella* spp., tilapia, *Vibrio cholerae*.

5.2 Introduction

Thailand is one of the major global producer freshwater fish. Tilapia are highlighted as the main cultivated fish in Thailand with annual production exceeds 200,000 tons per year (FAO, 2020). Due to the increasing of tilapia production under intensification of farming, this circumstance can induce fish are more susceptible to bacterial infection (Sundberg et al., 2016). *Aeromonas* and *Vibrio cholerae* are important bacteria, which are natively inhabitant in aquatic environment and in particular role of foodborne pathogens. *A. hydrophila* could be either the main causative agent of motile *Aeromonas* septicemia (MAS) or co-infection with other viruses in fish (Stratev and Odeyemi, 2017; Nicholson et al., 2020). A report of *A. hydrophila* implicated in foodborne diarrheal outbreak was documented (Silva et al., 2017). The presence of *V. cholerae* in freshwater fish has been concerned, because it can cause cholera, a severe diarrheal disease with devastating death in humans (Elimian et al., 2019). *Salmonella* is one of the top five bacterial pathogens

causing foodborne illnesses in humans (Lee and Wendy, 2017). Even though *Salmonella* is harmless in fish, these bacteria can contaminate to fish, which were associated with many foodborne disease outbreaks (Liu et al., 2018). Various foodborne pathogens can be detected in tilapia and pose a risk of foodborne infection through fish consumption.

Antimicrobial resistance (AMR) has been increasing global concerned due to its limited therapeutic options resulting in failure of antimicrobial treatment. AMR bacteria found in fish and aquatic environment may originate from different sources, including runoff, wastewater, and sewage. This contamination contributed the AMR problem in aquaculture being an important One Health issue. However, AMR data in fish and aquatic environment is poorly studied. Extended spectrum β-lactamase (ESBL) producing bacteria, which are resistant to most of penicillins and cephalosporins, can co-select to other AMR genes (Zeynudin et al., 2018; Gawish et al., 2021). Multidrug resistance (MDR) in *A. hydrophila*, *Salmonella* spp., and *V. cholerae* isolated from fish has been reported (Hounmanou et al., 2016; Saharan et al., 2020; Prabha et al., 2021). The infection of these ESBL and MDR bacteria should be concerned, because the treatment of these bacterial infection may ineffective.

Colistin is a last-line antimicrobial that is used to treat severe Gram-negative bacterial infection. In general, colistin has been commonly used in livestock production such as pigs and poultry, while it has not been used in aquaculture (Apostolakos and Piccirillo, 2018; Pungpian et al., 2021). Thus, the emergence of colistin resistance has been commonly reported in livestock. However, previous studies reported the presence of *mcr-1* and *mcr-3* in fish (Lv et al., 2018; Tuo et al., 2018; Saharan et al., 2020; Le et al., 2021). Quinolones are effective antimicrobials for

Gram-negative bacteria infection (Pham et al., 2019). Their analogs were applied in humans and aquatic animals. For example, ciprofloxacin is commonly used for treatment of gastrointestinal and urinary tracts infection in humans, while oxolinic acid and enrofloxacin are approved to treat columnaris and MAS in aquatic animals (Baoprasertkul et al., 2012). The mutations of Quinolone Resistance Determining Region (QRDR), especially, *gyrA* and *parC* region, and the plasmid-encoded quinolone resistance (PMQR) are major mechanism conferred to quinolone resistance; however, other mechanisms conferred to quinolone resistance in aquatic animals has not been clearly described (Chenia, 2016). Therefore, this study aimed to examine genotypic and phenotypic AMR characteristics, virulence genes, and ESBL production of *A. hydrophila*, *Salmonella* spp., and *V. cholerae* isolated from hybrid red tilapia and cultivation water, and to determine *gyrA* and *parC* mutations in the QRDR of ciprofloxacin-resistant isolates.

5.3 Materials and methods

5.3.1 Bacterial strains

A total of 278 isolates, including *A. hydrophila* (n = 15), *Salmonella* (n = 188) and *V. cholerae* (n = 75) (Table 13) were collected from bacterial stock in the Department of Veterinary Public Health, Faculty of Veterinary Science from Chulalongkorn University. In brief, these isolates were collected from fresh hybrid red tilapia and cultivation water in Kanchanaburi province from October 2019 to November 2020. The fish samples were divided in carcass rinse, intestine, meat, and liver and kidney (Table 13). All isolates were stored in 20% glycerol at -80 ºC. The isolates of *V. cholerae* were performed sero-grouping by slide-agglutination test with polyvalent *V. cholerae* O1, monoclonal *V. cholerae* O139, and monoclonal *V.*

cholerae O141 from available commercial antiserum (S&A reagents lab, Bangkok, Thailand).

Table 13. Number of bacterial isolates tested in this study (n = 278).

Sample type	No. of bacterial isolate		
	A. hydrophila	*Salmonella*	*V. cholerae*
Cultivation water	5	106	34
Carcass rinse	10	24	10
Intestine	0	57	23
Meat	0	0	2
Liver and kidney	0	1	6
Total	**15**	**188**	**75**

5.3.2 Antimicrobial susceptibility test

Agar dilution method was used to determine antimicrobial susceptibility to twelve antimicrobial agents, including ampicillin, chloramphenicol, ciprofloxacin, enrofloxacin, florfenicol, gentamycin, oxolinic acid, oxytetracycline, streptomycin, sulfamethoxazole, tetracycline, and trimethoprim following a standard protocol (CLSI, 2015). The antimicrobials were chosen based on the common antimicrobials used in human and aquatic animals. The clinical breakpoints of *A. hydrophila*, *Salmonella*, and *V. cholerae* were determined based on standard protocols (CLSI, 2014; CLSI, 2016; EUCAST, 2018; EUCAST, 2020). *Staphylococcus aureus* ATCC 29213, *E. coli* ATCC 25922, and *Pseudomonas aeruginosa* ATCC 27853 were used as quality control strains.

5.3.3 Detection of ESBL production

The detection of ESBL production was performed by disc diffusion method according to CLSI guideline (CLSI, 2015). In screening test, susceptibility to three antibiotic disks of ceftazidime (30 µg), cefotaxime (30 µg), and cefpodoxime (10 µg) (Oxoid, England, UK) were tested. The isolates that resisted to at least one of these cephalosporins were further confirmed using combination disk diffusion method. For ESBL confirmation, ceftazidime (30 µg), cefotaxime (30 µg), and these two disks with clavulanic acid were used. The difference of inhibition zone between single cephalosporin and cephalosporin containing clavulanic acid is greater than 5 mm was positive ESBL isolates.

5.3.4 DNA preparation and PCR

DNA template was prepared by whole-cell boiling method (Lévesque et al., 1995). Briefly, the pure bacterial isolates were streaked onto nutrient agar (Difco, MD, USA). The plates were incubated at 37 ºC for 18-24 hr. Then, a single colony was picked and inoculated in an Eppendorf tube containing 150 µl of rNase free water. The well-mixed suspension was heated for 10 min and immediately placed on ice. The suspension was centrifuged at 11,000 rpm for 5 min, and the supernatant was used as DNA template.

All primers of virulence and AMR genes were listed in Table 14. *A. hydrophila* (aerolysin: *aero*; hemolysin: *hly*), *Salmonella* (*invA*), and *V. cholerae* (hemolysin gene: *hlyA*, cholera toxin gene: *ctx*, and toxin co-regulated toxin: *tcpA*) were determined their virulence. Resistance genes were chosen corresponding to AMR phenotype, $bla_{TEM,}$ bla_{SHV}, bla_{CTX-M}, and bla_{PSE} corresponding to β-lactam resistance and ESBL production; bla_{NDM} and bla_{OXA} corresponding to carbapenem resistance; *catA*, *catB*, *floR*, and *cmlA* corresponding to phenicol resistance; *ermB* corresponding to erythromycin resistance; *qnrA*, *qnrB*, *qnrS*, *aac(6')-Ib-cr*, and *qepA* corresponding to

quinolone resistance; *aadA1*, *aadA2*, and *aac(3)IV* corresponding to gentamicin resistance; *tetA*, *tetB,* and *tetD* corresponding to tetracycline resistance; *strA* and *strB* corresponding to streptomycin resistance; *sul1*, *sul2*, and *sul3* corresponding to sulfonamide resistance; *dfrA1* and *dfrA12* corresponding to trimethoprim resistance; *mcr-1* to *mcr-5* corresponding to colistin resistance. Integrons (*int1*, *int2,* and *int3*) and the SXT element (int_{SXT}) were also detected.

The final PCR volume (50 µl) was prepared following the manufacturer's instruction. A 5 µl of template DNA, 25 µl of TopTaq Master Mix (Qiagen®, Stockach, Germany), 2 µl of each forward and reverse primer, and 5 µl of coralLoad, and 11 µl of sterile rNase free water were used. The PCR amplification was carried out on Tpersonal combi model (Biometra®, Göttingen, Germany). The PCR product was separated on 1.5% (w/v) agarose gel, and stained with Redsafe™ nucleic acid staining solution (Intron Biotechnology, Seongnam, Republic of Korea). The results were photographed by Omega Fluor™ gel documentation system (Aplegen, CA, USA).

5.3.5 Determination of nucleotide sequences of QRDR

Salmonella isolates (n = 11) were performed nucleotide sequencing for detection of mutation in the QRDR. The *gyA* and *parC* were amplified by PCR using the primer listed in Table 14. The *Salmonella* isolates were selected based on ciprofloxacin resistance rate and type of sample, including intestine (n = 4), and cultivation water (n = 2), and carcass rinse (n = 2) with the susceptible to ciprofloxacin (n = 3, negative control). The amplicons of *gyA* and *parC* were submitted for purification and nucleotide sequencing (Bionics Co., LTD., Gyeonggi-Do, Republic of Korea). The amino acid sequences were aligned and analyzed using Molecular Evolutionary Genetic Analysis (Mega) software version 11 (Tamura et al., 2021). The reference sequences from the GenBank database are available at the National Centre for Biotechnology Information (NCBI) (https://www.ncbi.nlm.nih.gov).

Table 14. Primers used to detect AMR and virulence genes of *A. hydrophila* (n = 15), *Salmonella* spp. (n = 188), and *V. cholerae* (n = 75).

