Doctoral Thesis

Molecular Genetic Analysis of Antimicrobial Resistance in Foodborne Bacteria

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Graduate School of Integrated Sciences for Life

Hiroshima University

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STATEMENT OF ORIGINALITY AND DECLARATION

I hereby declare that this dissertation is the product of my own research work, except for references to publications where due acknowledgement has been appended in the text. I confirm that neither part of this work nor material in substantial content has been previously submitted towards the award of doctoral degree or related diploma in an academic institution or institute of higher education.

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Chapter 1

1. General introduction

Antimicrobial resistance (AMR) has emerged, and is recognized as one of the leading public health challenges of the 21st century. AMR is defined as the ability of microorganisms to survive and proliferate under the influence of an antimicrobial agent (Abushaheen et al., 2020). The UK government and the Welcome Trust have commissioned "The Review on Antimicrobial resistance," and estimated that by 2050, nearly 10 million people per year are at risk because of the increasing incidence of AMR, which could cost USD 100 trillion economic output (O'Neill 2014; O'Niell, 2016). Knowing the global trends of AMR and current magnitude of occurrence are crucial aspects to evaluate pathogen control around the world. Literature claims suggest that, if left unattended, AMR will continue to spread beyond imagination and could facilitate the evolution of new resistance mechanisms in bacterial pathogens which may be much more fatal in the future than the existing superbugs (Murray et al., 2022).

Antibiotics are generally used to treat bacterial infections in clinical settings around the world. In addition, the increasing demand for animal protein has driven farmers to apply antibiotics as prophylaxis and growth enhancers (Kasimanickam et al., 2021). As they are frequently used in therapeutics, bacteria have a higher chance of developing more intricate resistance mechanisms against those antimicrobials. Consequently, newly evolved strains appear to resist or jeopardize the efficacy of recommended treatment options against many infections, causing profound consequences such as morbidity and mortality as well as clinical complications (Abushaheen et al., 2020; Khameneh et al., 2016). Understanding bacterial resistance mechanisms and the behavior of mobile genetic elements, would help find effective antimicrobials to fight the escalating life-threatening drug resistant infections. The

problem is not solved when researchers only rely on clinical data to monitor the spread and trends of AMR. Given that microbes freely move (Shankar, 2016), or can be transmitted to humans through the consumption of contaminated food products, areas where AMR surveillance seems to be minimally implemented should be targeted and monitored as such.

Although several efforts to implement AMR surveillance programs along the food supply chain have experienced overall increase over decades, AMR rates vary between geographical regions and among different species. Moreover, human activities and urbanization as well as acquisition of genetic elements by the so-called superbugs are key players in the increasing trends of AMR around the globe. In Japan, two national AMR surveillance programs implemented by the Ministry of Health, Labour, and Welfare for 20 years (from 1999 and 2000) showed that AMR bacteria are on the rise and have chronologically remained endemic (Suzuki, 2021). Therefore, monitoring the spread and distribution of AMR at the local, regional, and national level would be a crucial part of control strategy. In this context, this research primarily explored AMR originating from supermarket retail meat and seafood products in a One Health perspective. Specifically, this study aimed to:

1. Isolate different bacterial species from supermarket retail meat and seafood samples
2. Investigate the occurrence of antimicrobial resistance determinants in the isolates
3. Characterize antimicrobial resistance among the isolates
4. Compare the rate of antimicrobial resistance among meat isolates recovered in 2009 and 2021
5. Characterize foodborne bacteria carrying mobile colistin resistance (*mcr*) genes

1.2. Review of related literature

1.2.1 Origin of antibiotic resistance

Although the origins of antibiotic resistance have long remained elusive, it was thought that antibiotic tolerance or the development of resistance was driven by the successful use of antibiotics in the clinical settings and in farming processes (Davies and Davies, 2010; Vanderbroucke-Grauls and Kluytmans, 2022). It is obvious that most antibiotic compounds are derived from nature, and resistance to these compounds is due to evolutionary events, leading to the emergence of resistance genes which circulate between different ecological niches such as humans, animals, and the environmental (Fig. 1) (Vanderbroucke-Grauls and Kluytmans, 2022). In addition, by definition, bacterial pathogens are susceptible to an antibiotic compound but develop resistance afterwards owing to the occurrence of mutations and genetic transfer mechanisms (Martinez, 2014; Martinez and Baquero, 2000). Antibiotic resistance due to mutation usually occurs in genes encoding the targets of the antibiotic, genes encoding their transporters, and genes encoding repressors of the antibiotic transporters (Martinez, 2014).

In horizontal gene transfer, genetic material from one bacterial cell is transferred to the other. It is suggested that this might have evolved from environmental bacteria, since it was not normally present in human pathogens before antibiotic administration (Arnold et al., 2022). Since many antibiotics are synthesized from nature or environmental microorganisms, it has also been suggested that the evolution of resistance genes originated from antibiotic producers, thus, they acquired genes to protect themselves from their own antibiotic production that would kill them (Benveniste and Davies, 1973).

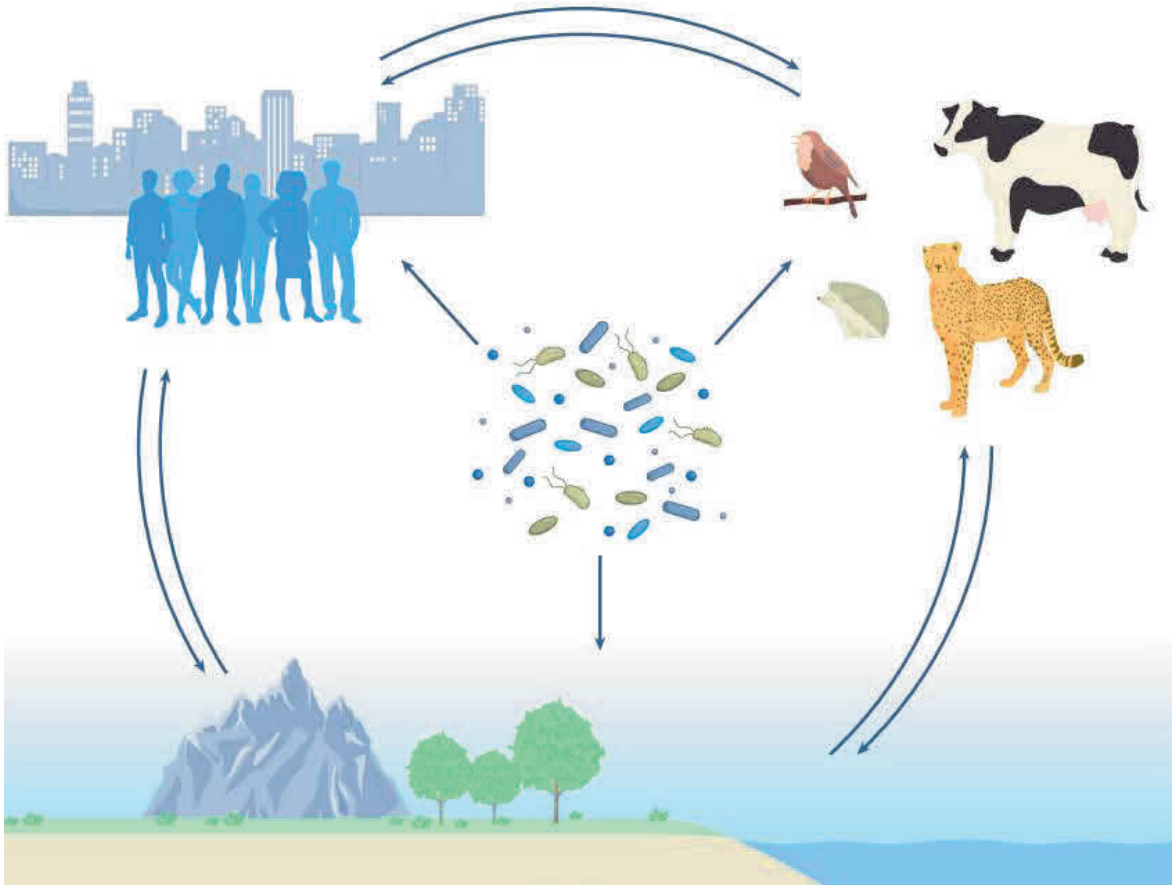

Fig 1. Antimicrobial resistance genes and resistant bacteria are continuously exchanged between humans, animals, and the environment. Bacteria are omnipresent and encounter antimicrobial agents made by humans or present in nature (for example, made by fungi). Resistance, therefore, can develop everywhere, and resistant strains or resistance genes can be exchanged between humans, wild animals, livestock, and the environment. Increased use of antimicrobial agents, in combination with growing populations of humans, growing populations of livestock, and closer contact of humans with animals, all promote the global spread of antimicrobial resistance. To combat this global health challenge, a One Health approach is needed (Vanderbroucke-Grauls and Kluytmans, 2022). Reused with permission from Nature Medicine (ref 211752). Copyright 2022, Springer Nature America Inc.

1.2.2 Mechanisms of antimicrobial resistance

The mechanisms related to antibiotic resistance in bacteria can be grouped into two types: 1) intrinsic resistance and 2) acquired resistance. Intrinsic resistance occurs when a particular bacterium uses natural means or its biological properties against the compound while acquired resistance results from acquisition of resistance genes, or mutations in chromosomal DNA sequence (Khameneh et al., 2016). Bacteria can develop resistance using specific mechanisms such as antibiotic inactivation by enzyme breakdown, the expression of efflux pumps, alterations to the antibiotic's intended target, or reduced cell membrane permeability (Irfan et al., 2022; Darby et al., 2023). Detailed explanation of the mechanisms is indicted in Figure 2 and elaborated below:

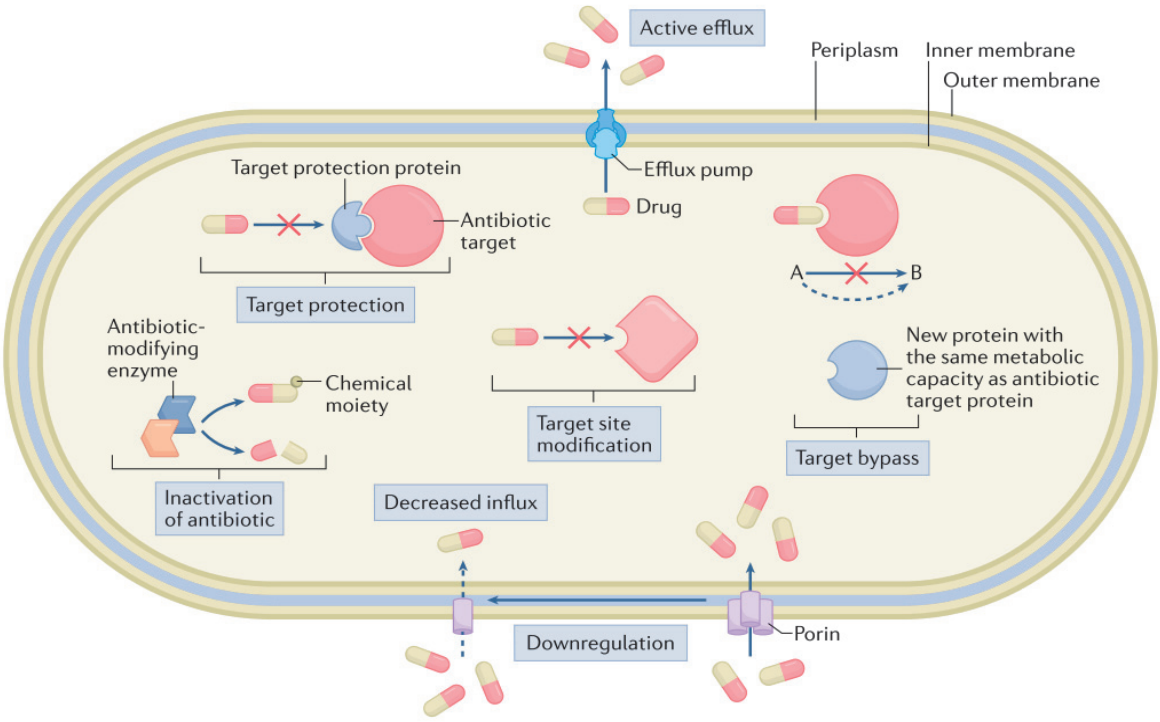

Figure 2. Overview of the molecular mechanisms of antibiotic resistance (Darby et al., 2023). Reused with permission from Nature Reviews Microbiology, (ref 211753), Copyright 2022. Springer Nature Limited.