Gene	Primer	Oligonucleotide sequences	Product size (bp)	Reference
Virulence genes				
A. hydrophila				
aer	*Aer*-F	CTACTTTTGCCGGCGAGCGG	953	(Ahmed et al., 2018)
	Aer-R	TGATTCCCGAAGGCACTCCC		
ah	*AH*-F	GAAAGGTTGATGCCTAATACGTA	625	(Ahmed et al., 2018)
	AH-R	CGTGCTGGCAACAAAGGACAG		
aero	*aero*-F	CACAGCCAATATGTCGGTGAAG	326	(Singh et al., 2008)
	aero-R	GTCACCTTCTCGCTCAGGC		
hly	*hly*-F	CTATGAAAAAACTAAAAATAACTG	1500	(Yousr et al., 2007)
	hly-R	CAGTATAAGTGGGGAAATGGAAAG		
***Salmonella* spp.**				
invA	*invA*-F	GTGAAATTATCGCCACGTTCGGGCAA	284	(Kumar et al., 2015)
	invA-R	TCATCGCACCGTCAAAGGAACC		
V. cholerae				
ompW	*ompW*-F	CACCAAGAAGGTGACTTTATTGTG	588	(Sathiyamurthy et al., 2013)
	ompW-R	GAACTTATAACCACCCGCG		
hlyA	*hlyA*-F	GGCAAACAGCGAAACAAATACC	481	(Imani et al., 2013)
	hlyA-R	CTCAGCGGGCTAATACGGTTTA		
ctx	*ctx*-F	CAGTCAGGTGGTCTTATGCCAAGAGG	167	(Wong et al., 2012)
	ctx-R	CCCACTAAGTGGGCACTTCTCAAACT		
tcpA	*tcpA*-F	CACGATAAGAAAACCGGTCAAGAG	453	(Singh et al., 2002)
	tcpA-R	CGAAAGCACCTTCTTTCACGTTG		
Resistance gene				
bla_{TEM}	bla_{TEM}-F	GCGGAACCCCTATTT	964	(Olesen et al., 2004)
	bla_{TEM}-R	TCTAAAGTATATATGAGTAAACTTGGT CTGAC		
bla_{SHV}	bla_{SHV}-F	TTCGCCTGTGTATTATCTCCCTG	854	(Hasman et al., 2005)
	bla_{SHV}-R	TTAGCGTTGCCAGTGYTG		

Table 14. Primers used to detect AMR and virulence genes of *A. hydrophila* (n = 15), *Salmonella* spp. (n = 188), and *V. cholerae* (n = 75). (Continue)

Gene	Primer	Oligonucleotide sequences	Product size (bp)	Reference
Resistance gene				
bla_{CTX-M}	bla_{CTX-M}-F	CGATGTGCAGTACCAGTAA	585	(Batchelor et al., 2005)
	bla_{CTX-M}-R	AGTGACCAGAATCAGCGG		
bla_{NDM}	bla_{NDM}-F	GGTTTGGCGATCTGGTTTTC	621	(Poirel et al., 2011)
	bla_{NDM}-R	CGGAATGGCTCATCACGATC		
bla_{PSE}	bla_{pse}-F	GCTCGTATAGGTGTTTCCGTTT	575	(Li et al., 2013)
	bla_{pse}-R	CGATCCGCCGATGTTCCATCC		
bla_{OXA}	bla_{OXA}-F	ACACAATACATATCAACTTCGC	813	(Costa et al., 2006)
	bla_{OXA}-R	AGTGTGTGTTTAGAATGGTGATC		
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		
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		
ermB	*ermB*-F	AGACACCTCGTCTAACCTTCGCTC	640	(Raissy et al., 2012)
	ermB-R	TCCATGTACTACCATGCCACAGG		
dfrA1	*dfrA1*-F	GGAGTGCCAAAGGTGAACAGC	367	(Shahrani et al., 2014)
	dfrA1-R	GAGGCGAAGTCTTGGGTAAAAAC		
dfrA12	*dfrA12*-F	TTCGCAGACTCACTGAGGG	330	(Chuanchuen et al., 2008a)
	dfrA12-R	CGGTTGAGACAAGCTCGAAT		

Table 14. Primers used to detect AMR and virulence genes of *A. hydrophila* (n = 15), *Salmonella* spp. (n = 188), and *V. cholerae* (n = 75). (Continue)

Gene	Primer	Oligonucleotide sequences	Product size (bp)	Reference
catA	*catA*-F	CCAGACCGTTCAGCTGGATA	454	(Chuanchuen et al., 2008a)
	catA-R	CATCAGCACCTTGTCGCCT		
catB	*catB*-F	CGGATTCAGCCTGACCACC	461	(Chuanchuen et al., 2008a)
	catB-R	ATACGCGGTCACCTTCCTG		
cmlA	*cmlA*-F	TGGACCGCTATCGGACCG	641	(Chuanchuen et al., 2008a)
	cmlA-R	CGCAAGACACTTGGGCTGC		
strA	*strA*-F	TGGCAGGAGGAACAGGAGG	405	(Chuanchuen et al., 2008a)
	strA-R	AGGTCGATCAGACCCGTGC		
strB	*strB*-F	GGCAGCATCAGCCTTATAATTT	470	(Mala et al., 2017)
	strB-R	GTGGATCCGTCATTCATTGTT		
tetA	*tetA*-F	GGCGGTCTTCTTCATCATGC	502	(Khan et al., 2019)
	tetA-R	CGGCAGGCAGAGCAAGTAGA		
tetB	*tetB*-F	CGCCCAGTGCTGTTGTTGTC	615	(Chuanchuen et al., 2008a)
	tetB-R	CGCGTTGAGAAGCTGAGGTG		
tetD	*tetD*-F	AAACCATTACGGCATTCTGC	787	(Kumai et al., 2005)
	tetD-R	GACCGGATACACCATCCATC		
addA1	*addA1*-F	CTCCGCAGTGGATGGCGG	631	(Chuanchuen et al., 2008a)
	addA1-R	GATCTGCGCGCGAGGCCA		
addA2	*addA2*-F	CATTGAGCGCCATCTGGAAT	500	(Chuanchuen et al., 2008b)
	addA2-R	ACATTTCHCTCATCGCCGGC		
aac(3)IV	*aac(3)IV*-F	GTGTGCTGCTGGTCCACAGC	627	(Stoll et al., 2012)
	aac(3)IV-R	AGTTGACCCAGGGCTGTCGC		
aac(6')-Ib-cr	*aac(6')-Ib-cr*-F	TTGCGATGCTCTATGAGTGGCTA	482	(Park et al., 2006)
	aac(6')-Ib-cr-R	CTCGAATGCCTGGCGTGTTT		
qepA	*qepA*-F	GCAGGTCCAGCAGCGGGTAG	199	(Yamane et al., 2008)
	qepA-R	CTTCCTGCCCGAGTATCGTG		

Table 14. Primers used to detect AMR and virulence genes of *A. hydrophila* (n = 15), *Salmonella* spp. (n = 188), and *V. cholerae* (n = 75). (Continue)

Gene	Primer	Oligonucleotide sequences	Product size (bp)	Reference
floR	*floR*-F	ATGGTGATGCTCGGCGTGGGCCA	800	(Ying et al., 2019)
	floR-R	GCGCCGTTGGCGGTAACAGACACCGTGA		
mcr-1	*mcr-1-F*	AGTCCGTTTGTTCTTGTGGC	320	(Rebelo et al., 2018)
	mcr-1-R	AGATCCTTGGTCTCGGCTTG		
mcr-2	*mcr-2-F*	CAAGTGTGTTGGTCGCAGTT	715	(Rebelo et al., 2018)
	mcr-2-R	TCTAGCCCGACAAGCATACC		
mcr-3	*mcr-3-F*	AAATAAAAATTGTTCCGCTTATG	929	(Rebelo et al., 2018)
	mcr-3-R	AATGGAGATCCCCGTTTTT		
mcr-4	*mcr-4-F*	TCACTTTCATCACTGCGTTG	1116	(Rebelo et al., 2018)
	mcr-4-R	TTGGTCCATGACTACCAATG		
mcr-5	*mcr-5-F*	ATGCGGTTGTCTGCATTTATC	1644	(Rebelo et al., 2018)
	mcr-5-R	TCATTGTGGTTGTCCTTTTCTG		
Integrons				
int1	*int1-F*	CCTGCACGGTTCGAATG	497	(Kitiyodom et al., 2010)
	int1-R	TCGTTTGTTCGCCCAGC		
int2	*int2-F*	GGCAGACAGTTGCAAGACAA	247	(Kitiyodom et al., 2010)
	int2-R	AAGCGATTTTCTGCGTGTTT		
int3	*int3-F*	CCGGTTCAGTCTTTCCTCAA	155	(Kitiyodom et al., 2010)
	int3-R	GAGGCGTGTACTTGCCTCAT		
Integrative and conjugative element				
int_{SXT}	int_{SXT}-F	GCTGGATAGGTTAAGGGCGG	592	(Kitiyodom et al., 2010)
	int_{SXT}-R	CTCTATGGGCACTGTCCACATTG		
QRDR				
gyrA	*gyrA*-F	GCTGAAGAGCTCCTATCTGG	436	(Chuanchuen and
	gyrA-R	GGTCGGCATGACGTCCGG		Padungtod, 2009)
parC	*parC*-F	GTACGTGATCATGGATCGTG	390	(Chuanchuen and
	parC-R	TTCCTGCATGGTGCCGTCG		Padungtod, 2009)

5.3.6 Statistical analyses

Descriptive statistics was used to describe the prevalence of AMR, virulence genes, integrons, and SXT element of the bacteria isolates. Logistic regression analyses were carried out to determine the association between the AMR and their determinants. Two-sided hypothesis testing with a *P*-value ≤ 0.05 based on the likelihood ratio test were used. All statistical analysis was performed using Stata version 14.0 (StataCorp, College Station, TX, USA)

5.4 Results

5.4.1 Phenotype and genotype of AMR, virulence genes, and ESBL production in *A. hydrophila*

In this study, the isolates of *A. hydrophila* was retrieved from carcass rinse and cultivation water. All the isolates were resistant to ampicillin (100%) (Table 15). Out of 15 isolates, four isolates (26.7%) resisted to oxytetracycline, tetracycline, trimethoprim, and three isolates (20.0%) resisted against oxolinic acid. The prevalence of MDR was 26.7%, while none of ESBL production was detected. Among six AMR resistance patterns, resistance to AMP (53.3%) was the most common pattern. AMP-OTC-TET-TRI and AMP-OXO were found in two isolates at the same rate (13.3%) (Appendix B, Table S1).