Alteration of drug target

The modification of a drug's target can be achieved by target replacement (e.g. acquisition of chimeric penicillin-binding proteins can lead to β-lactam resistance), enzymatic modification of the target (e.g. vancomycin resistance is due to restructuring of the cell wall), or target mutation (e.g. resistance to quinolone is due to mutations in bacterial topoisomerases) (Martinez, 2014).

Expression of efflux pumps

Efflux pumps are transport proteins that expel or pump drug from within cells into the surrounding environment (Webber and Piddock, 2003). Thus, the antibiotic concentration within the intracellular environment decreases below inhibitory level which becomes non-toxic to bacterial survival. Five major families of efflux transporters have been identified in prokaryotic kingdom: MATE (multidrug and toxic efflux), RND (resistance-nodulation-division), MF (major facilitator), SMR (small multi-drug resistance), and ABC (ATP binding cassette) (Lomovskaya et al., 2001).

Reduced permeability

For an antimicrobial agent to be effective against the target bacteria, it needs to penetrate the cell envelope to reach its target. The outer membrane, especially of gram-negative bacteria is an intricate organelle that can act as a barrier, while still allowing nutrient uptake (Darby et al., 2023). During bacterial growth, the permeability of the outer membrane changes, influencing the amount of drug that can be translocated across the membrane. The so-called OmpF and OmpC porins identified in *Enterobacteriaceae* allow small hydrophilic β-lactam antibiotics to enter bacterial cell (Whittle et al., 2021). Repression of porin genes prevents the entry of antibiotics into bacterial cells. Additionally, mutations occurring in the

porin proteins can change the channel's diameter which consequently blocks the translocation of drugs across bacterial membrane (Bajaj et al., 2016; Whittle et al., 2021).

Inactivation of drugs by enzymes

This mechanism is by far the most common, where bacteria produce enzymes to hydrolyze or modify the chemical structure of antibiotics—can be divided into two mechanisms: inactivation by degrading the antibiotic and modification by the transfer of a functional group. Both mechanisms are widespread among *Enterobacteriaceae* and gram-positive bacteria due to indirect or horizontal gene transfer mechanisms (Darby et al., 2023). Some examples of drug inactivation include the action of β-lactamases on β-lactam antibiotics and the inactivation of tetracyclines by tetracycline hydroxylases (Tooke et al., 2019). β-lactamases are enzymes that hydrolyze the amide bond of β-lactam ring in β-lactam drugs.

1.2.3 Major groups of antibiotic resistance genes and their nomenclature

Many genes are involved in antibiotic resistance among bacteria. The production of β-lactamases is most frequently detected in a Gram-negative bacterial isolate that demonstrates resistance to a β-lactam antibiotic. These β-lactamases are classified into 1) Ambler molecular classification, which is based on protein sequence and 2) functional classification, which groups β-lactamases according to the functional similarities of substrate hydrolysis (Ambler, 1980; Bush et al., 1995). The Ambler classification comprises of four molecular classes, A, B, C, and D. Classes A, C, and D use active site serine to hydrolyze their substrates, whereas class B β-lactamases are metalloenzymes that utilize active-site zinc ion to hydrolyze β-lactams (Bush and Bradford, 2019). Nonetheless, a new classification scheme was proposed, where the enzymes are divided into three groups: group 1 (class C) cephalosporinases; group

2 (classes A and D) broad-spectrum, inhibitor-resistant, and extended-spectrum β-lactamases and serine carbapenemases; and group 3 metallo-β-lactamases (Bush and Jacoby, 2010).

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Chapter 2

Comparative molecular profiling of antimicrobial resistance and phylogenetic characterization of multidrug-resistant *Escherichia coli* isolated from meat sources in 2009 and 2021 in Japan

1. Summary

The global spread of antimicrobial resistance (AMR) is alarming. *Escherichia coli* is a gram-negative bacterium that causes healthcare-associated infections and is a major threat to public health. Currently, no comprehensive antimicrobial surveillance of multidrug-resistant *E. coli* of diverse phylogroups along the meat value chain has been implemented in Higashihiroshima, Japan. Therefore, by employing the One Health approach, 1,183 bacterial isolates, including 303 recovered from meat samples in 2009, were screened for the presence of antimicrobial resistance determinants using multiplex PCR and DNA sequencing techniques. Seventy-seven non-duplicate *E. coli* isolates that harbored AMR genes were subjected to antimicrobial susceptibility testing and the detection of integrons. Phylogenetic characterization, which has not been previously investigated, was used to assign *E. coli* to one of the eight phylogroups.

Twenty-six out of 33 (78.8%) and 34 out of 44 (77.3%) *E. coli* isolates from 2009 and 2021 exhibited multidrug resistance (MDR) phenotypes, respectively. The most common clinical resistance was observed against ampicillin, tetracycline, kanamycin, sulfamethoxazole/trimethoprim, cefotaxime, and chloramphenicol. Overall, 22.1% (17/77) of the *E. coli* isolates carried an extended-spectrum β-lactamase (ESBL)-encoding gene and showed the ESBL-resistant phenotypes. For the two isolation years, AmpC/ESBL prevalence decreased from 42.4% in 2009 to 20.5% in 2021. The identified AMR genes included *bla*$_{\text{CTX-M-1}}$, *bla*$_{\text{CTX-M-2}}$, *bla*$_{\text{CTX-M-14}}$, *bla*$_{\text{CTX-M-15}}$, and *bla*$_{\text{SHV-12}}$ (ESBL-types); *bla*$_{\text{SHV-1}}$, *bla*$_{\text{TEM-1}}$, *bla*$_{\text{TEM-135}}$, and *bla*$_{\text{TEM-176}}$ (narrow-spectrum types); *bla*$_{\text{CMY-4}}$, *bla*$_{\text{ADC-32}}$, *bla*$_{\text{ADC-216}}$, *bla*$_{\text{ACT-48}}$, and

bla$_{\text{ACT-51}}$ (AmpC types); and integrons. All *E. coli* isolates were negative for carbapenemase-encoding genes, whereas one isolate from 2009 carried *mcr-5.1* allele. Approximately 52% of *E. coli* isolates identified in 2009 were assigned to phylogroup A compared to the 20.5% in 2021. Notably, the highest proportions of *E. coli* phylogroups exhibiting MDR were groups A, B1, and F, suggesting that members of these groups are mostly associated with drug resistance. This study highlights the role of meat as a significant reservoir of MDR *E. coli* and potential source for transmission of AMR genes. Furthermore, the findings emphasize the importance of continuous monitoring to track the changes in the spread of antimicrobial resistance in the food chain.

Keywords: Multidrug resistance, *E. coli*, Extended-spectrum β-lactamase, Phylogroup, Meat, Japan.

2. Introduction

Escherichia coli, a member of the *Enterobacteriaceae* family, naturally belongs to the normal commensal flora of warm-blooded animals and humans (Bendary et al., 2022). Hence, *E. coli* is versatile, with some strains causing extra-intestinal and systemic infections in a broad range of hosts (Pereira et al., 2013). Owing to its ubiquity in nature, *E. coli* is commonly used as a fecal indicator of food hygiene, including water quality assessment, to certify the effectiveness of sanitation in the food industry (Sodagari et al., 2021). It has been classified as i) commensal, ii) intestinal pathogenic, or iii) extra-intestinal pathogenic. Based on phylogenetic classification, the species can be assigned to one of the following phylogroups: A, B1, B2, or D (Carlos et al., 2010). In a new classification, Clermont et al. (2013) proposed four additional phylogroups: C, E, F, and *Escherichia* cryptic clade I. Members of the phylogroup differ in genotypic and phenotypic characteristics. Extra-intestinal pathogenic strains mostly belong to phylogenetic groups B2 and D, whereas

commensal *E. coli* strains usually belong to groups A and B1 (Corzo-Ariyama et al., 2019; Pereira et al., 2013).

E. coli infections are mostly treatable. However, due to evolutionary transformation and variation, the bacterium has developed novel resistance mechanisms against important antimicrobials, including the last-line antibiotics used in clinical practice. For instance, the problematic mechanisms in *E. coli* correspond to the acquisition and silent dissemination of exogenous genes through horizontal gene transfer of mobile genetic elements (plasmids, transposons, insertion sequences, or integrons) and genomic islands (Ouchar Mahamat et al., 2021; Partridge et al., 2018; Xedzro et al., 2022). As a result, the development of resistance to multiple antimicrobials has emerged, which is challenging for the existing therapeutic options. Multidrug resistance (MDR) has become a concern and one of the most serious global health threats of this century. MDR has been associated with increased mortality and morbidity due to treatment failures and increased healthcare costs (Catalano et al., 2022).

Treatment of bacterial infections requires the use of antibiotics (Zhang et al., 2021). Currently, the recommended treatment option for severe infections caused by MDR bacterial pathogens, including *E. coli*, is extended-spectrum cephalosporins and quinolones (Laird et al., 2021) as resistance to older antimicrobials has continuously been increasing over the years. Since the introduction of expanded-spectrum cephalosporins in the early 1980s, microbial resistance through extended-spectrum β-lactamases (ESBLs) has become a growing concern (Livermore and Hawkey, 2005; Saravanan et al., 2018). ESBLs are enzymes that can hydrolyze penicillin, oxyimino-cephalosporins (third and fourth generation), and monobactams, such as aztreonam, but spare cephamycin or carbapenems (Poirel et al., 2018; Saravanan et al., 2018). However, they are inhibited by β-lactamase inhibitors, such as clavulanic acid. (Ouchar Mahamat et al., 2021). TEM and SHV are among the first reported ESBLs. CTX-M-type ESBLs have also emerged and have been identified in *E. coli* isolates

of human and animal origin (Poirel et al., 2018). Animals colonized by ESBL-producing *E. coli* could be a significant source for the dissemination of ESBL genes through the food chain, further exacerbating the problem of antimicrobial resistance.

Antimicrobial resistance (AMR) continues to be a global crisis, and is increasingly being reported in humans, animals, and the environment. It has been estimated that by 2050, AMR will cause millions of human deaths, financial burden, and a major reduction in livestock production if alternative measures to prevent the spread of AMR are not implemented (Islam et al., 2021; Orubu et al., 2020). Globally, the increased consumption of meat is of particular concern because meat represents a substantial proportion of the human diet and is expected to grow in the future (Casella et al., 2017). This issue has intensified the common use of antibiotics in animal husbandry, not only for the treatment of bacterial infections, but also as growth promoters and prophylaxis to increase animal stock production (Gawish et al., 2021) to feed the growing population of this century.