The presence of *aero* and *hly* was observed in all *A. hydrophila* isolates (n = 15) (Table 15). All AMR genes and their determinants were reported from fish carcass rinse (Table 15). The most common resistance genes were *mcr-3* (20.0%), and the presence of *floR*, *qnrS*, *sul1*, *sul2*, and *dfrA1* was resistance at the same rate (13.3%) (Table 15, Figure 5). One isolate (6.7%) of *A. hydrophila* was positive to *int1*.

Table 15. AMR and virulence genes of *A. hydrophila* isolates from cultivation water (n = 5) and fish carcass rinse (n = 10).

	Resistance (%)		
	Cultivation water (n = 5)	**Carcass rinse (n = 10)**	**Grand total (n = 15)**
Antimicrobials			
Ampicillin	5 (100.0)	10 (100.0)	15 (100)
Chloramphenicol	0 (0)	0 (0)	0 (0)
Ciprofloxacin	0 (0)	0 (0)	0 (0)
Enrofloxacin	0 (0)	0 (0)	0 (0)
Florfenicol	0 (0)	0 (0)	0 (0)
Gentamicin	0 (0)	0 (0)	0 (0)
Oxolinic acid	0 (0)	3 (30.0)	3 (20.0)
Oxytetracycline	1 (20.0)	3 (30.0)	4 (26.7)
Streptomycin	0 (0)	0 (0)	0 (0)
Sulfamethoxazole	0 (0)	0 (0)	0 (0)
Tetracycline	1 (20.0)	3 (30.0)	4 (26.7)
Trimethoprim	1 (20.0)	3 (30.0)	4 (26.7)
MDR	1 (20.0)	3 (30.0)	4 (26.7)
Virulence genes			
aero	5 (100)	10 (100)	15 (100)
hly	5 (100)	10 (100)	15 (100)
AMR genes*			
floR	0 (0)	2 (20.0)	2 (13.3)
qnrS	0 (0)	2 (20.0)	2 (13.3)
sul1	0 (0)	2 (20.0)	2 (13.3)
sul2	0 (0)	2 (20.0)	2 (13.3)
dfrA1	0 (0)	2 (20.0)	2 (13.3)
mcr-3	0 (0)	3 (30.0)	3 (20.0)
Integrons*			
int1	0 (0)	1 (10.0)	1 (6.7)

*This table showed only positive isolates. Non-detected genes screened in *A. hydrophila* in this study were: bla_{TEM}, bla_{SHV}, bla_{CTX-M}, bla_{NDM}, bla_{PSE}, bla_{OXA}, *sul3*, *qnrA*, *qnrB*, *ermB*, *dfrA12*, *catA*, *catB*, *cmlA*, *strA*, *strB*, *tetA*, *tetB*, *tetD*, *addA1*, *addA2*, *aac(3)IV*, *aac(6')-Ib-cr*, *qepA*, *mcr-1*, *mcr-2*, *mcr-4*, *mcr-5*, *int2*, *int3*, and int_{SXT}

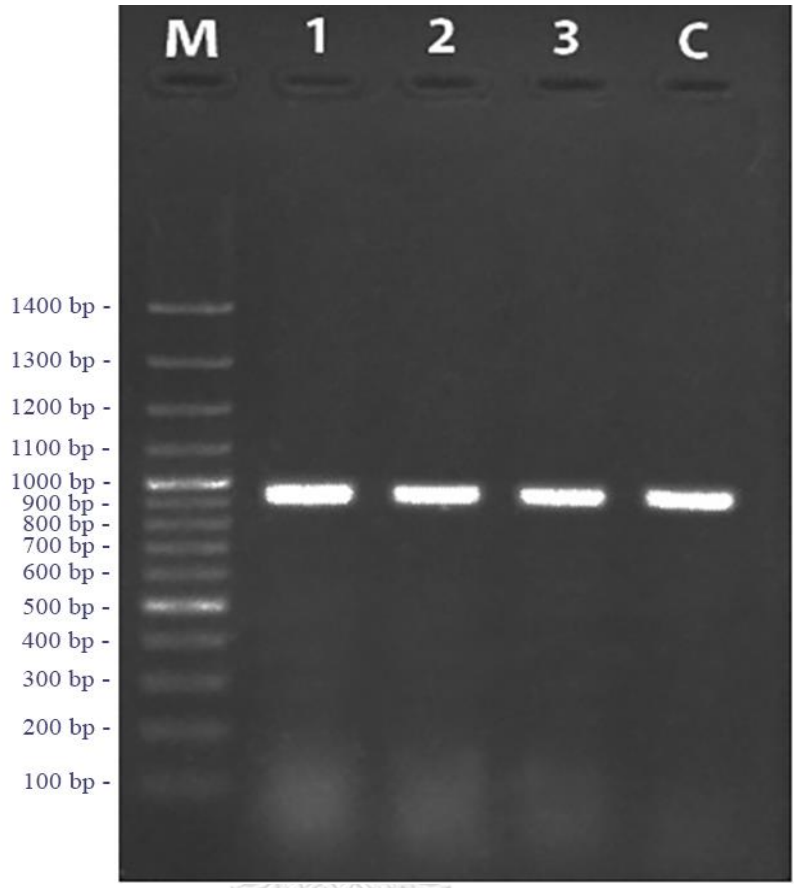


Figure 5. Colistin resistance genes of three *A. hydrophila* isolated from hybrid red tilapia. Lane M, molecular weight marker (Gene Ruler 100 bp DNA Ladder Plus, Thermo Fisher); lane 1-3: positive *mcr-3* of *A. hydrophila*, and lane C: positive control for *mcr-3* with molecular size 929 bp.

5.4.2 Phenotype and genotype of AMR, virulence genes, and ESBL production in *Salmonella*

All *Salmonella* isolates (n = 188) were resistant to at least one antimicrobial (Table 16). More than 72% of the *Salmonella* isolates exhibited MDR. High resistance rates were found in ampicillin (79.3%), followed by oxolinic acid (75.5%), oxytetracycline (71.8%), tetracycline (70.7%). All *Salmonella* isolates were sensitive to gentamicin (100%). Only two isolates (1.1%) were resistant to trimethoprim. Out of 35 AMR patterns, the most two common were AMP-CHP-FFC-OTC-OXO-TET (20.7%)

and AMP-CHP-ENR-FFC-OTC-OXO-TET (18.6%) (Appendix B, Table S2). None of ESBL production was detected and the prevalence of MDR was 72.3%.

The majority of *Salmonella* contained *qnrS* (65.4%), *tetA* (64.9%), bla_{TEM} (63.8%), and *floR* (55.9%) (Table 16). Other AMR genes were *tetB* (1.6%), *strA* (1.6%), *sul2* (1.6%), and *sul1* (1.1%), respectively. None of colistin resistance genes were observed in this collection. The cultivation water exhibited high resistance than any other sample types. Among fish samples, most of AMR phenotype and genotype, except florfenicol, were examined in fish intestine.

Table 16. AMR and virulence genes of *Salmonella* isolates (n = 188) from hybrid red tilapia and cultivation water.

	Resistance (%)				
	Cultivation water (n = 106)	Carcass rinse (n = 24)	Intestine (n = 57)	Liver and kidney (n = 1)	Grand total (n = 188)
Antimicrobials					
Ampicillin	97 (91.5)	13 (54.2)	38 (66.7)	1 (100)	149 (79.3)
Chloramphenicol	78 (73.6)	11 (45.8)	28 (49.1)	1 (100)	118 (62.8)
Ciprofloxacin	23 (21.7)	3 (12.5)	14 (24.6)	0 (0)	40 (21.3)
Enrofloxacin	43 (40.6)	6 (25.0)	20 (35.1)	0 (0)	69 (36.7)
Florfenicol	69 (65.1)	12 (50.0)	22 (38.6)	1 (100)	104 (55.3)
Gentamicin	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Oxolinic acid	86 (81.1)	16 (66.7)	40 (70.2)	0 (0)	142 (75.5)
Oxytetracycline	88 (83.0)	13 (54.2)	34 (59.6)	0 (0)	135 (71.8)
Streptomycin	2 (1.9)	0 (0)	2 (3.5)	0 (0)	4 (2.1)
Sulfamethoxazole	3 (2.8)	0 (0)	1 (1.8)	0 (0)	4 (2.1)
Tetracycline	88 (83.0)	13 (54.2)	32 (56.1)	0 (0)	133 (70.7)
Trimethoprim	2 (1.9)	0 (0)	0 (0)	0 (0)	2 (1.1)
MDR	89 (84.0)	13 (54.2)	34 (59.6)	0 (0)	136 (72.3)
Virulence genes					
invA	106 (100)	24 (100)	57 (100)	1 (100)	188 (100)
AMR genes*					
bla_{TEM}	75 (70.8)	10 (41.7)	34 (59.6)	1 (100)	120 (63.8)
floR	71 (67.0)	10 (41.7)	24 (42.1)	0 (0)	105 (55.9)
qnrS	79 (74.5)	13 (54.2)	31 (54.4)	0 (0)	123 (65.4)
tetA	78 (76.3)	13 (54.2)	31 (54.4)	0 (0)	122 (64.9)
tetB	2 (1.9)	0 (0)	1 (1.8)	0 (0)	3 (1.6)
strA	2 (1.9)	0 (0)	1 (1.8)	0 (0)	3 (1.6)
sul1	2 (1.9)	0 (0)	0 (0)	0 (0)	2 (1.1)
sul2	2 (1.9)	0 (0)	1 (1.8)	0 (0)	3 (1.6)

*This table showed only positive isolates. Non-detected genes screened in *Salmonella* in this study were: bla_{SHV}, bla_{CTX-M}, $bla_{NDM,}$ bla_{PSE}, bla_{OXA}, *sul3*, *qnrA*, *qnrB*, *ermB*, *dfrA1*, *dfrA12*, *catA*, *catB*, *cmlA*, *strB*, *tetD*, *addA1*, *addA2*, *aac(3)IV*, *aac(6')-Ib-cr*, *qepA*, *floR*, *mcr-1*, *mcr-2*, *mcr-3*, *mcr-4*, *mcr-5*, *int1*, *int2*, *int3*, and $int_{SXT.}$

5.4.3 Phenotype and genotype of AMR, virulence genes, and ESBL production in *V. cholerae*

All *V. cholerae* isolates (n = 75) were non-agglutinating vibrios (NAGs) with O1 and O139. Moreover, they exhibited non-agglutination to O141. All of *V. cholerae* isolates were susceptible to all tested antimicrobials. Based on the virulence of *V. cholerae*, only *hlyA* was detected in all *V. cholerae*.

The predominant AMR genes in *V. cholerae* was *sul1* (12.0%) (Table 17). About 4% of *V. cholerae* isolates harbored *catB*, *qnrS*, *tetA*, *tetB*, *strA*, and *dfrA1*. Colistin resistance genes, integrons, and SXT element of *V. cholerae* were not found in this study.

Table 17. AMR and virulence genes of *V. cholerae* isolates (n = 75).

	Prevalence (%)					
	Cultivation water (n = 34)	Fish carcass rinse (n = 10)	Intestine (n = 23)	Meat (n = 2)	Liver and kidney (n = 6)	Total (n = 75)
Virulence genes						
hlyA	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
ctx	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
tcpA	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
AMR genes*						
catB	2 (5.9)	1 (10.0)	0 (0)	0 (0)	0 (0)	3 (4.0)
qnrS	2 (5.9)	1 (10.0)	0 (0)	0 (0)	0 (0)	3 (4.0)
tetA	2 (5.9)	1 (10.0)	0 (0)	0 (0)	0 (0)	3 (4.0)
tetB	2 (1.9)	0 (0)	1 (1.8)	0 (0)	0 (0)	3 (4.0)
strA	2 (1.9)	0 (0)	1 (1.8)	0 (0)	0 (0)	3 (4.0)
sul1	8 (23.5)	1 (10.0)	0 (0)	0 (0)	0 (0)	9 (12.0)
dfrA1	2 (5.9)	1 (10.0)	0 (0)	0 (0)	0 (0)	3 (4.0)

*This table showed only positive isolates. Non-detected genes screened in *V. cholerae* in this study were: bla_{TEM}, bla_{SHV}, bla_{CTX-M}, bla_{NDM}, bla_{PSE}, bla_{OXA}, *sul2*, *sul3*, *qnrA*, *qnrB*, *ermB*, *dfrA12*, *catA*, *cmlA*, *strB*, *tetD*, *addA1*, *addA2*, *aac(3)IV*, *aac(6')-Ib-cr*, *qepA*, *floR*, *mcr-1*, *mcr-2*, *mcr-3*, *mcr-4*, *mcr-5*, *int1*, *int2*, *int3*, and int_{SXT}.

5.4.4 Sequencing

The determination of QRDR in eight *Salmonella* isolates that serovars included Saintpaul (n = 5), Neukoelln (n = 1), Virchow (n = 1), and Chartes (n = 1) (Table 18). The six isolates were detected a point mutation in *gyrA* from C to A at position 248 (Ser83Tyr).

Table 18. Mutations of *gyrA* in QRDR in ciprofloxacin resistant *Salmonella* isolates (n = 8).

Sample type	Serovar (n)	*gyrA* mutation	PMQR	Other AMR genotypes
Cultured water	Neukoelln (1)	-	*qnrS*	bla_{TEM}, *tetA*, *floR*
	Chartes (1)	C248A	*qnrS*	bla_{TEM}, *tetA*, *floR*
Carcass rinse	Virchow (1)	-	*qnrS*	bla_{TEM}, *tetA*
	Saintpaul (1)	C248A	*qnrS*	bla_{TEM}, *tetA*
Intestine	Saintpaul (4)	C248A	*qnrS*	bla_{TEM}, *tetA*

5.4.5 The association between the phenotypic and genotypic AMR

The result of logistic regression showed there was statistically significant association of tetracycline resistance and the presence of *tetA* in all isolates (OR 259.0, CI 52.3-1283.6, *P*-value < 0.0001), Sulfamethoxazole resistance and the presence of *sul2* (OR 90.3, CI 5.6-1455.1, *P*-value = 0.001), and streptomycin resistance and the presence of *strA* (OR 273.0, CI 1.8-42372.4, *P*-value = 0.029).

5.5 Discussion

5.5.1 Phenotype and genotype of AMR, virulence genes, and ESBL production in *A. hydrophila* isolates

The complete resistance of *A. hydrophila* (100%) to ampicillin found in this study was consistent with previous studies in tilapia and other aquatic animals due to its intrinsic resistance (Stratev and Odeyemi, 2016; Lee and Yoon, 2021). In this study, the majority of bacterial isolates were resistant to tetracycline, trimethoprim, and oxolinic acid, which were in accordance with previous reports (Chenia, 2016; El-ghareeb et al., 2019). *A. hydrophila* can persist in high concentrations of these antimicrobials in environment through the up-regulation of efflux pump and

production of anti-oxidative agents (Yu et al., 2021). Oxolinic acid is a one of fluoroquinolones, which has been widely used in aquatic animals (OIE, 2019). This antimicrobial can tolerate to strong sunlight and persist for a long-term freshwater (Louros et al., 2020). Resistance to quinolones in *A. hydrophila* were contributed from many mechanisms, including chromosomal mutation of QRDR, efflux pump, and PMQR, which was similar to the mechanisms of quinolone resistance in *Enterobacteriaceae*, such as *Escherichia coli* and *Salmonella.* (Chenia, 2016; Yang et al., 2017). Thus, further study of quinolone resistance mechanism in *A. hydrophila* should be investigated. The MDR of *A. hydrophila* in this study were 26.7%. Class 1 integron was detected in one non-MDR isolate. Although integrons and efflux pump were confirmed as vital roles in the development of MDR in *A. hydrophila* (Deng et al., 2016; Lo et al., 2022). Other mechanism in *A. hydrophila* conferred to MDR development should be determined.

Colistin had been widely used as feed additive in food-producing animals in Thailand for centuries until it was banned in 2017 (Olaitan et al., 2021). However, colistin resistance genes still existed. Transference of colistin-resistant bacteria from livestock to aquatic animals may accelerate the horizontal gene transfer of mobile genetic elements to autochthonous aquatic bacteria. This study is a first report of colistin resistance gene (*mcr-3*) in fish in Thailand. The isolate positive for colistin resistance gene confirmation from a previous study with the same primer pairs, was used as a positive control (Pungpian et al., 2021). Previous studies reported of *mcr-3* were detected in *E. coli* and *Salmonella* isolated from pigs and pork, and clinical isolates of humans (Luk-In et al., 2021; Pungpian et al., 2021). This raised a One Health concern of widely distribution of colistin resistance isolates in diverse sectors. Distribution of *mcr-1*, *mcr-3*, and *mcr-4* in bacteria isolated from fish were summarized (Figure 6). Therefore, the source of *mcr-3* should be investigated to

reduce the dissemination of colistin resistance genes in aquatic animals and environment. Although detection of *floR*, *sul1*, *sul2*, and *dfrA1* in this study was not correlate with their phenotypes, these genes were previously reported in *Aeromonads* isolated from freshwater fish and aquatic environment (Piotrowska and Popowska, 2014; Hayatgheib et al., 2021). In this study, 100% of *A. hydrophila* contained *aero* and *hly*, which were higher than a previous study in freshwater fish in Egypt (Ahmed et al., 2018). These virulence genes were reported in foodborne disease outbreak of diarrheal patients in Brazil (Silva et al., 2017). This study indicated that the resistance genes and virulence factors circulated in these hybrid red tilapia. Therefore, strategies to reduce the AMR distribution in aquaculture should be addressed.

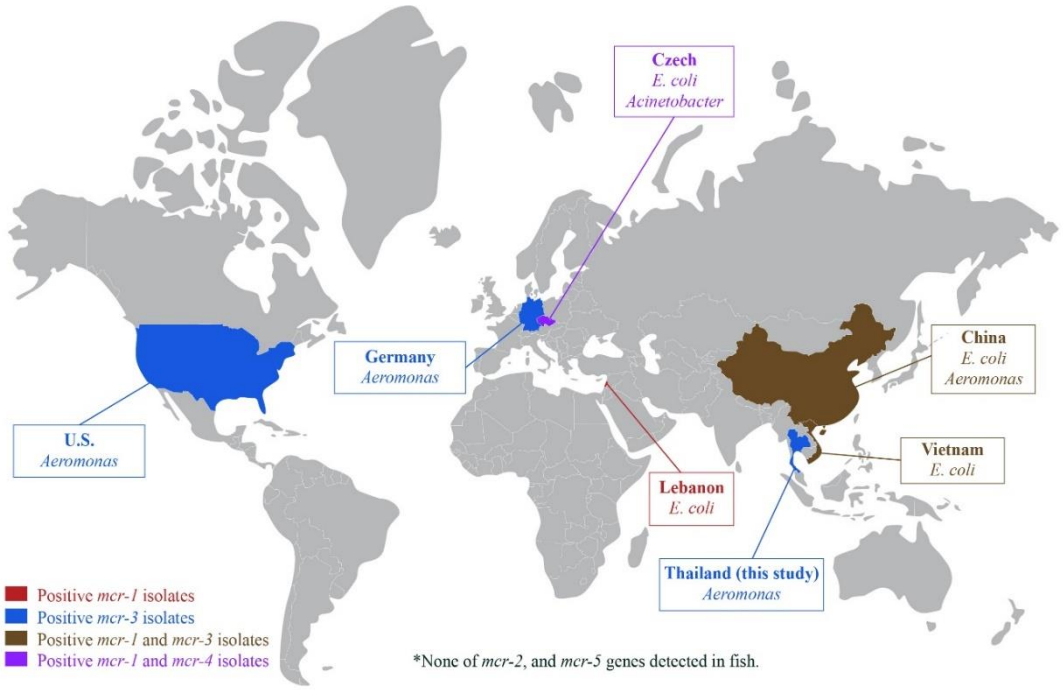


Figure 6. Distribution of colistin resistance gene *mcr-1*, *mcr-3*, and *mcr-4* in bacteria isolated from hybrid red tilapia

5.5.2 Phenotype and genotype of AMR, virulence genes, and ESBL production in *Salmonella* isolates

All *Salmonella* isolates were resistant to at least one antimicrobial and more than 75% of them were MDR, which was in agreement with previous studies of freshwater fish in India and Egypt (Cunha-Neto et al., 2019; Saharan et al., 2020; Gawish et al., 2021). This signified the MDR *Salmonella* in freshwater fish. The *Salmonella* isolates in this study was highly resistant to ampicillin (79.3%) due to intrinsically resistant to penicillins. β-lactam antimicrobials were approved for treatment of streptococcosis in tilapia (Baoprasertkul et al., 2012). β-lactam production is the major mechanism of β-lactam resistant *Salmonella*, which has been reported in fish worldwide (Jongjareanjai et al., 2009; Agoba et al., 2017). Oxytetracycline is one of common antimicrobials used in tilapia farms in Thailand, because it is effective to treatment of many endemic fish diseases, such as aeromoniasis, and francisellosis (Baoprasertkul et al., 2012). This study exhibited high resistance to oxolinic acid (75.5%), and oxytetracycline (71.8%). Frequent use of these antimicrobial groups can create selective pressure resulting in development of AMR and MDR bacteria. No resistant to gentamicin and low resistance to trimethoprim (1.1%) were observed in the *Salmonella* isolates in this study, which was similar to a previous study in Kenya (Wanja et al., 2020).