In Japan, antibiotics such as penicillin, tetracycline, and streptomycin have been approved for use as prophylaxis and growth promoters in animal husbandry for several years (Harada and Asai, 2010). Their intensive use has allowed antimicrobial-resistant bacteria to develop and spread from food animals to humans (Sadek et al., 2021; Verraes et al., 2013). Some reports have shown that food-producing animals are potential reservoirs of antimicrobial-resistant organisms, including *E. coli* (Kanokudom et al., 2021; Mateus-Vargas et al., 2017). Moreover, extensive investigations involving genetic characterization and antimicrobial surveillance have reported the existence of extended-spectrum β-lactamase-producing *E. coli* (ESBL-E) in food animals, including poultry (Casella et al., 2017; Nakayama et al., 2022; Sornsenee et al., 2022; Zhang et al., 2021). To track the incidence of ESBL-E, a strategic One Health approach, which focuses on preventing the spread of antimicrobial resistance among different local ecosystems, was introduced. Despite its public

health significance, the prevalence of MDR *E. coli* and/or ESBL-E in retail meat in Japan has been reported only in a few areas, including Aichi prefecture (Kawamura et al., 2014), Osaka prefecture (Nahar et al., 2018), and Nagano prefecture (Hayashi et al., 2018). Since the consumption of meat products is rapidly growing in Japan (Sasaki et al., 2022), it is of utmost importance to know the prevalence of antimicrobial-resistant *E. coli* for food safety reasons to assess their significance in human health and to track the changes in the spread of MDR bacteria to humans. Moreover, there exist data and year gap in studies on AMR in food-producing animals in the current region of study, suggesting that the prevalence status of AMR is unknown, limiting accessibility to data for future monitoring of AMR trends. Such data are also needed to assess whether antimicrobial resistance policy implementations are effective. Accordingly, the present study comparatively investigated the prevalence and genetic characteristics leading to drug resistance, as well as the phylogenetic characterization of *E. coli* isolated from retail meat sources in 2009 and 2021 in Higashihiroshima, Japan.

3. Materials and methods

3.1 Description of study location and sampling procedure

The study was conducted in Higashihiroshima, Hiroshima Prefecture, Japan (Fig 3). According to Japanese census data, the city had a total of 196,608 inhabitants as of October 2020, Hiroshima (Japan): Prefecture, Cities, Towns, and Villages–Population Statistics, Charts and Map (citypopulation.de). Higashihiroshima has a land size of 635.32 km^2 and is characterized by cold winters and hot humid summers.

A simple random sampling method was used to select sampling locations. During the period from May to December 2021, 90 meat samples were purchased from 22 retail supermarkets. Each supermarket was randomly visited up to three times during the study period. During sampling, domestically produced and imported meat samples (raw chicken,

beef, pork, and wild meat) in different portions were obtained. Of those 90 meat samples, 58 were domestic products (37 chicken meat, 10 beef, 9 pork, and 2 wild meat samples) and 32 were imported ones (chicken meat: 11 from Brazil, 1 from USA, and 1 from Thailand; beef: 8 from USA, 1 from Canada, and 1 from Mexico; pork: 7 from USA, 1 from Canada, and 1 from New Zealand). Samples were gently packaged in transparent polyethylene bags and immediately transported under cooling conditions (on ice) to the laboratory. Microbiological analysis was carried out within 3 to 5 h of sample collection.

3.2 Microbiological techniques and analysis

Using sterile forceps and scissors, 25 g of each sample was aseptically dissected into different sections and placed in a sterile stomacher bag. Homogenization was immediately performed with 225 mL sterile buffered peptone water (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan) for 60 s. Thereafter, a 100 µL homogenate from each sample was directly spread onto MacConkey agar (Eiken Chemical Co., Ltd., Tochigi, Japan) containing ampicillin (100 µg/mL), streptomycin (50 µg/mL), meropenem (2 µg/mL), or colistin (2 µg/mL). The plates were then incubated at 37 ºC for 24 h. Overnight enrichment assays were also performed by transferring a 1 mL-aliquot of each suspension into 9 mL Luria-Bertani (LB) broth (Nacalai Tesque, Inc., Kyoto, Japan) with or without ampicillin and streptomycin. For capturing β-lactamase producers, ampicillin broth cultures were surface streaked on MacConkey supplemented with ampicillin (100 µg/mL), or meropenem (2 µg/mL), while streptomycin broth cultures were plated on MacConkey containing streptomycin (50 µg/mL). Homogenate samples that did not show bacterial growth after enrichment were also plated on respective antibiotic plates. In all cases, such cultures did not produce colonies on agar and were not selected for further screening. In order to avoid inducible resistance against colistin, enrichment with colistin was not performed. Instead, cultures without antibiotics were plated

Fig. 3. Map of the sampling location in Higashihiroshima, Japan. Sampling locations are marked by stars.

on MacConkey containing colistin (2 μg/mL), followed by incubation under experimental conditions. Subsequently, 3 to 5 morphologically distinct colonies from each plate were preferentially selected. The colonies were re-cultured on individual MacConkey agar plates with the same antibiotic supplementation, as described above. In total, 880 antibiotic-resistant bacterial isolates were obtained and purified on LB agar without antibiotics.

In addition, 303 bacterial isolates obtained from 95 meat samples (24 retail supermarkets) collected in 2009 were included in subsequent analyses. The isolates were recovered by enriching 500 μL meat homogenate in 5 mL LB broth without antibiotics (37 ºC for 15 h), followed by surface streaking on MacConkey without antibiotics. Of those 95 samples, 65 were domestic products (29 chicken meat, 18 beef, and 18 pork samples) and 30 were imported ones (chicken meat: 6 from Brazil and 2 from China; beef: 6 from Australia, 1 from Brazil, 1 from New Zealand, 1 from Mexico, and 1 China; pork: 3 from USA, 2 from Denmark, 2 from Canada, 1 from Mexico, 1 from China, 1 from Thailand, 1 from Australia, and 1 Canadian wild meat sample). The supermarkets visited are in the same region, though some vary from those visited in 2021.

3.3 Bacterial genomic DNA preparation

Total genomic DNA was extracted from the boiled lysate, as previously described (Ahmed et al., 2014) with minor modifications. Briefly, overnight LB broth culture was centrifuged at 13,000 rpm for 2 min. The cells were harvested, resuspended in 1x TE buffer (1 M Tris-HCl, pH 8.0 and 0.5 M EDTA), and the turbidity (OD_{600}) was adjusted to approximately 0.1. The OD-adjusted suspension was lysed at 100 ºC for 10 min. The resulting solution was centrifuged, and the supernatant was stored at -20 ºC and used as the DNA template.

3.4 Molecular screening to detect ESBLs and other AMR genes

Molecular screening of 1,183 isolates including those recovered in 2009 was conducted to identify ESBLs (*bla*$_{SHV}$, *bla*$_{TEM}$, and *bla*$_{CTX-M}$), other β-lactamase-encoding genes (*bla*$_{OXA-1}$, *bla*$_{OXA-2}$, *bla*$_{OXA-5}$, *bla*$_{OXA-9}$, and AmpC-type β-lactamases), integrons (class 1, class 2, and class 3), and aminoglycoside acetyltransferase gene [*aac(6′)-Ib*] using a newly established eicosaplex/octaplex PCR system as previously described (Soliman et al., 2023). Briefly, PCR was performed using the Tks Gflex^{TM} DNA polymerase (Takara Bio Inc., Shiga, Japan) in a 2 µM dNTPs and 10 µM primer concentration under the following cycling conditions: initial denaturation at 98 ºC for 1 min, 35 cycles of denaturation at 98 ºC for 10 s, annealing at 50 ºC for 15 s, extension at 68 ºC for 30 s, and a final extension at 68 ºC for 15 s. PCR detection of mobile colistin resistance genes, *mcr-1* to *mcr-9* (for all isolates from 2009 and only 2021 isolates selected on colistin-containing MacConkey agar) and carbapenemases was also performed. PCR amplicons were analyzed on 3% agarose gel (FastGene NE-A02 agarose gel), stained with ethidium bromide, and visualized under ultraviolet light. Suspected isolates possessing antimicrobial resistance genes were selected and confirmed by single PCR using specific primers. PCR amplicons were purified using ExoSAP-IT (Thermo Fisher Scientific, Japan) and submitted for sequencing on an ABI automated 3730xl DNA sequencer (GATC Biotech, Konstanz, Germany) to identify antimicrobial resistance genes.

3.5 Amplification of variable regions of class 1 and 2 integrons

The variable regions of the integrons were amplified using primers complementary to the 5′ and 3′ conserved segments of class 1 integrons (5′ CS and 3′ CS) (Ahmed, et al., 2007) and class 2 integrons (hep74 and hep51) (Ahmed, et al., 2007). Class 3 integrons were not included in this section since none of the isolates possessed such integrase gene. The PCR amplification conditions were as follows: initial denaturation at 98 ºC for 1 min, 30 cycles of

denaturation at 98 ºC for 10 s, annealing at 50 ºC for 15 s, extension at 68 ºC for 2 min, and a final extension at 68 ºC for 15 s. All primers used are listed in Table S1 in appendices.

3.6 Bacterial identification by 16S rRNA gene sequencing

Amplification of 16S rRNA fragments was carried out on selected isolates that were positive for an antimicrobial resistance gene. The universal primers 27F and 1492R, which generate a 1,500-bp DNA fragment, were used. PCR products were cleaned with ExoSAP-IT and processed for sequencing to identify bacterial species. For *E. coli* selection, if more than one isolate from the same sample carried identical AMR genes, only one representative was selected. Subsequently, a total of 77 non-duplicate isolates were identified as *E. coli* and were used for further experiments.

3.7 Phenotypic antimicrobial susceptibility testing

All *E. coli* isolates were assayed for antimicrobial susceptibility using the Kirby-Bauer method, according to the interpretative criteria described by the Clinical Laboratory Standards Institute (CLSI, 2018). The minimum inhibitory concentration (MIC) of colistin for *mcr*-positive isolate was performed by following EUCAST guidelines (EUCAST, 2023). Overnight cultures were obtained from LB agar plates and suspended in sterile saline (0.85% w/v NaCl). Turbidity was adjusted to 0.5 McFarland standards. Thereafter, the surface of the Mueller-Hinton agar plates was streaked with cotton swabs already dipped into the standardized inocula. Antibiotic disks (Eiken Chemical Co., Ltd., Tochigi, Japan) were aseptically placed on inoculated Mueller-Hinton agar plates using sterile forceps. The isolates were tested against 15 antimicrobial agents from 12 different classes, as follows: ampicillin (10 µg), cefotaxime (30 µg), ceftazidime (30 µg), cefepime (30 µg), cefoxitin (30 µg), aztreonam (30 µg), meropenem (10 µg), amikacin (30 µg), kanamycin (30 µg), tetracycline

(30 µg), chloramphenicol (30 µg), ciprofloxacin (5 µg), sulfamethoxazole-trimethoprim (23.75/1.25 µg), colistin (10 µg), and fosfomycin (50 µg). The plates were incubated at 37 ºC for 16–18 h and the results were recorded based on the interpretative chart supplied with the antimicrobial agent. *E. coli* ATCC 25922 was used as quality control. Multidrug resistance was defined as resistance to at least one antimicrobial agent in three or more classes (Magiorakos et al., 2012). Isolates that showed intermediate resistance (buffer zone) were classified as being resistant to prevent reporting as susceptible (Rodloff, 2008).