None of ESBL-producing *Salmonella* were detected in this study; however, the widespread of ESBL-producing bacteria in livestock animals and clinical strains of humans were reported in Thailand (Lay et al., 2021). Infection with resistance *Salmonella* can deteriorate clinical signs due to limitation of available therapeutic options. In this study. the predominant resistance genes were *qnrS* (65.4%) which is PMQR genes conferred quinolone resistance and enhanced QRDR mutation (Correia et al., 2017). The distribution of PMQR genes in the *Salmonella* isolates should be

further investigated in aquaculture. Abundance of *tetA* (64.9%) and *floR* (55.9%) was observed in this study, which was similar to previous studies in farmed fish in Brazil and environmental water in China (Zhou et al., 2019; Ferreira et al., 2021; Ye et al., 2021). Due to the high prevalence of AMR bacteria in fish intestine observed in this study, the removal of tilapia's intestine properly should be performed to reduce cross-contamination and enhance fish safety consumption.

5.5.3 Phenotype and genotype of AMR, virulence genes, and ESBL production in *V. cholerae* isolates

Infection of *V. cholerae* is a major public concern, because it is a causative agent of pandemic cholera. Serogroup O1 and O139 have been reported in cholera outbreaks with high mortality worldwide, while serogroup O141 caused sporadic cases of cholera-like diarrhea (Ghosh et al., 2016; Elimian et al., 2019; Hounmanou et al., 2022). All *V. cholerae* isolates found in this study were belonging to non O1/O139 and, non O141, which were abundant in fish and aquatic environment (Halpern and Izhaki, 2017; Schwartz et al., 2019). Infection of the non O1/O139 and non O141 *V. cholerae* can affect human heath, because these *V. cholerae* harbored virulence genes (Bakhshi et al., 2012). Infection of these *V. cholerae* isolates can cause watery diarrhea in humans as previously reported (Baker-Austin and Oliver, 2018).

Virulence genes (*hlyA, ctx*, and *tcpA*) were examined in this study. The *hlyA* gene encoded pore-forming toxin leading to cytotoxicity and cell vacuolation of intestinal cells leading to fluid leakage (Ramamurthy et al., 2020). All *V. cholerae* in this study carried *hlyA*, which was similar to previous study in fish, shellfish, and environmental samples (Shan et al., 2022). The *ctx* gene encoding cholera enterotoxin causes massive secretion of electrolytes and water to intestinal lumen leading to severe fluid loss, while *tcpA* gene acted as a promotor for pilus formation

inducing bacterial colonization in host's intestine (Ng et al., 2016; Silva and Benitez, 2016). In this study, no detection of *ctx* and *tcpA* indicated that *V. cholerae* were not cholera-causing strains. However, infection of *V. cholerae* can pose a risk of diarrhea due to the effect of *hlyA*.

In this study, none of the isolates were resistant to antimicrobials. Low prevalence of AMR was previously reported in environmental non-cholera *V. cholera* strains (Bier et al., 2015). In contrary, previous study showed high prevalence of resistant *V. cholerae* in fish (Fu et al., 2020). In this study, *sul1*, *catB*, *qnr*, *tetA*, *tetB*, *strA*, and *dfrA1* were detected, which were in accordance with a previous study in Thailand (Mala et al., 2017). The presence of *sul* (12.0%) observed in this study raised a particular concern, because sulfamethoxazole is suggested as an antibiotic of choice for cholera treatment (Leibovici-Weissman et al., 2014).

Overall, *tetA* was the predominant AMR genes observed in *Salmonella* and *V. cholerae* in this study. This gene can confer high phenotypic resistance of tetracycline and oxytetracycline. It is obvious that there was statistical association between tetracycline resistance and the presence of *tetA* under logistic regression analysis. High detection of *tetA* was consistency to previous studies in freshwater fish and environmental water (Muziasari et al., 2017; Hayatgheib et al., 2021). Other *tet* genes, such as *tetL*, *tetO*, and *tetW* were also previously reported in aquaculture (Harnisz et al., 2015; Muziasari et al., 2017).

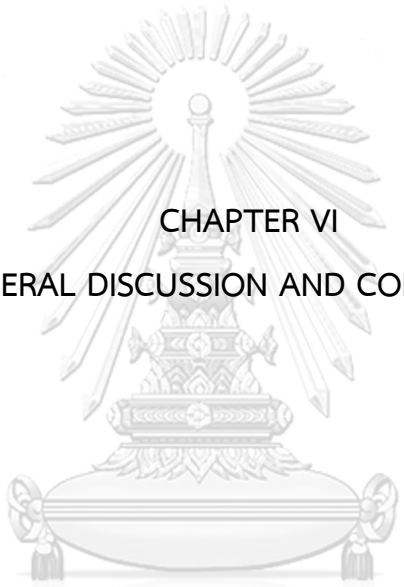
Only the *Salmonella* isolates in this study were resistant to ciprofloxacin with single point mutation in *gyrA* (Ser83Tyr) was observed in this study. This was in accordance with a previous study of *Salmonella* isolated from chicken, pork, and clinical isolates of human (Sinwat et al., 2018). Other PMQR genes mediated quinolone resistance such as *aac(6')-Ib-cr*, and *qepA* were negative in this study. This

implied that mutation in *gyrA* and presence *qnr*S were the factors mediating to quinolone resistance in *Salmonella* this study.

In conclusions, this study highlighted that ubiquitous AMR pathogens inhabited in aquaculture. Major sources of AMR bacteria should be evaluated to better understand their distribution in aquaculture. Quantitative AMR detection is highly recommended to monitor AMR trend in aquaculture. Laboratory capacity building and harmonized standard protocols should be developed to generate comparable AMR data. Good personal hygiene and sanitation practices are highly required to ensure fish safety consumption. Surveillance and monitoring of AMR in aquaculture under One Health can reduce AMR contamination.

CHAPTER VI

GENERAL DISCUSSION AND CONCLUSION



6.1 General discussion

Part 1 Bacterial pathogens and factors associated with *Salmonella* contamination in hybrid red tilapia (*Oreochromis* spp.) in a cage culture system

Thailand is one of the main tilapia producers in the world, few studies regarding to food safety of tilapia in Thailand were documented. Most of them were conducted in diseased fish or disease outbreak investigation (Nicholson et al., 2020). Tilapia can harbor a variety of bacteria, including fish pathogens (i.e *A. hydrophila*) and gut microflora (i.e *V. cholerae*), and contaminated bacteria (i.e. *Salmonella* and *E. coli*). Bacterial contamination in tilapia were mainly retrieved from anthropogenic wastes, agricultural production, and wastewater discharge. *A. hydrophila*, *V. cholerae*, and *Salmonella* were major bacteria zoonoses found in tilapia (Cortés-Sánchez et al., 2019; Ferreira et al., 2021). The occurrence of these bacteria in tilapia can possibly increase the risk of food-borne diseases in humans.

Various *Aeromonas* species were mostly reported in fish and aquatic environment, such as *A. veronii*, *A. dhakensis*, and *A. schubertii*. *A. veronii* have been the dominant species were related to variuos outbreaks in Thailand (Dong et al., 2015; Sakulworakan et al., 2021). *A. dhakensis* and *A. veronii* were the major pathogens reported in freshwater fish in Malaysia (Radu et al., 2003; Azzam-Sayuti et al., 2021). These later two *Aeromonas* app. are zoonotic pathogens that exhibit morphological characteristics similar to *A. hydrophila.* Thus, identification of *Aeromonas* app. required molecular-based methods with species-specific primers. (Rasmussen-Ivey et al., 2016).

CLSI recommends to include *A. hydrophila* to perform AST in aquatic animals (CLSI, 2020). In this study, the overall prevalence of *A. hydrophila* was low at 2.5%, which is in a normal range in healthy fish. This finding was supported by previous studies indicated that the prevalence of *A. hydrophila* in healthy fish was 2.3%

(Mzula et al., 2019). During *A. hydrophila* outbreaks in fish, the prevalence can rise to 25.0-33.3% (Ahmed et al., 2018; Sonkol et al., 2020). In this study, the prevalence of *A. hydrophila* in cultivation water was high at 12.5%, while the lower prevalence was observed in previous studies (Elbehiry et al., 2019). However, high prevalence of *A. hydrophila* in cultivation water were observed in Egypt (El-Gohary et al., 2020). The detection of high prevalence of *A. hydrophila* in this study were at risk for foodborne outbreak.

V. cholerae are ubiquitous in freshwater environment similar to *A. hydrophila* (Laviad-Shitrit et al., 2018); *V. cholerae* are gut microbiota, so it is not surprisingly that these bacteria could be found in intestinal tracts of fish (Halpern and Izhaki, 2017). This study observed the high prevalence of *V. cholerae* in the intestine (20.8%) and cultivation water (38.3%). The prevalence of *V. cholerae* in this study was higher than previous reports in China, which observed in fish (8.1%) and cultivation water (13.4%) (Yan et al., 2019). However, the prevalence of *V. cholerae* in this study may underestimate due to unable to detect, because this pathogen has different stages in the aquatic environment such as free-living, synergistic living with zooplanktons, and vegetative stage (viable but non-cultural (VBNC) stage). VBNC bacteria cannot be detected by a conventional bacterial isolation method, but they require additional supplements, such as catalase or sodium pyruvate for vegetative stage resuscitation (Mizunoe et al., 2000; Imamura et al., 2015). Molecular techniques, such as realtime-PCR can be used to detect the VBNC bacteria (Casasola Rodríguez et al., 2018). Characterization of all *V. cholerae* stages are required to examine overall prevalence of *V. cholerae*.

In this study, all *V. cholerae* isolates were non O1/O139 *V. cholerae*. Among many serogroups of *V. cholerae* serogroup O1 and O139 were important due to their

ability to produce cholera toxin. Thailand is endemic area for cholera outbreaks that have been occurred in refugee camps (Phares et al., 2016). However, serogroup O1 was observed in shrimp and environmental water in Thailand (Mala et al., 2017; Siriphap et al., 2017). Fish can be a potential reservoirs of *V. cholerae* serogroups O1 and O139 (Hounmanou et al., 2019; Yan et al., 2019). Exposure to contaminated fish and freshwater with virulent *V. cholerae* had a risk of cholera. However, *V. cholerae* serogroup non O1/O139 were capable of virulence causing sporadic cases in human. Infection of serogroup non O1/O139 *V. cholerae* exhibited either enteric (diarrhea) or extra-intestinal symptoms (bacteremia or wound infection) (Baker-Austin and Oliver, 2018; Schwartz et al., 2019). Infection of non O1/O139 with enteric symptoms in humans was confirmed in coastal area in Thailand (Tulatorn et al., 2018). This evidence suggested that *V. cholerae* both O1/O139 and non O1/O139 can transmit to human.

Salmonella is a zoonotic pathogen that can be found in fish worldwide. In this study, the highest prevalence of *Salmonella* (58.3%) was observed in cultivation water, which was consistency to a previous study in fish pond from Bangladesh (87.5%) (Ava et al., 2020). However, the prevalence of *Salmonella* in freshwater ranged from 11.9-20.6% (Traoré et al., 2014; Antaki et al., 2016). This indicated fecal contamination in cultivation water in these tilapia farms. However, all fish meat in this study were absent of *Salmonella*, which met the national microbiological standard of fish intended for human consumption in Thailand (BQSF, 2017). Among tilapia samples, this study found the highest prevalence of *Salmonella* in tilapia intestine (38.3%).

Salmonella serovar Saintpaul, Escanaba, Neukoelln, and Papuana were reported in tilapia and cultivation water. Budiati (2016) reported *Salmonella* serovar

Agona, Bovis-mobificans, Corvallis, Mikawashima, and Typhimurium isolated from tilapia in Malaysia. Tekale (2015) and Li (2017) reported *Salmonella* serovar Weltevreden in India and China. A study in Egypt found *Salmonella* serovar Enteritidis, Typhimurium, Kentucky, Infantis, Virchow, Paratyphi B, Senftenberg, and Anatum (Gawish et al., 2021). This data indicated that *Salmonella* serovars found in tilapia were different based on geographical distribution. Apart from *S.* Saintpaul reported in humans and pork from Thailand, other serovars found in this study, including Escanaba, Neukoelln, and Papuana have not been reported in in Thailand (Sinwat et al., 2015; Pungpian et al., 2021). A report of *S.* Escanaba was observed in poultry in India, while serovar Neukoelln and Papuana have not been detected in previous publications (Negi et al., 2015). Serovar Weltevreden did not observe in this study. Notably, serovar Weltevreden were detected in human clinical strains and different aquatic animals, such as shrimp, oyster, tuna, and tilapia. (Li et al., 2017; Hassan et al., 2018; Atwill and Jeamsripong, 2021).

The presence of *E. coli* in fish and cultivation water indicated the existence of microbial pollutant from fecal contamination. In this study, the highest prevalence and concentration of *E. coli* was observed in fish intestine. Therefore, the cross-contamination during fish preparation should be aware. In this study, most of *E. coli* in fish meat were within the limits that must lower than 10 MPN/25 g (BQSF, 2017). For cultivation water, this study observed 75.8% of the samples were contaminated with *E. coli*, which was similar to previous study of *E. coli* in fish pond in Bangladesh (Ava et al., 2020). Currently, the regulatory for microbiological standard in cultivation water was restricted in seawater used in marine aquaculture. Thus, microbiological standard in freshwater aquaculture should be drafted to reduce the bacterial contamination in fish. None of *E. coli* isolates in this study carried *stx1* and *stx2*, which can be found in Shiga toxin-producing *E. coli* (STEC). Previous publications of

STEC have been reported in tilapia from Egypt and Brazil (Saqr et al., 2016; Cardozo et al., 2018). The detection of virulence genes in *E. coli* should be confirmed after *E. coli* isolation to monitor the emergence of toxigenic *E. coli* in hybrid red tilapia.

The environmental parameters, including water and weather parameters, are directly associated with bacterial contamination in hybrid red tilapia and cultivation water. This study found that the humidity and maximum wind gust associated with the presence of *Salmonella* in the sample. Previous studies showed that water temperature, humidity, and DO were positively associated with the presence of *E. coli* (Ismail et al., 2016; Gołas et al., 2022). These conditions were preferable for mesophilic bacteria and led to bacterial multiplication. The weather conditions are mainly affected to introduced bacteria including *Salmonella* and *E. coli* to the fish farms. (Lebel et al., 2015). The high levels of rainfall and temperature was positively correlated with pathogens found in cage-culture tilapia (Lebel et al., 2015). The rain assisted fecal contaminants discharge to cultivation site, and high temperature can promote the bacteria growth. Moreover, the high temperature also affected fish health that contributed to lower fish feeding as a result of susceptibility of disease infection. Determination of weather and water conditions should be examined to identify potential predictors of bacterial contamination in cultured fish.

The Thai Agricultural Standard on Good Agricultural Practices (GAP) for tilapia farms was initiated to reduce bacterial contamination in tilapia (GAP, 2010). This standard suggested the good practices from pre-harvest processes, such as farm site selection and water quality assessment, fish management, until post-harvest process. Selection of no or low microbial pollution site, and using proper disinfection and sanitation in fish farms are required to prevent bacterial cross-contamination from

environment to cultivation area. Closed containment aquaculture systems should be implemented to promote food safety for hybrid red tilapia consumption.

Part 2 Molecular epidemiology of AMR and extended-spectrum ß-lactamase production of *E. coli* isolated from farm-raised hybrid red tilapia

AMR is an emerging public health threat impacting the worldwide population. Aquaculture is largely dependent on natural water resources, which are susceptible to the acquisition of microbial and AMR contaminants, and transferable mobile genetic elements from the environment. Aquaculture contributed to a major part of AMR development and distribution (Preena et al., 2020). This study highlighted high resistance to ampicillin, oxytetracycline, tetracycline, and oxolinic acid in the *E. coli* isolates. *E. coli* in this study were mainly resistant to ampicillin (63.1%), because some of ß-lactam antibiotic has been licensed to use in aquatic animals in Thailand. This data was in agreement with the resistance of ß-lactam antibiotic examined in animal and human isolates from Thailand (Pungpian et al., 2021). A previous study reported that amoxycillin was commercially available in fish stores in Thailand (Saengsitthisak et al., 2021). In this study, the resistance to oxytetracycline (58.6%), oxolinic acid (57.4%), and enrofloxacin (33.6%) was observed. This is possible that these antimicrobials are widely used in aquaculture. Additionally, these antimicrobials have been approved by Thailand FDA (Baoprasertkul et al., 2012).

ESBL-producing *E. coli* was observed in this study less than 5%, which much lower than previous reports in Vietnam and Tanzania (Hon et al., 2016; Moremi et al., 2016). In this study, half of *E. coli* isolates were MDR (53.8%), which was similar to previous studies in India and Malaysia (Saharan et al., 2020; Dewi et al., 2022). This addressed the dissemination of ESBL-producing and MDR *E. coli* in different

geographical distribution. In this study, the most prevalent resistance genes found in *E. coli* were bla_{TEM}, *qnrS*, *tetA*, and *tetB*, which corresponded to their phenotypes. bla_{TEM} is a common ß-lactamase gene that conferred resistance to ß-lactam antibiotics. More than half of the isolates (58.0%) carried bla_{TEM}, which is lower than the previous studies in Tanzania (63.3%) and Egypt (100%). (Moremi et al., 2016; Saqr et al., 2016). The presence of bla_{CTX-M} in this study agreed with previous study in frozen tilapia imported from Thailand to Saudi Arabia and tilapia farms in Thailand (Elhadi, 2016; Thongkao and Sudjaroen, 2019). Based on sequencing data, all bla_{CTX-M} were subtyped as $bla_{CTX-M-55}$, which was not commonly reported in tilapia. However, $bla_{CTX-M-55}$ has been commonly reported in food-producing animals. In contrary, $bla_{CTX-M-1}$, $bla_{CTX-M-9}$, and $bla_{CTX-M-15}$ were commonly observed in tilapia (Moremi et al., 2016; Hassen et al., 2020; Hoa et al., 2020).

In terms of the type of sample, fish meat exhibited high resistance to ampicillin, which was similar to previous study observed in tilapia meat from Egypt (Saqr et al., 2016). Furthermore, fish intestine showed the highest phenotypic resistances in all tested antimicrobials raising public health concern. Fish intestine functionally provided the optimal conditions for bacteria survival and promote horizontal transfer (Fu et al., 2020). Treatment of bacterial infection using medicated feed impacted on the bacterial community in fish gut microflora and increased their high efflux pump expression (Sáenz et al., 2019). This can develop cross resistance to other antimicrobials. In this study, bla_{CTX-M} can be found in fish sample but non-detected in cultivation water. Based in the results of this study, fish intestine is recommended to use as a representative sample for AMR monitoring and surveillance in fish.