3.8 Phylogenetic groupings

Phylogenetic characterization was performed using quadruplex PCR, as previously described (Clermont et al., 2013). The amplification targets were *arpA*, *chuA*, *yjaA*, and DNA fragment TspE4.C2. The phylogroup was assigned based on the presence/absence of a gene target in the order *arpA*|*chuA*|*yjaA*|TspE4.C2. Where an isolate was not specifically assigned, additional testing was performed to correctly assign phylogroups. For instance, if an isolate yields A/C or D/E quadruplex genotypes, phylogroup screening using allele-specific C and E primers were used to differentiate between A and C or D and E, respectively. *E. coli* ATCC 25922 was used as a quality control strain for phylogenetic group B2. Nucleic acid-free water was used as the negative control.

3.9 Statistical analysis and BLAST search

The Pearson's chi-square test or Fisher's exact test was used to test for any significant differences in genotypic and phenotypic features in *E. coli* according to the two isolation years. *p*-values of <0.05 were considered statistically significant for comparisons. Statistical computations were performed using IBM SPSS version 29.0 (SPSS Inc., Chicago, IL, USA). Phylogroup distribution was recorded in Microsoft Excel 365 and presented as percentages.

The map was generated using QGIS software version 3.28. All sequenced data were subjected to a similarity search using the Basic Local Alignment Search Tool (BLAST) program available at the National Center for Biotechnology Information (NCBI) database.

4. Results

4.1 Prevalence of *E. coli* isolated from retail meat samples

In this study, 77 non-duplicate *E. coli* isolates carrying antimicrobial resistance (AMR) genes were detected. Of these, 33 (34.7%) isolates were recovered from 95 meat samples collected in 2009, whereas 44, representing 48.9% were isolates recovered from 90 meat samples collected in 2021. Of the 33 *E. coli* isolates obtained in 2009, the AmpC/ESBL-producers were detected in 14 (42.4%), whereas 9 (20.5%) of these producers were detected in isolates from 2021 (Table 1). The results showed that the occurrence of AmpC/ESBL-producing *E. coli* was significantly higher (p-value = 0.037) in 2009 than in 2021, despite a lower prevalence of *E. coli*. We found a 22% decrease in meat contamination by AmpC/ESBL-carriers over the two years of our study. In addition, the prevalence of *E. coli* was higher in chicken meat samples than in beef or pork, whereas none was detected in wild meat samples (deer, boar, and horse) in the two isolation years (Table 1).

Table 1. Number of *E. coli* (n=77) and AmpC/ESBL-producing isolates (n=23) recovered from various meat samples

Isolation year	Meat category	No. of samples	No. of isolates (%)	
			E. coli	*AmpC/ESBL-producers
2009	Chicken	37	26 (70.3)	12 (46.2)
	Beef	28	4 (14.3)	1 (25.0)
	Pork	29	3 (10.3)	1 (33.3)
	Wild meat	1	0 (0.0)	0 (0.0)
	Total	**95**	**33 (34.7)**	**14 (42.4)**
2021	Chicken	50	43 (86.0)	9 (20.9)
	Beef	19	0 (0.0)	0 (0.0)
	Pork	19	1 (5.3)	0 (0.0)
	Wild meat	2	0 (0.0)	0 (0.0)
	Total	**90**	**44 (48.9)**	**9 (20.5)**

*Statistical difference between AmpC/ESBL-producers recovered in 2009 and 2021 (p<0.05).

4.2 Prevalence of β-lactamase-encoding genes and other AMR genes in *E. coli* isolated from various meat samples

Among the 77 *E. coli* isolates identified to harbor AMR genes, 16 (20.8%) and 44 (57.1%) carried the $bla_{\text{CTX-M}}$ and bla_{TEM} resistance genes, respectively. The $bla_{\text{CTX-M}}$ gene was found only in chicken isolates, whereas bla_{TEM} was detected in isolates recovered from pork and chicken meat samples. Only one isolate (1.3%) each from chicken and pork tested positive for bla_{SHV} (Table 2). Additionally, the AmpC-type β-lactamases (bla_{CMY}, bla_{ACT}, and bla_{ADC}) representing 7.8% of the total *E. coli* isolates were found in six isolates recovered from chicken, beef, and pork. These β-lactamases were only detected in meat samples from 2009. Finally, the modular gene cassette arrays of class 1 and class 2 integrons that confer resistance to aminoglycosides, trimethoprim, chloramphenicol, and quaternary ammonium compounds were identified in 27 (35.1%) and 3 (3.9%) of the *E. coli* isolates, respectively (Table 2). None of the isolates possessed class 3 integrons.

4.3 Comparative analysis between the prevalence of resistance genotypes identified in meat samples from 2009 and 2021

Figure 4 illustrates the prevalence rates of resistance genes in 2009 and 2021. Of the 98 detected AMR genes, 17 (17.4%) were the ESBL-types. Comparatively, the prevalence rates of ESBLs among AMR genes detected in 2009 and 2021 were 19.0% and 16.1%, respectively (*p*-value >0.05). Furthermore, PCR and DNA sequencing of β-lactamase-encoding genes detected high prevalence of the bla_{TEM} gene (44.9%), of which the majority (60.7%) occurred in isolates from 2021 (Fig. 4). Regarding the other β-lactamase genes, the prevalence rates in 2009 and 2021 respectively, are as follows: $bla_{\text{CTX-M}}$ (16.7% and 16.1 %), bla_{SHV} (4.8% and 0.0%), and AmpC-type β-lactamases (14.3% and 0.0%). Integrase gene cassette arrays were found to be more prevalent in isolates obtained in 2009 than in those obtained in 2021.

Table 2. Distribution of ESBLs and other AMR genes in *E. coli* isolated from various meat samples (n=77)

Meat category	*β-lactamase-encoding genes					*Integron cassettes	
	ESBL-types (No.)		Narrow-spectrum-types (No.)		AmpC-types (No.)	Class 1 (No.)	Class 2 (No.)
	bla$_{CTX-M}$	*bla*$_{SHV}$	*bla*$_{SHV}$	*bla*$_{TEM}$			
Chicken	*bla*$_{CTX-M-1}$ (1)	*bla*$_{SHV-12}$ (1)	-	*bla*$_{TEM-1}$ (38)	*bla*$_{CMY-4}$ (2)	*aadA1* (16)	*dfrA1-sat2* (2)
	bla$_{CTX-M-2}$ (11)			*bla*$_{TEM-135}$ (3)	*bla*$_{ACT-51}$ (1)	*aadA5-dfrA17* (1)	*dfrA1-sat2-aadA2* (1)
	bla$_{CTX-M-14}$ (1)			*bla*$_{TEM-176}$ (1)	*bla*$_{ACT-48}$ (1)	*aadA12* (3)	
	bla$_{CTX-M-15}$ (2)					*dfrA17* (1)	
	bla$_{CTX-M-like}$ (1)					*dfrA12-DCP* (1)	
						aadB-orf1-cmlA (1)	
Beef	-	-	-		*bla*$_{ADC-216}$ (1)	*aadA1* (3)	
Pork	-	-	*bla*$_{SHV-1}$ (1)	*bla*$_{TEM-1}$ (2)	*bla*$_{ADC-32}$ (1)	*aadA5-dfr17* (1)	-
Wild meat	-	-	-		-	-	-
Total	**16 (20.8%)**	**1 (1.3%)**	**1 (1.3%)**	**44 (57.1%)**	**6 (7.8%)**	**27 (35.1%)**	**3 (3.9%)**

n= the total number of non-duplicate *E. coli* isolates

*Prevalence was expressed as a percentage of n

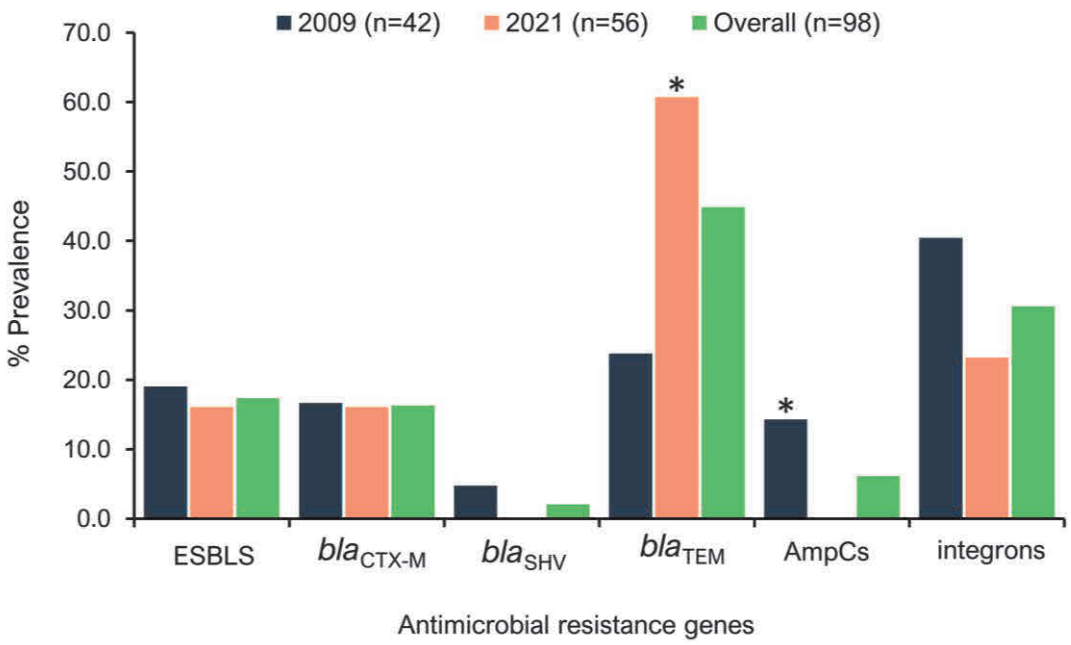

Fig. 4. Comparison between the prevalence of resistance genotypes detected in retail meat samples from 2009 and 2021. Statistical differences (p-value <0.05) in the prevalence of resistance genes are indicated by asterisks.

4.4 Antibiogram profiles of *E. coli*

The sensitivity of the 77 *E. coli* isolates to 15 antimicrobial agents commonly used in therapeutic practice was assessed. All the isolates were susceptible to amikacin and meropenem. Of note, it was observed that the resistance of the isolates to the antimicrobials was higher in 2009 than in 2021, except for ampicillin, kanamycin, ciprofloxacin, and sulfamethoxazole/trimethoprim. Interestingly, decreased resistance to antimicrobials was observed between 2009 and 2021 as follows (significance indicated by p-value in brackets): cefotaxime, 48.5 % vs. 22.7% (p-value = 0.018); ceftazidime, 18.2 % vs.6.8%; cefepime, 12.1% vs. 4.6%; cefoxitin, 21.2% vs. 4.6% (p-value = 0.033); aztreonam, 33.3% vs. 9.1 % (p-value = 0.008); chloramphenicol, 36.4% vs. 22.7%; colistin, 3.0% vs. 0.0%; and fosfomycin, 12.1% vs. 9.1% (Fig. 5). Notably, one *E. coli* isolate that showed phenotypic resistance to colistin (disk method) carried mobile colistin resistance gene *mcr-5.1* allele (MIC, 8 µg/mL) and exhibited MDR to critically important antimicrobials (CIAs) (Table 3).