Quinolone resistance is one of major public health concerned since this antimicrobial is commonly used in fish and human. In this study, two major mechanisms relating to quinolone resistance, including mutations in QRDR and the presence of PMQR determinants, were investigated. All resistant isolates to ciprofloxacin had *gyrA* mutations, including Ser83Leu and/or Asp87Asn, together with/without *parC* mutation compared with no mutation in the susceptible isolates. Mutation at Ser83Leu was similar to a previous study in *E. coli* isolates (Sellera et al., 2018), but it was different from *Aeromonas* isolates from South America (Chenia, 2016). The second point mutation of *gyrA* at Asp87Asn was commonly co-existed with a major mutation at Ser83 resulting in double point mutation (Yeh et al., 2017; Shaheen et al., 2021). Mutation in *parC*, Ser80Iso, which was observed in this study was in agreement with previous studies in fish and aquatic environment (Johnning et al., 2015; Chenia, 2016). Previous study indicated that the isolates carrying double *gyrA* mutations with *parC* mutation (triple mutations) was 2,000-fold greater than the susceptible isolates (van der Putten et al., 2018). However, this study found that triple mutations contained low resistance to ciprofloxacin (MIC 2-16 µg/ml). The predominant PMQR determinant observed in this study was *qnrS.* However, the role of *qnrS* in quinolone resistance is still unclear, because some high ciprofloxacin resistance isolates did not carry *qnr* genes.

Even though *E. coli* is not the major pathogen in fish, AMR *E. coli* can transmit their resistance genes to other bacteria in aquatic environment. Deployment of One Health in AMR and implementation of antibiotic stewardship should be performed. The emergence of AMR in aquaculture and possibly spread to the environment need effectively and timely control for preventing dissemination of AMR. Antibiotic stewardship and rational use of antimicrobials should be carried out in the

aquaculture. Multisectoral collaboration under the One Health is required to prevent and control of AMR in aquaculture.

Part 3 Determination of the phenotype and genotype of AMR, virulence genes, and ESBL production of *A. hydrophila*, *Salmonella* spp., and *V. cholerae* isolated from hybrid red tilapia and cultivation water

A. hydrophila is an important fish pathogen and zoonotic foodborne pathogen found in aquatic environment. In this study, all *A. hydrophila* isolates were resistance to ampicillin. The resistance to ampicillin was mediated by chromosome and plasmid. The intrinsic resistance to ß-lactam antimicrobials of *A. hydrophila* contributed by chromosomally mediated class C, and class D ß-lactamase (Chen et al., 2012). The production of ß-lactamase of *Aeromonas* was species-specific; therefore, the interpretation of ß-lactam resistance should be tailored for each *Aeromonas* species.

Plasmid and integrons are key roles in quinolone resistance of *A. hydrophila* (Deng et al., 2016). The *qnrS* was mainly observed in this study although all *A. hydrophila* isolates were susceptible to ciprofloxacin, enrofloxacin, and oxolinic acid. Detection of *qnrS* in *A. hydrophila* was consistent with previous studies with no detection of *qnrA* and *qnrB* (Chenia, 2016; Deng et al., 2016). Previous study of PMQR genes (*qnrB* and *qnrS*) were detected in *Aeromonas* (Wimalasena et al., 2017). The role of *qnr* in quinolone resistance and other PMQR genes in *Aeromonas* should be evaluated. In this study, colistin resistance (*mcr-3*) were detected in *A. hydrophila.* Other studies also reported colistin resistance genes, including *mcr*-1, *mcr-3*, and *mcr-4* were identified in *Aeromonas* spp. among fish and cultivation water (Eichhorn et al., 2018; Shen et al., 2018; Hassan et al., 2020; Liu et al., 2020; Tekedar et al.,

2020). Intrinsic resistance combined with other plasmid-borne resistance made *A. hydrophila* vulnerable developing MDR.

Salmonella is a major bacterial agent that was introduced to aquatic environment. This study indicated the common AMR in the *Salmonella* isolates were ampicillin, oxolinic acid, oxytetracycline, and tetracycline, which were similar to the observation among the *E. coli* isolates. bla_{TEM}, *qnrS*, *tetA*, and *floR* were the most frequent resistance genes observed in this study. Chloramphenicol was banned in food animals and aquatic animals due to its toxicity, while florfenicol is an antibiotic of choice for treatment against *Aeromonas* in tilapia (Assane et al., 2019). However, florfenicol was banned in Thailand. Four phenicol resistance genes examined in this study were *floR*, *cmlA*, *catA*, and *catB*, but only *floR* was detected. Previous studies indicated that *floR* was predominantly detected in fish and cultivation water in China (Zhou et al., 2019; Ye et al., 2021). The findings of *floR* raise serious public health concern due to plasmid-borne resistance. The horizontal plasmid transfer of these AMR genes was easily spread to other bacteria in aquaculture.

None of *V. cholerae* isolates in this study were serogroup O1, O139, and O141. *V. cholerae* serogroup O141 was newly discovered due to their distinct genetic clade from other serogroups and causing cholera similar to serogroup O1 and O139 (Hounmanou et al., 2022). Serogroup O141 was occasionally reported in aquatic animals and freshwater (Loeck et al., 2018; Fang et al., 2019). Therefore, serogroup O141 should be included to be identified together with serogroup O1 and O139. All *V. cholerae* observed in this study were susceptible to tested antimicrobials. It was postulated that genetic modification of susceptible *V. cholerae* during bacterial colonization in host cells which co-occurrence with the deletion of multiple mobile genetic elements (Das et al., 2016). However, susceptible isolates can become

resistant isolates by expression of their resistance genes or acquisition of other AMR genes, and genetic determinants. Therefore, performing AST should be continuously monitored.

The common resistance genes of *sul1*, *catB*, *qnr*, *tetA*, *tetB*, *strA*, and *dfrA1* were observed in *V. cholerae* in this study, which was inconsistent with previous studies indicated that *V. cholerae* were mostly resistant to sulfonamide and ampicillin (Hossain et al., 2018; Fu et al., 2020). In this study, *sul1* was the predominant gene in agreement with a previous study (Baron et al., 2016). High prevalence of *tetA*, *strA*, and *dfrA1* was similar to a previous study in fish (Fri et al., 2018). This study did not detect any integrons or SXT element. In contrary, SXT element was detected in fish in South Africa and environmental water in Thailand (Mala et al., 2017). The results of this study indicated the necessary of AMR monitoring in *Vibrio* in aquaculture is highly needed.

Overall, hybrid red tilapia were contaminated with indicator and pathogenic bacteria that may confer AMR phenotypes and genotypes. The emergence of ESBL, MDR, and colistin resistance genes was addressed in this study. To reduce the spread of AMR in the environment, effective strategies such as treatment of wastewater from communities and run-off agricultural sites, treatment of manure used for fertilizer, ban of using manure for fish feed, and optimal site management for agricultural and aquaculture, should be implemented.

6.2 Conclusion and suggestions

The results corresponding to the objectives of this study were achieved. This study revealed the contamination of indicator and pathogenic bacteria, and AMR bacteria in hybrid red tilapia and cultivation water. The dissemination and circulation

of AMR bacteria in fish and farm environment were concerned. The summary of the study objectives was described as follows:

Objective 1: To determine the prevalence of indicator and pathogenic bacteria, and environmental parameters associated with *Salmonella* contamination in hybrid red tilapia and cultivation water.

This study aimed to determine incidence of fecal coliforms, *E. coli*, *A. hydrophila*, *Salmonella* spp., *V. cholerae*, *V. vulnificus*, and *S. agalactiae* contamination in hybrid red tilapia and cultivation water, and to examine environmental factors affecting on *Salmonella* contamination. Tilapia and cultivation water were potential hotspots for fecal coliforms and *E. coli* contamination. In addition, the presence of *Salmonella*, *V. cholerae*, *A. hydrophila*, and *V. vulnificus* were observed in this study, while *S. agalactiae* was not detected. The similar rates of bacterial contamination observed among fish and cultivation water samples, indicating that cultivation water can be used for bacterial surveillance in aquaculture. *Salmonella* serovar Saintpaul was firstly addressed in hybrid red tilapia in this study. Serovar Neukoelln, Escanaba, and Papuana was firstly indicated in aquaculture of Thailand. The presence of *Salmonella* in hybrid red tilapia and cultivation water was statistical associated with the contamination of fecal coliforms, *E. coli*, *V. cholerae*, and *V. vulnificus*. This study highlighted genetic relatedness among *Salmonella* serovars isolated from grazing ducks reared in the proximity of the hybrid red tilapia farms, tilapia, and cultivation water suggesting grazing ducks may be a source of *Salmonella* contamination in hybrid red tilapia aquaculture. Proper preparation and fully cooked consumption of fish should be performed to ensure food safety.

Objective 2: To characterize phenotypic and genotypic AMR, virulence genes, and EBSL production of *E. coli* isolated from hybrid red tilapia and cultivation water.

This study observed AMR, virulence genes, and ESBL production of *E. coli* isolates. Most of *E. coli* isolates were resistant to at least one antimicrobial. MDR and ESBL-production were detected in *E. coli*. Most *E. coli* resisted to ampicillin, oxytetracycline, tetracycline, and oxolinic acid, which were frequently used in aquatic animals. AMR genes, including bla_{TEM}, *qnrS*, *tetA*, and *tetB* were commonly found in this study. The abundance of AMR genes was mainly found in fish intestine. Therefore, the intestine should be carefully removed before filleting a fish to reduce bacterial contamination. Class 1 integron was detected in *E. coli* isolates that can promote the AMR spreading in the aquaculture and environment. The majority of ß-lactamase and ESBL genes in *E. coli* was bla_{TEM-1} and $bla_{CTX-M-55}$, which were previously reported in pigs and wastewater in Thailand. None of *E. coli* isolates were detected *stx1* and *stx2*, which found in Shiga toxin-producing *E. coli* (STEC). This study indicated that hybrid red tilapia and cultivation water are potential reservoirs of AMR and their resistant determinants. To monitoring and surveillance of AMR in aquaculture, fish intestine is recommended to use for antimicrobial susceptibility. Strategies to reduce the risk of AMR in aquaculture should be examined under the One Health.

Objective 3: To determine the phenotypic and genotypic characteristics of AMR, virulence genes, and EBSL production of *A. hydrophila*, *Salmonella* spp., and *V. cholerae* isolated from hybrid red tilapia and cultivation water.