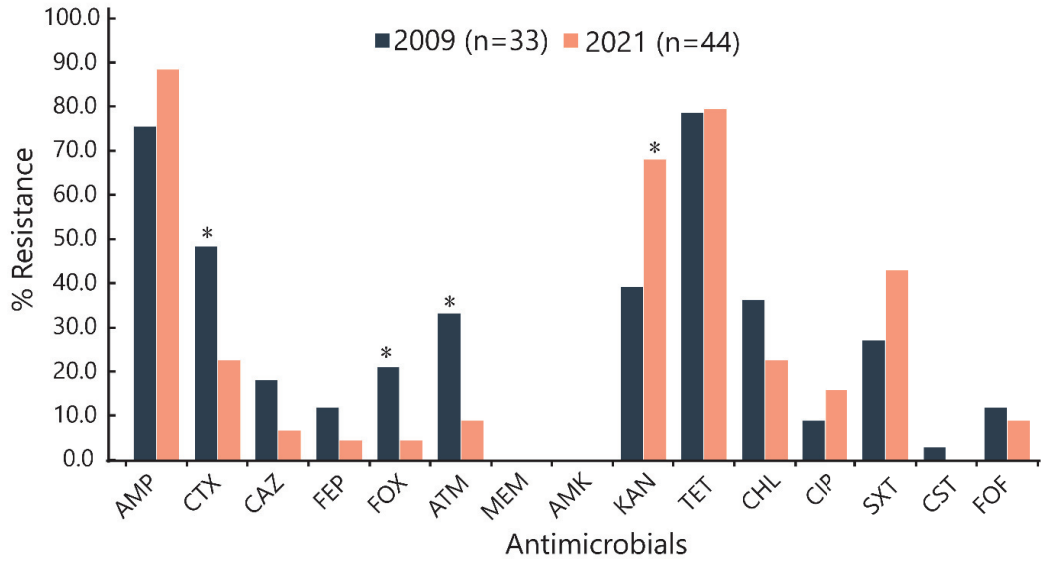

Fig. 5. Antimicrobial resistance profiles of *E. coli* isolated in 2009 and 2021. Intermediate-resistant isolates were reported as resistant. Statistical differences (*p*-value <0.05) in antibiotic resistance between the two isolation years are indicated by asterisks. AMP: ampicillin, CTX: cefotaxime, CAZ: ceftazidime, FEP: cefepime, FOX: cefoxitin, ATM: aztreonam, MEM: meropenem, AMK: amikacin, KAN: kanamycin, TET: tetracycline, CHL: chloramphenicol, CIP: ciprofloxacin, SXT: sulfamethoxazole/trimethoprim, CST: colistin, FOF: fosfomycin.

4.5 MDR patterns and phenotypic profiles of AmpC/ESBL-producing *E. coli*

This study revealed high prevalence rates of MDR among *E. coli* isolates. Sixty out of 77 (77.9%) isolates exhibited MDR phenotypes (Table 3). MDR was defined as the resistance of isolates to at least one antimicrobial agent in three or more classes (Magiorakos et al., 2012). Regarding the comparison between 2009 and 2021, MDR occurred in 26 (78.8%) and 34 (77.3%) of the isolates, respectively (Fig. 6). The results showed insignificant change (*p*-value >0.05) in MDR patterns between the two isolation years. In addition, all AmpC/ESBL-producers showed MDR and were all found to confer resistance to ampicillin and cefotaxime (third-generation cephalosporin), except one, but were susceptible to amikacin and meropenem (Table 4).

Table 3. Multidrug-resistance phenotypes and genetic variability of *E. coli* isolated from chicken meat, pork, and beef samples

No.	Isolate code	Source	Resistance phenotypes	Phylogroup	Resistance genotypes
1	B1-A009	Chicken	AMP, CTX, ATM, KAN, TET, SXT	C	bla_{TEM-1}, $bla_{CTX-M-1}$
2	BH1-S088	Chicken	AMP, CTX, CAZ, FEP, ATM, KAN, SXT, FOF	A	$bla_{CTX-M-15}$, bla_{TEM-1}, *aadA1*
3	BH2-S079	Chicken	AMP, KAN, SXT	A	bla_{TEM-1}, *aadA12*
4	H3-A056	Chicken	AMP, CTX, KAN, TET, SXT	D	bla_{TEM-1}, $bla_{CTX-M-14}$
5	K3-A121	Chicken	AMP, CTX, CAZ, ATM, FOF	B1	$bla_{CTX-M-15}$, bla_{TEM-1}
6	F5-A096	Chicken	AMP, KAN, TET	B1	bla_{TEM-1}, *aadA12*
7	HT1-S097	Chicken	AMP, CTX, KAN, TET, SXT	E	$bla_{CTX-M-2}$
8	HT3-S094	Chicken	AMP, CTX, FEP, ATM, TET, SXT	F	$bla_{CTX-M-2}$, *aadA1*
9	MT1-A128	Chicken	AMP, CTX, TET	E	$bla_{CTX-M-2}$
10	B3-S017	Chicken	AMP, KAN, TET, CHL, SXT	D	$bla_{TEM-135}$, *dfrA17*
11	K3-S089	Chicken	AMP, KAN, TET	A	bla_{TEM-1}, *aadA12*
12	E4-S74	Chicken	AMP, KAN, TET, CHL, CIP, SXT	B1	$bla_{TEM-135}$
13	H3-S026	Chicken	AMP, KAN, TET, CHL, SXT	E	bla_{TEM-1}, *dfrA12-DCP*
14	E3-A018	Chicken	AMP, CTX, KAN, TET, CIP	A	$bla_{CTX-M-2}$
15	HT1-A143	Chicken	AMP, KAN, TET	B1	$bla_{TEM-135}$
16	H1-A035	Chicken	AMP, KAN, TET	B1	$bla_{TEM-176}$
17	G4-A159	Chicken	AMP, KAN, TET	F	bla_{TEM-1}
18	K3-S086	Chicken	AMP, KAN, TC	A	bla_{TEM-1}
19	H3-A039	Chicken	AMP, KAN, TET, CHL, SXT	E	bla_{TEM-1}
20	HT2-A138	Chicken	AMP, KAN, TET	F	bla_{TEM-1}
21	J1-A076	Chicken	AMP, TET, CHL, SXT	B1	$bla_{CTX-M-like}$
22	I2-S048	Chicken	AMP, KAN, TET, CIP, SXT	F	bla_{TEM-1}
23	B5-A101	Chicken	AMP, KAN, TET, CHL	A	bla_{TEM-1}
24	A3-S003	Chicken	AMP, CTX, FOX, KAN, TET, CHL, SXT	F	bla_{TEM-1}
25	E3-A007	Chicken	AMP, KAN, TET	D	bla_{TEM-1}
26	B3-A013	Chicken	AMP, TET, CHL, CIP	B1	bla_{TEM-1}
27	BH1-A109	Chicken	AMP, CTX, CAZ, FOX, KAN, TET, CIP, SXT	E	*aadA1*
28	HT1-A145	Chicken	AMP, KAN, SXT	B1	bla_{TEM-1}
29	J2-S056	Chicken	AMP, KAN, TET, CIP, SXT	A	bla_{TEM-1}
30	B2-A022	Chicken	AMP, KAN, TET	F	bla_{TEM-1}
31	B6-A105	Chicken	AMP, KAN, TET, CHL	F	bla_{TEM-1}
32	B4-A102	Chicken	AMP, KAN, CIP	B1	bla_{TEM-1}
33	MT1-S108	Chicken	AMP, KAN, TET	F	bla_{TEM-1}
34	PA017	Pork	AMP, KAN, TET, CHL, SXT	B2	bla_{TEM-1}
35	37A-18A-2	Pork	AMP, TET, CHL, SXT	A	bla_{TEM-1}, *dfr17-aadA5*
36	3B-2B-1	Pork	CTX, FOX, ATM, CHL, FOF	A	bla_{ADC-32}
37	3E-2E-1	Beef	AMP, CTX, CAZ, FOX, ATM, TET, CHL, FOF	A	$bla_{ADC-216}$
38	8D-2D1-1	Beef	AMP, FOX, KAN, TET, SXT	F	*aadA1*
39	9D-2D1-2	Beef	AMP, KAN, TET, SXT	F	*aadA1*
40	138-2H-3	Chicken	AMP, CTX, FEP	A	$bla_{CTX-M-2}$
41	139-2H-4	Chicken	AMP, CTX, FEP, ATM, TET	B1	$bla_{CTX-M-2}$
42	156-9H-2	Chicken	AMP, CTX, CAZ, FOX, KAN, TET, CHL	B2	bla_{CMY-4}, bla_{TEM-1}, *aadB-orf1-cmlA*
43	158-10H-2	Chicken	AMP, KAN, TET, SXT	A	bla_{TEM-1}, *aadA1*
44	159-10H-3	Chicken	AMP, KAN, TET	F	bla_{TEM-1}
45	160-11H-1	Chicken	AMP, CTX, CAZ, ATM, SXT, CST	A	bla_{SHV-12}, *mcr-5.1*
46	161-11H-2	Chicken	AMP, TET, CIP	A	bla_{TEM-1}
47	162-11H-3	Chicken	AMP, KAN, TET	A	bla_{TEM-1}
48	165-12H-3	Chicken	AMP, CTX, CAZ, FOX, TET	B1	bla_{CMY-4}
49	166-13H-1	Chicken	AMP, TET, CHL, CIP, SXT	C	bla_{TEM-1}
50	171-14H-3	Chicken	TET, CHL, SXT	D	*dfrA1-sat2*
51	183-15H-1	Chicken	AMP, KAN, TET, SXT	B1	bla_{TEM-1}
52	198-22H-2	Chicken	AMP, CTX, ATM, TET	B1	bla_{TEM-1}
53	242-31J2-1	Chicken	KAN, TET, CIP, SXT	F	*dfrA1-sat2-aadA2*
54	261-33J2-1	Chicken	AMP, CTX, CAZ, FOX, ATM, KAN, TET, CHL, FOF	A	bla_{ACT-51}
55	263-33J2-3	Chicken	AMP, CTX, CAZ, FOX, ATM, KAN, CHL, FOF	A	bla_{ACT-48}, $bla_{ADC-216}$
56	267-34J1-2	Chicken	AMP, CTX, KAN, TET, CHL	A	$bla_{CTX-M-2}$, *aadA1*
57	269-34J2-2	Chicken	AMP, CTX, FEP, ATM, KAN, TET, CHL	A	$bla_{CTX-M-2}$, *aadA1*
58	286-36J2-1	Chicken	AMP, CTX, ATM, TET, CHL	E	$bla_{CTX-M-2}$, *aadA1*
59	301-36J2-1X	Chicken	AMP, CTX, FEP, ATM, KAN, TET, CHL	D	$bla_{CTX-M-2}$, *aadA1*
60	305-UN	Chicken	AMP, CTX, ATM	A	bla_{TEM-1}, *aadA1*

AMP: ampicillin, CTX: cefotaxime, CAZ: ceftazidime, FEP: cefepime, FOX: cefoxitin, ATM: aztreonam, MEPM: meropenem, AMK: amikacin, KAN: kanamycin, TET: tetracycline, CHL: chloramphenicol, CIP: ciprofloxacin, SXT: sulfamethoxazole/trimethoprim, CST: colistin, FOF: fosfomycin

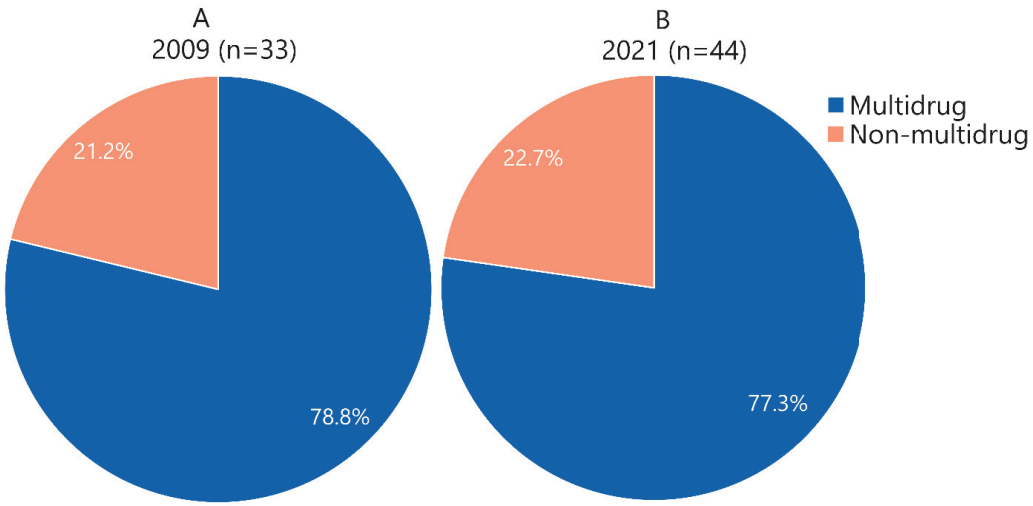

Fig. 6. Occurrence of multidrug resistance among *E. coli* isolates from retail meat samples.

4.6 Phylogroup classification of *E. coli* isolates

Phylogenetic characterization of 77 *E. coli* isolates revealed variable phylogroups. Phylogroup analysis of isolates obtained in 2009 showed that 17 (51.5%) belonged to group A, 5 (15.2%) to groups B1 or F, 1 (3.0%) to groups B2 or C or D, and 3 (9.1%) to group E (Fig. 7A). Conversely, in 2021, 9 (20.5%) belonged to group A, 12 (27.3%) to group B1, 11 (25.0%) to group F, 1 (2.3%) to group B2, 2 (4.6%) to group C, 3 (6.8%) to group D, and 6 (13.6%) to group E (Fig. 7B). The determination of *E. coli* phylogenetic groups revealed that phylogroup A occurred more frequently in 2009, followed by groups B1 and F, while the majority of the 44 isolates from 2021 were predominantly assigned to phylogroups A, B1, and F. Although the pathogenic groups (B2/D) were detected at a low frequency in both isolation years, it represents a significant risk factor to humans. The association between the *E. coli* phylogeny and MDR was determined (Fig. 8). In 2009, MDR occurred in 14 (42.4%) and 2 (6.1%) of isolates assigned to phylogroups A and E, respectively. All the isolates assigned to the remaining phylogroups were MDR. On the other hand, in 2021, 7 (15.9%), 9

(20.5%), 1 (2.3%), 5 (11.4%), and 8 (18.2%) of phylogroups A, B1, C, E, and F showed MDR phenotypes, respectively. All the four isolates assigned to pathogenic B2/D (9.1%) group also exhibited MDR traits.

Table 4. Phenotypic antimicrobial susceptibility of AmpC/ESBL-producing *E. coli* (n=23) isolated from retail meat samples

No.	Isolate code	AMR genes	Antimicrobials														
			AMP	CTX	CAZ	FEP	FOX	ATM	MEM	AMK	KAN	TET	CHL	CIP	SXT	CST	FOF
1	B1-A009	*bla*$_{TEM-1}$, *bla*$_{CTX-M-1}$	**(R)**	**(R)**	(S)	(S)	(S)	**(R)**	(S)	(S)	**(R)**	**(R)**	(S)	(S)	**(R)**	(S)	(S)
2	BH1-S088	*bla*$_{CTX-M-15}$, *bla*$_{TEM-1}$, *aadA1*	**(R)**	**(R)**	**(R)**	**(R)**	(S)	**(R)**	(S)	(S)	**(R)**	(S)	(S)	(S)	**(R)**	(S)	**(R)**
3	H3-A056	*bla*$_{TEM-1}$, *bla*$_{CTX-M-14}$	**(R)**	**(R)**	(S)	(S)	(S)	(S)	(S)	(S)	**(R)**	**(R)**	(S)	(S)	**(R)**	(S)	(S))
4	K3-A121	*bla*$_{CTX-M-15}$, *bla*$_{TEM-1}$	**(R)**	**(R)**	**(R)**	(S)	(S)	**(R)**	(S)	(S)	(S)	(S)	(S)	(S)	(S)	(S)	**(R)**
5	HT1-S097	*bla*$_{CTX-M-2}$	**(R)**	**(R)**	(S)	(S)	(S)	(S)	(S)	(S)	**(R)**	**(R)**	(S)	(S)	**(R)**	(S)	(S)
6	HT3-S094	*bla*$_{CTX-M-2}$, *aadA1*	**(R)**	**(R)**	(S)	**(R)**	(S)	**(R)**	(S)	(S)	(S)	**(R)**	(S)	(S)	**(R)**	(S)	(S)
7	MT1-A128	*bla*$_{CTX-M-2}$	**(R)**	**(R)**	(S)	(S)	(S)	(S)	(S)	(S)	(S)	**(R)**	(S)	(S)	(S)	(S)	(S)
8	E3-A018	*bla*$_{CTX-M-2}$	**(R)**	**(R)**	(S)	(S)	(S)	(S)	(S)	(S)	**(R)**	**(R)**	(S)	**(R)**	(S)	(S)	(S)
9	J1-A076	*bla*$_{CTX-M-like}$	**(R)**	(S)	(S)	(S)	(S)	(S)	(S)	(S)	(S)	**(R)**	**(R)**	(S)	**(R)**	(S)	(S)
10	3B-2B-1	*bla*$_{ADC-32}$	(S)	**(R)**	(S)	(S)	**(R)**	**(R)**	(S)	(S)	(S)	(S)	**(R)**	(S)	(S)	(S)	**(R)**
11	3E-2E-1	*bla*$_{ADC-216}$	**(R)**	**(R)**	**(R)**	(S)	**(R)**	**(R)**	(S)	(S)	(S)	**(R)**	**(R)**	(S)	(S)	(S)	**(R)**
12	138-2H-3	*bla*$_{CTX-M-2}$	**(R)**	**(R)**	(S)	**(R)**	(S)	(S)	(S)	(S)	(S)	(S)	(S)	(S)	(S)	(S)	(S)
13	139-2H-4	*bla*$_{CTX-M-2}$	**(R)**	**(R)**	(S)	**(R)**	(S)	**(R)**	(S)	(S)	(S)	**(R)**	(S)	(S)	(S)	(S)	(S)
14	156-9H-2	*bla*$_{CMY-4}$, *bla*$_{TEM-1}$, *aadB-orf1-cmlA*	**(R)**	**(R)**	**(R)**	(S)	**(R)**	(S)	(S)	(S)	**(R)**	**(R)**	**(R)**	(S)	(S)	(S)	(S)
15	160-11H-1	*bla*$_{SHV-12}$, *mcr-5.1*	**(R)**	**(R)**	**(R)**	(S)	(S)	**(R)**	(S)	(S)	(S)	(S)	(S)	(S)	**(R)**	**(R)**	(S)
16	165-12H-3	*bla*$_{CMY-4}$	**(R)**	**(R)**	**(R)**	(S)	**(R)**	(S)	(S)	(S)	(S)	**(R)**	(S)	(S)	(S)	(S)	(S)
17	174-2H-1X	*bla*$_{CTX-M-2}$	**(R)**	**(R)**	(S)	(S)	(S)	(S)	(S)	(S)	(S)	(S)	(S)	(S)	(S)	(S)	(S)
18	261-33J2-1	*bla*$_{ACT-51}$	**(R)**	**(R)**	**(R)**	(S)	**(R)**	**(R)**	(S)	(S)	**(R)**	**(R)**	**(R)**	(S)	(S)	(S)	**(R)**
19	263-33J2-3	*bla*$_{ADC-26}$, *bla*$_{ACT-48}$	**(R)**	**(R)**	**(R)**	(S)	**(R)**	**(R)**	(S)	(S)	**(R)**	(S)	**(R)**	(S)	(S)	(S)	**(R)**
20	267-34J1-2	*bla*$_{CTX-M-2}$, *aadA1*	**(R)**	**(R)**	(S)	(S)	(S)	(S)	(S)	(S)	**(R)**	**(R)**	**(R)**	(S)	(S)	(S)	(S)
21	269-34J2-2	*bla*$_{CTX-M-2}$, *aadA1*	**(R)**	**(R)**	(S)	**(R)**	(S)	**(R)**	(S)	(S)	**(R)**	**(R)**	**(R)**	(S)	(S)	(S)	(S)
22	286-36J2-1	*bla*$_{CTX-M-2}$, *aadA1*	**(R)**	**(R)**	(S)	(S)	(S)	**(R)**	(S)	(S)	(S)	**(R)**	**(R)**	(S)	(S)	(S)	(S)
23	301-36J2-1X	*bla*$_{CTX-M-2}$, *aadA1*	**(R)**	**(R)**	(S)	**(R)**	(S)	**(R)**	(S)	(S)	**(R)**	**(R)**	**(R)**	(S)	(S)	(S)	(S)

AMP: ampicillin, CTX: cefotaxime, CAZ: ceftazidime, FEP: cefepime, FOX: cefoxitin, ATM: aztreonam, MEM: meropenem, AMK: amikacin, KAN: kanamycin, TET: tetracycline, CHL: chloramphenicol, CIP: ciprofloxacin, SXT: sulfamethoxazole/trimethoprim, CST: colistin, FOF: fosfomycin. **R**: resistant, S: susceptible

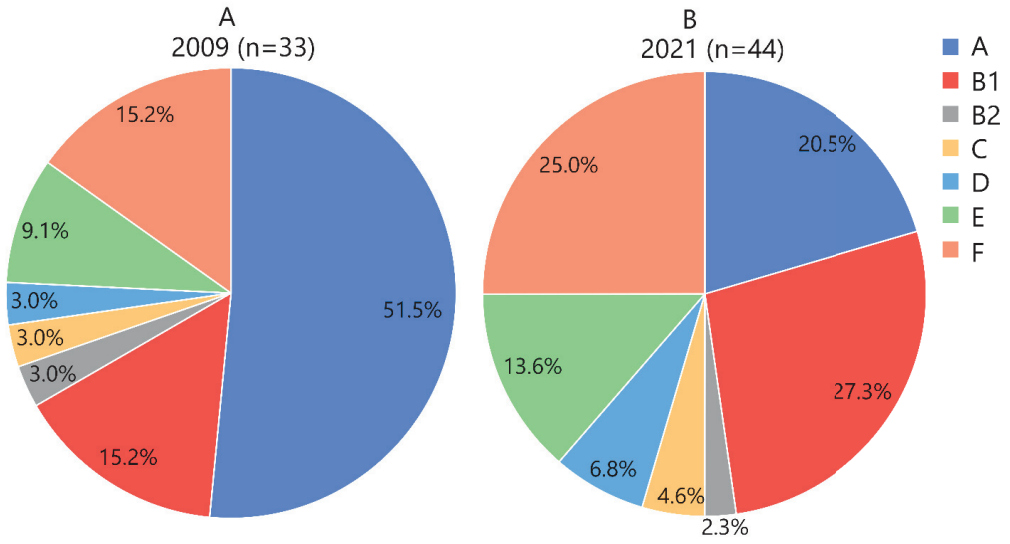

Fig. 7. Occurrence of variable commensal and pathogenic phylogenetic groups of *E. coli* isolates from retail meat samples.