The isolates of *A. hydrophila*, *Salmonella* spp., and *V. cholerae* were characterized for AMR phenotype and genotype, virulence genes, and ESBL production. High prevalence of AMR in zoonotic bacteria in hybrid red tilapia and

cultivation water was observed in this study. The major finding of this study was emerging of *mcr-3* in the *A. hydrophila* isolates. *A. hydrophila* highly resisted against oxolinic acid, oxytetracycline, tetracycline, and trimethoprim. *Salmonella* exhibited high resistance to ampicillin, oxolinic acid, oxytetracycline, chloramphenicol, and florfenicol. However, all *V. cholerae* isolates detected in this study were not serogroups O1, O139, and O141, which were commonly found in environment. The *V. cholerae* isolates were susceptible to all tested antimicrobials. Most of AMR genotypes was mostly related to their corresponding phenotypes. Diverse AMR bacteria found in aquaculture is of public health significance. This is an urgent need to provide effective intervention and a close monitoring to mitigate AMR in aquaculture.

The overall results from three chapters highlighted high contamination of indicator and pathogenic bacteria isolated from hybrid red tilapia and cultivation water. Multiple AMR phenotypes and genotypes were detected in aquaculture. This study also highlighted the emergence of colistin resistance gene in hybrid red tilapia aquaculture, which is a critical threat to human health.

6.3 Suggestions and further studies

6.3.1. AMU and AMR

AMU for treatment in aquaculture should be selected based on a result of antimicrobial susceptibility test. Rational AMU and antibiotic stewardship should be promoted in aquaculture. Guidelines of AMU in freshwater fish should be initiated Using unregistered or prohibited antimicrobial drugs must be banned. Education of AMU and AMR is required for aquaculture farmers to enhance their awareness. Furthermore, implementation of GAP farms should be carried out to reduce microbial contamination in aquaculture and maximize food safety from fish.

6.3.2. Reduction of risk of bacterial contamination

Good personal hygiene and sanitation practices are needed to reduce bacterial infection of humans. Appropriate fish rinsing before and after evisceration must be done. To reduce the cross-contamination, different measures such as disinfection of knives, cutting boards, and utensils, sanitization of the fish processing area, and using proper storage practices before cooking should be performed. Consumption of raw or inadequate cooking fish should be avoided to reduce the risk of acquiring zoonotic pathogens.

6.3.3. Enhancing laboratory capacity and harmonized standard protocols

Building laboratory capacity for isolation of bacteria and identification of AMR should be established in local and regional levels to monitor AMR in aquaculture. Standard protocols of bacterial isolation and antimicrobial susceptibility test are required for harmonized AMR results.

6.3.4. Phenotypic and genotypic characterization of AMR and their determinants

Phenotypic and genotypic characterization of AMR is highly recommended for antimicrobial susceptibility test. Also, whole-genome sequencing (WGS) is suggested to undertaken in the AMR isolates for future surveillance. This is because WGS is useful to identify whole DNA sequence of a bacteria, locate AMR genes on either chromosome or plasmid, examine virulence genes, and explore the potential mechanisms of resistance. High resolution of genetic information regarding AMR with rapid detection will be useful for timely prevention and control of AMR.

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APPENDICS

APPENDIX A

Parts of this dissertation have been processed for publication as follow:

List of international publication

1. Thaotumpitak, V., Sripradite, J., Atwill, E.R., Tepaamorndech, S. and Jeamsripong, S., 2022. Bacterial pathogens and factors associated with *Salmonella* contamination in hybrid red tilapia (*Oreochromis* spp.) cultivated in a cage culture system. (Accepted to Food Quality and Control). https://doi.org/10.1093/fqsafe/fyac036.

List of local conference

1. Thaotumpitak, V., Sripradite, J., Atwill, E.R., and Jeamsripong, S., 2021. Distribution of *Escherichia coli* and *Salmonella* in hybrid red tilapia (*Oreochromis* spp.) and cultured water. The 2021 National RGJ and RRI Conferences, 14th June, 2021, Bangkok, Thailand.

APPENDIX B

Table S1. AMR pattern for *A. hydrophila* isolates (n = 15) from cultivation water and carcass rinse.

Resistance pattern	No. of isolate (%)		
	Cultivation water (n = 5)	Carcass rinse (n = 10)	Total (n = 15)
AMP	4 (80.0)	4 (40.0)	8 (53.3)
AMP-OTC-TET-TRI	1 (20.0)	1 (10.0)	2 (13.3)
AMP-OTC-TRI	0 (0)	1 (10.0)	1 (6.7)
AMP-OTC-OXO-TET	0 (0)	1 (10.0)	1 (6.7)
AMP-OXO	0 (0)	2 (20.0)	2 (13.3)
AMP-TRI	0 (0)	1 (10.0)	1 (6.7)

AMP, ampicillin; OTC, oxytetracycline; OXO, oxolinic acid; TET, tetracycline; TRI, trimethoprim

Table S2. AMR pattern for *Salmonella* spp. isolates (n = 188) from hybrid red tilapia and cultivation water

Resistance pattern	No of isolates (%)				
	Cultivation water (n = 106)	Fish carcass rinse (n = 24)	Intestine (n = 57)	Liver and kidney (n = 1)	Total (n = 188)
Susceptible	4 (3.8)	8 (33.3)	11 (19.3)	0 (0)	23 (12.2)
AMP-CHP-CIP-ENR-FFC-OTC-OXO-TET	1 (0.9)	1 (4.2)	2 (3.5)	0 (0)	4 (2.1)
AMP-CHP-CIP-ENR-OTC-OXO-TET	6 (5.7)	0 (0)	4 (7.0)	0 (0)	10 (5.3)
AMP-CHP-CIP-FFC-OTC-OXO-TET	3 (2.8)	0 (0)	0 (0)	0 (0)	3 (1.6)
AMP-CHP-CIP-OTC-OXO-TET	2 (1.9)	0 (0)	0 (0)	0 (0)	2 (1.1)
AMP-CHP-ENR-FFC-OTC-OXO-STR-TET	0 (0)	0 (0)	1 (1.8)	0 (0)	1 (0.5)
AMP-CHP-ENR-FFC-OTC-OXO-SMZ-TET	1 (0.9)	0 (0)	0 (0)	0 (0)	1 (0.5)
AMP-CHP-ENR-FFC-OTC-OXO-TET	25 (23.6)	5 (20.8)	5 (8.8)	0 (0)	35 (18.6)
AMP-CHP-FFC	8 (7.5)	0 (0)	3 (5.3)	1 (100)	12 (6.4)
AMP-CHP-FFC-OTC	1 (0.9)	0 (0)	0 (0)	0 (0)	1 (0.5)
AMP-CHP-FFC-OTC-OXO	0 (0)	0 (0)	1 (1.8)	0 (0)	1 (0.5)
AMP-CHP-FFC-OTC-OXO-TET	27 (25.5)	5 (20.8)	7 (12.3)	0 (0)	39 (20.7)
AMP-CHP-FFC-OXO-STR	0 (0)	0 (0)	1 (1.8)	0 (0)	1 (0.5)
AMP-CHP-FFC-TET	3 (2.8)	0 (0)	0 (0)	0 (0)	3 (1.6)
AMP-CHP-OTC-OXO-TET	1 (0.9)	0 (0)	1 (1.8)	0 (0)	2 (1.1)
AMP-CHP-OXO	0 (0)	0 (0)	1 (1.8)	0 (0)	1 (0.5)
AMP-CHP-OXO-TET	0 (0)	0 (0)	1 (1.8)	0 (0)	1 (0.5)
AMP-CIP-ENR-OTC-OXO-SMZ-TET	0 (0)	0 (0)	1 (1.8)	0 (0)	1 (0.5)
AMP-CIP-ENR-OTC-OXO-TET	8 (7.5)	0 (0)	6 (10.5)	0 (0)	14 (7.4)
AMP-CIP-ENR-OTC-OXO-TET-TRI	1 (0.9)	0 (0)	0 (0)	0 (0)	1 (0.5)
AMP-CIP-FFC-OTC-OXO-TET	0 (0)	1 (4.2)	0 (0)	0 (0)	1 (0.5)
AMP-CIP-OTC-OXO-TET	1 (0.9)	1 (4.2)	1 (1.8)	0 (0)	3 (1.6)
AMP-CIP-TET	1 (0.9)	0 (0)	0 (0)	0 (0)	1 (0.5)
AMP-ENR-OTC-OXO-TET	0 (0)	0 (0)	1 (1.8)	0 (0)	1 (0.5)
AMP-FFC-OTC-OXO-TET	0 (0)	0 (0)	1 (1.8)	0 (0)	1 (0.5)
AMP-OTC-OXO-SMZ-STR-TET	1 (0.9)	0 (0)	0 (0)	0 (0)	1 (0.5)

Table S2. AMR pattern for *Salmonella* spp. isolates (n = 188) from hybrid red tilapia and cultivation water. (Continue)

Resistance pattern	No of isolates (%)				
	Cultivation water (n = 106)	Fish carcass rinse (n = 24)	Intestine (n = 57)	Liver and kidney (n = 1)	Total (n = 188)
AMP-OTC-OXO-TET-TRI	1 (0.9)	0 (0)	0 (0)	0 (0)	1 (0.5)
AMP-OTC-SMZ-STR-TET	1 (0.9)	0 (0)	0 (0)	0 (0)	1 (0.5)
AMP-OTC-OXO-TET	5 (4.7)	0 (0)	0 (0)	0 (0)	5 (2.7)
AMP-OTC-TET	0 (0)	0 (0)	1 (1.8)	0 (0)	1 (0.5)
CHP-FFC	0 (0)	0 (0)	1 (1.8)	0 (0)	1 (0.5)
ENR-OTC-OXO	1 (0.9)	0 (0)	0 (0)	0 (0)	1 (0.5)
OTC	2 (1.9)	0 (0)	1 (1.8)	0 (0)	3 (1.6)
OTC-OXO	0 (0)	0 (0)	1 (1.8)	0 (0)	1 (0.5)
OXO	2 (1.9)	3 (12.5)	5 (8.8)	0 (0)	10 (5.3)
Total	106 (56.4)	24 (12.8)	57 (30.3)	1 (0.5)	188 (100)

AMP, ampicillin; CHP, chloramphenicol; CIP, ciprofloxacin; ENR, enrofloxacin; FFC, florfenicol; OTC, oxytetracycline; OXO, oxolinic acid; STR, streptomycin; SMZ, sulfamethoxazole; TET, tetracycline

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