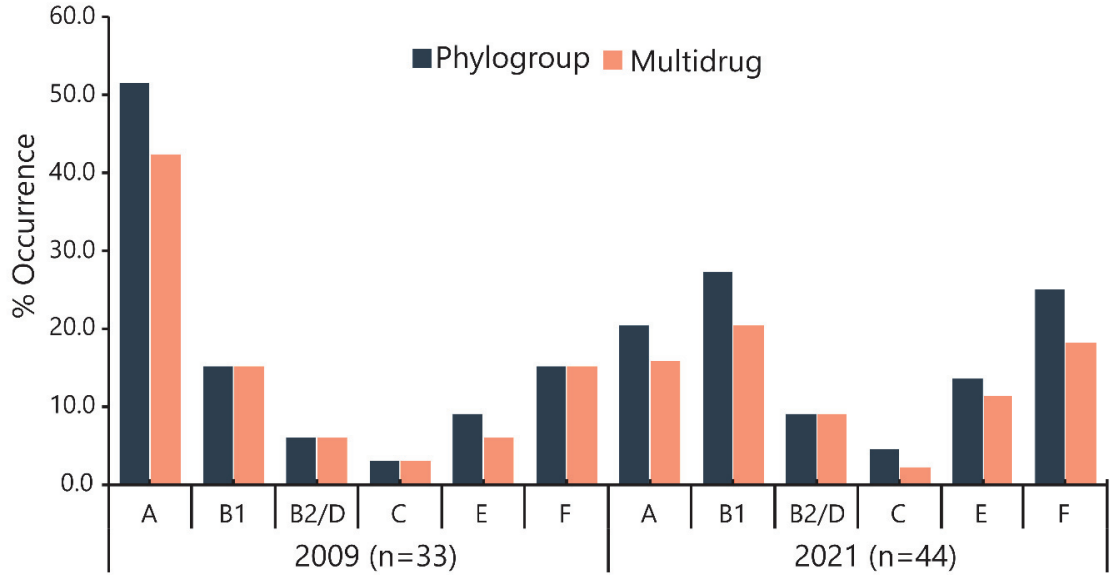

Fig. 8. Summary of phylogenetic groupings and the association between phylogroup and multidrug resistance.

4.7 Distribution of AMR genes according to phylogenetic groups

The detected AMR genes included *bla*$_{CTX-M}$ (16.3%), *bla*$_{SHV}$ (2.0%), *bla*$_{TEM}$ (44.9%), AmpC-type β-lactamases (6.1%), and integrons (30.6%). Interestingly, these genes were closely associated with a particular *E. coli* phylogeny. The *bla*$_{CTX-M}$ genes were mostly associated with A (6.1%) and E (4.1%) phylogroups. The *bla*$_{TEM}$ genes were frequently identified in A (13.3%) and B (13.3%), and to some extent F (8.2%) phylogroup. In addition, integrons were also found to be more associated with A (11.2%) and F (8.2%), while none of B2, C, D, E, or F was positive for *bla*$_{SHV}$/AmpC. This study revealed high prevalence of AMR genes (37.8%) in isolates assigned to phylogroup A (Table 5).

Table 5. Distribution of antimicrobial resistance genes including ESBLs detected in *E. coli* isolates according to phylogenetic groups

Genotype/ Phylogroup	Antimicrobial resistance genes (n=98)					Total
	bla$_{CTX-M}$	*bla*$_{SHV}$	*bla*$_{TEM}$	AmpC	Integrons	
A (%)	6 (6.1)	2 (2.0)	13 (13.3)	5 (5.1)	11 (11.2)	37 (37.8)
B1 (%)	3 (3.1)	0 (0.0)	13 (13.3)	1 (1.0)	2 (2.0)	19 (19.4)
B2 (%)	0 (0.0)	0 (0.0)	2 (2.0)	0 (0.00)	1 (1.0)	3 (3.1)
C (%)	1 (1.0)	0 (0.0)	2 (2.0)	0 (0.00)	1 (1.0)	4 (4.1)
D (%)	1 (1.0)	0 (0.0)	3 (3.1)	0 (0.00)	2 (2.0)	6 (6.1)
E (%)	4 (4.1)	0 (0.0)	3 (3.1)	0 (0.00)	5 (5.1)	12 (12.2)
F (%)	1 (1.0)	0 (0.0)	8 (8.2)	0 (0.00)	8 (8.2)	17 (17.3)
Total (%)	**16 (16.3)**	**2 (2.0)**	**44 (44.9)**	**6 (6.1)**	**30 (30.6)**	**98 (100)**

5. Discussions

One of the most alarming issues of this century is the continuous spread of infections caused by resistant microorganisms, particularly multidrug-resistant bacterial pathogens (Ammar et al., 2021; Bendary et al., 2022). Unfortunately, there are insufficient reports on comprehensive antimicrobial surveillance in certain parts of Japan. Moreover, to the best of the authors' knowledge, no report has phylogenetically assigned *E. coli* isolated from meat in Higashihiroshima, Japan, using Clermont's phylogroup classification, including the newly proposed groups. Therefore, the prevalence of *E. coli* and its phylogenetic classification were assessed, as well as the variability of MDR bacteria among animal hosts.

In this study, 77 *E. coli* isolates were obtained from 185 meat samples including chicken, beef, pork, and wild meat. The prevalence rates of *E. coli* in samples from 2009 and 2021 were 34.7% and 48.9%, respectively (Table 1). Such an increase in the prevalence rate in 2021 highlights the possibility of increased cross-contamination or reduced implementation of strict manufacturing protocols in abattoir settings due to the large production scale to meet the increasing demand for meat consumption in recent years. However, the observed prevalence rates of *E. coli* in the two isolation years were lower compared to those previously reported in France (91.7%) (Casella et al., 2017), Thailand (78%) (Sornsenee et al., 2022), and Cameroon (73.33%) (Fanjip et al., 2022). Disparities in sampling methods, sampling sizes, isolation protocols, geographical location, or seasonal factors could account for the varied prevalence of *E. coli* among different countries. Meanwhile, AmpC/ESBL production among the *E. coli* isolates in the present study revealed that ESBL-producers were more prevalent in samples obtained in 2009 (42.4%, 14/33) compared to those obtained in 2021 (20.5%, 9/44) (Table 1). Moreover, multiple studies have genotyped ESBL-producing *E. coli* recovered from Japanese retail meat samples. Hiroi et al. (2012) reported 48 ESBL-producing *E. coli* isolates from broilers, cattle, and pigs in the

Shizuoka Prefecture, Japan. In a study conducted in Osaka, Japan, Nahar et al. (2018) reported a total of 91% and 100% ESBL-producing *E. coli* from domestic and imported retail meat samples, respectively. Very recently, Nakayama et al. (2022) also reported 52% ESBL-producing *E. coli* in chickens. Our study revealed lower rates of such producers compared with other studies. In 2015, the World Health Assembly adopted a global action plan on AMR and called on each country to develop a National Action Plan (NAP) to prevent the global spread of AMR (World Health Organization, 2022). Japan has implemented NAP on antimicrobial resistance since April 2016, by outlining specific targets for antimicrobial use including those applied in livestock production (The government of Japan, 2020). It is possible that such action plan is impacting in certain prefectures of Japan as well as other countries, which might be associated with the reduced prevalence of ESBL-producing *E. coli* in 2021. Furthermore, the different regions of study and isolation years might have contributed to the varied patterns in the prevalence rates.

PCR and DNA sequencing revealed that $bla_{\text{CTX-M}}$ genes ($bla_{\text{CTX-M-1}}$, $bla_{\text{CTX-M-2}}$, $bla_{\text{CTX-M-14}}$, $bla_{\text{CTX-M-15}}$, and $bla_{\text{CTX-M-like}}$) were common ESBL-types and were mostly associated with chicken isolates (Table 2). This observation was expected, and our results are in line with Hayashi et al. (2018) and Kawamura et al. (2014), who frequently detected $bla_{\text{CTX-M}}$ in chicken meat samples in Japan. Similarly, a recent report in Singapore showed a high prevalence of chicken-derived CTX-M-producing *E. coli* (Guo et al., 2021). Cormier et al. (2019) frequently detected $bla_{\text{CTX-M-1}}$ variants in chicken meat in Canada. Furthermore, all 29 *E. coli* isolates recovered from retail chicken meat in South Korea carried the $bla_{\text{CTX-M}}$ gene and were found to hydrolyze cefotaxime (Kim et al., 2018). Such common detection of CTX-M producers mainly resulted from the worldwide off-label use of ceftiofur, an extended-spectrum cephalosporin of veterinary importance administered *in vivo* to treat bacterial infections in chickens (Casella et al., 2017). It is also possible that the frequency of

horizontal gene transfer of mobile genetic elements plays a significant role in the rapid dissemination of ESBLs (Verraes et al., 2013) including *bla*$_{\text{CTX-M}}$ variants among chickens. It is worth mentioning that since wild meat is not commonly retailed in the current region of study, we were unable to obtain representative number, which reduced the probability of detecting AMR genes. As such, we identified this as a limitation to our study.

Interestingly, our study could not detect ESBL-producing *E. coli* in pork or beef (Tables 1 and 2). Hence, cephalosporin resistance was not observed in these isolates. The Nippon AMR One Health Report (NAOR) 2020 reported the resistance rate of cefotaxime among *E. coli* isolates from healthy cattle and pigs to be 0%. The results of the present study are in line with those of the NAOR. Of note, all the *bla*$_{\text{CTX-M}}$ genes detected in 2009 were *bla*$_{\text{CTX-M-2}}$ and represented 16.7% of the total AMR genes analyzed in that year. Comparative genotypic analysis between 2009 and 2021 showed a 3% decrease (19.0% vs. 16.1%) (p-value >0.05) in ESBL prevalence among the total AMR genes analyzed (Fig. 4). Therefore, our data to some extent confirmed that the acquisition and transmission of ESBL-resistance traits of *E. coli* to food animals did not significantly occur between the two years of study in Higashihiroshima, Japan. However, there was a significant difference (p-value = 0.00) in the prevalence of the *bla*$_{\text{TEM}}$ gene (60.7%) recorded in 2021 compared to the 23.8% detected in 2009, suggesting that the continuous use of penicillin is triggering the selective pressure on *E. coli* populations, resulting in the rapid evolution and dissemination TEM enzymes along the meat value chain. Integrons are site-specific recombination systems that carry modular gene cassette structures and are capable of integrating into core bacterial genomes (Zhu et al., 2014). They confer antimicrobial resistance, particularly to aminoglycosides, trimethoprim, chloramphenicol, and quaternary ammonium ions. The common types of integron gene cassettes were mostly from class 1 and occurred in 27 (35.1%) of *E. coli* isolates (Table 2). Such high detection of class 1 integrons is not surprising, since they are described as the most

prevalent and are linked to antimicrobial resistance in gram-negative bacteria (Otero-Olarra et al., 2020). Among the 98 AMR genes, approximately 31% were integron positive, with higher prevalence occurring in 2009 (40.5%) in comparison to that in 2021 (23.2%) (Fig. 4) but without significant difference (p>0.05).

The rapid and global spread of resistance mechanisms in gram-negative bacteria is the main cause of increased antimicrobial resistance. In this context, we investigated the susceptibility of these isolates to antimicrobials commonly used in therapeutic practices. All isolates demonstrated resistance to at least one antimicrobial. Maximum resistance to ampicillin, tetracycline, kanamycin, and sulfamethoxazole/trimethoprim was observed, and was found to be higher in 2021 than in 2009. These antimicrobials, as well as other aminoglycosides and, to a lesser extent, fluoroquinolones and chloramphenicol, have been used for animal production in Japan for many years (Harada and Asai, 2010). The continuous use of such antimicrobials may be one cause of the elevated resistance in 2021. In another study, high resistance to ampicillin, tetracycline, chloramphenicol, and cefoxitin was recorded (Bendary et al., 2022). Furthermore, a study conducted by Nahar et al. (2018) in Osaka, Japan also reported high rates of resistance, especially against ampicillin, tetracycline, and chloramphenicol. Nonetheless, there was a higher prevalence of resistance to third and fourth generation cephalosporins, as well as cefoxitin and aztreonam, in 2009 than in 2021 (Fig. 5). There was a significant difference (p<0.05) in resistance against cefotaxime, cefoxitin, aztreonam, and kanamycin between the two years. According to NAOR 2020, third generation cefotaxime resistance rates among *E. coli* from healthy chickens increased from 1.5% in 2012 to 4.8% in 2013 but have steadily decreased to 4.1% and 2.2% in 2014 and 2015, respectively. In the following year, there was a temporal increase in the rate of resistance to cefotaxime (5.7%), which decreased again to 4.7% in 2017 and 3.2% in 2018. The decreasing trend in cephalosporin resistance observed in the current comparative study is

in line with that of NAOR. It is also possible that the implementation of voluntary restrictions on the use of third generation cephalosporins in vaccine manufacturing in the poultry industry since 2012 has resulted in a decreased incidence of cephalosporin resistance among *E. coli* isolates from chickens (Hayashi et al., 2018; Hiki et al., 2015). Meanwhile, in the same NAOR 2020 statistics, resistance to ampicillin, tetracycline, and kanamycin among *E. coli* isolated from healthy animals (cattle, pig, and chickens), ranged from 11.6% to 36.1%, 26.5% to 55.4%, and 0.0% to 43.9%, respectively, in contrast to those recorded in this study. Even though the resultant level of resistance may vary depending on the location, experimental technique, or animal species under investigation, this report could be of significance when implementing measures to curb the spread of antimicrobial resistance. Alarmingly, MDR occurred in 60 of 77 (77.9%) *E. coli* isolates (Table 3). Comparative phenotypic analysis showed that there was insignificant difference (p-value >0.05) in the occurrence of MDR among *E. coli* isolates between 2009 (78.8%) and 2021 (77.3%), suggesting that there is a high propensity for increased MDR *E. coli* transmission in the future if the existing preventive measures are compromised. Molecular characterization of *E. coli* in 2009 in Hiroshima prefecture reported that 28 out of 69 (40.6%) isolates showed MDR (Ahmed et al., 2009), which was lower compared to those isolated in the same year in Higashihiroshima. In this study, we selected only *E. coli* isolates possessing AMR genes, which could be a cause of the high MDR patterns. Additionally, the high prevalence can be ascribed to multiple resistance mechanisms such as the overexpression of multidrug efflux pumps and enzymatic hydrolysis of drugs, leading to the displacement of a functional chemical group (Catalano et al., 2022).

Phylogenetic analysis, performed according to the Clermont scheme, revealed considerable variability among the *E. coli* isolates tested (Fig. 7). The most prevalent phylogroups were A, B1, and F, whereas phylogroup B2 was the least commonly identified in

the two isolation years. Notably, there was a higher proportion of human commensal A phylogroups in 2009 compared to that in 2021. This signifies that contamination by human transmission has continuously declined, possibly due to the extra hygienic practices implemented in abattoir settings. Moreover, our findings also revealed that the avirulent B1 and F groups, which are mostly associated with animal hosts, predominated in 2021, indicating that animal-animal transmission of such *E. coli* phylogroups may continue to increase if stringent control measures are not implemented in the animal industry. A similar study reported a major proportion of A and B1 phylogroups among eight proposed groups in the Clermont scheme (Sarowska et al., 2022). These phylogroups are mostly commensal and frequently identified in human and animal hosts. The majority of extra-intestinal *E. coli* strains belong to phylogroups B2 or D, and they appear to possess more virulent factors than A and B1; nevertheless, some members in the latter groups have been found to be associated with systemic infections (Corzo-Ariyama et al., 2019). Although the detection of pathogenic extra-intestinal *E. coli* in this study was low, its existence in food represents a significant risk factor and highlights the potential source of cross-contamination in the human food value chain. Furthermore, MDR frequently occurred in all the phylogroups. The isolates assigned to the avirulent A and B1 phylogroups were characterized by high MDR patterns, followed by F in the two isolation years (Fig. 8). These findings are in accordance with a previous study that reported that avirulent A and B1 groups have a greater propensity to develop MDR (Mataseje et al., 2009). Notably, all *E. coli* isolates assigned to groups B2 or D exhibited MDR (Table 3 and Fig 8) and were found to carry either the β-lactamase gene or integron cassette arrays (Table 5). It is worth mentioning that pathogenic B2 has a lower trend towards antimicrobial resistance (Pereira et al., 2013) and the low detection in this study leaves its pattern of resistance unclear, even though all isolates of the group showed MDR phenotypes.

6. Conclusions

In summary, our findings revealed a high prevalence of MDR *E. coli* with the detection of a higher occurrence of AmpC/ESBLs in 2009 compared to the 2021 isolation year. Comparative characterization of *E. coli* phylogenetic groups and their association with antimicrobial resistance was confirmed for the first time in Higashihiroshima, Japan. Our findings suggest a potential transmission route for AmpC/ESBLs genes and MDR bacteria of diverse phylogroups from retail meat to humans. Furthermore, this study highlights the role of meat as a reservoir of *E. coli* and emphasizes the importance of continuous monitoring to track the changes in the spread of antimicrobial resistance in the food chain. It could also be of practical importance for food processors to undertake corrective and preventive measures regarding the implementation of safety management systems and good manufacturing practices in Higashihiroshima and Japan as a whole. Future research avenues should consider large sample sizes from more sampling locations to draw a definitive conclusion.

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Chapter 3

Molecular and phenotypic characterization of antimicrobial resistance among members of Enterobacterales and non-Enterobacterales isolated from retail meat in Japan

1. Summary

β-lactamase mediated antimicrobial resistance (AMR) in Gram-negative bacteria has been recognized as a significant public health threat that requires immediate attention. Herein, we aimed to characterize the genetic determinants of AMR, including β-lactamases in Gram-negative bacterial isolates recovered from retail meat in Higashihiroshima, Japan. Eicosaplex/octaplex PCR was performed to detect the AMR determinants present in meat isolates collected in 2009 and 2021. Mobile colistin resistance genes (*mcr*) and acquired carbapenemases were also investigated, and the susceptibility of the isolates to antimicrobials was assessed. Plasmid replicon typing, which has not been previously investigated, was performed to characterize any plasmids that might be associated with horizontal gene transfer of extended-spectrum β-lactamases (ESBLs) in ESBL-producing *Escherichia coli*.

The predominant bacterial isolates included *E. coli*, *Rhanella* spp., *Klebsiella* spp., *Salmonella* spp., *Enterobacter* spp., *Hafnia* spp., *Serratia* spp., *Citrobacter* spp., *Morganella* spp., *Acinetobacter* spp., *Pseudomonas* spp., and *Aeromonas* spp. Within 120 isolates of 2009, 72% (n=86) genetic determinants of β-lactam resistance were identified, while 74% (93/125) were found in the 2021 isolates. Extended-spectrum β-lactam resistance determinants ($bla_{\text{CTX-M}}$ or bla_{SHV}) were found in 10 (12/120) and 29.6% (37/125) of 2009 and 2021 isolates, respectively, with $bla_{\text{RHAN-1/2}}$ (9.6%) of the CTX-M group being the most prevalent ESBL resistance gene in 2021. AmpC β-lactamases were detected in 41.7 (50/120) and 15.2% (19/125) of 2009 and 2021 isolates, respectively. We found 6 colistin-resistant isolates carrying the *mcr* gene, whereas a single strain of *Shewanella xiamenesis* isolated in 2021

harbored *bla*$_{\text{OXA-48-like}}$ carbapenemase-encoding gene. Overall, the most common clinical resistance was observed against ampicillin, cefotaxime, cefoxitin, aztreonam, kanamycin, tetracycline, sulfamethoxazole/trimethoprim, chloramphenicol, and fosfomycin. PCR-based replicon typing revealed that the most common plasmid incompatibility groups in ESBL-producing *E. coli* were IncI1, IncFIB, and IncFrepB. To the best of our knowledge, this study reports the first molecular characterization of AmpC β-lactamases within members of Enterobacterales and non-Enterobacterales isolated from retail meat in Higashihiroshima, Japan.

Our study suggests the potential transfer of AMR determinants from food to humans, which warrants particular attention in meat and food industries. To overcome this threat, it is important to develop strategies for the periodic surveillance of AMR along the meat chain and to understand the genetic basis of transmission.

Keywords: Enterobacterales, Non-Enterobacterales, Extended-spectrum β-lactamase, Meat, Japan

2. Introduction

The global resurgence of antimicrobial resistance (AMR) has been widely recognized. Over the decades, antimicrobials have been widely used in veterinary medicine for therapeutic and prophylactic purposes to improve animal yield (Gawish et al., 2021; Wang et al., 2022). However, the inappropriate use of antimicrobials has resulted in an elevated incidence of multidrug-resistant microorganisms (Cooper et al., 2020), owing to selective pressure favoring the evolution and dissemination of antimicrobial-resistant bacteria in animal and human populations (Abd El-Aziz et al., 2021; Watanabe et al., 2023). AMR is a public health issue owing to the continuous occurrence of multidrug resistance (MDR) traits, particularly among gram-negative bacteria in healthcare settings (Galarde-López et al., 2022). MDR Gram-negative bacteria have been reported as one of the most life-threatening situations worldwide because they can cause a variety of difficult-to-treat infections (Khalifa et al., 2019; Worku et al., 2022). Without effective antimicrobial applications in humans, mortality and spread of infectious diseases will continue to increase beyond imagination. Hence, AMR surveillance programs have been useful in providing updated information on the emergence and changes in AMR in the food and animal production continuum.

Extended-spectrum β-lactamases (ESBLs), plasmid-mediated quinolone resistance genes, and AmpC β-lactamases have been frequently detected in Enterobacterales, rendering clinical resistance to broad-spectrum cephalosporins and fluoroquinolones (Janatova et al., 2014). There is growing evidence of the occurrence of bacteria expressing resistance to β-lactams and fluoroquinolones in animal populations (Hayashi et al., 2018; Khalifa et al., 2019; Worku et al., 2022). Notably, the rapid dissemination of ESBLs is considered as a global concern because it poses a greater threat to the human food supply chain, hospitals, and community settings (Burgess et al., 2021; Hayashi et al., 2018). ESBLs include a group of β-lactamase-encoding genes that hydrolyze penicillin, oxyimino-cephalosporins (third and

fourth generation cephalosporins), and monobactams, but spare carbapenems. They are inhibited by β-lactamase inhibitor, such as clavulanic acid. The major ESBL groups include TEM, SHV, and CTX-M and are associated with the occurrence of MDR phenotypes among gram-negative bacteria (Hayashi et al., 2018; Kłos et al., 2022). Currently, the recommended treatment options for complicated infections caused by MDR bacterial pathogens include extended-spectrum cephalosporins and quinolones (Adel et al., 2021), as the resistance to older antimicrobials continues to increase. However, the continuous spread of ESBLs has challenged the effectiveness of critically important antimicrobials (CIA), resulting in severe treatment failure. Thus, the potential risks to public health posed by MDR Gram-negative bacteria necessitate the need for an integrative One Health concept for antimicrobial surveillance along the meat value chain.

AmpC β-lactamases have received limited attention, but are now identified as a problem in Gram-negatives due to their high hydrolytic activities on third generation cephalosporins (Rensing et al., 2019; Sukmawinata et al., 2020). Bacteria overexpressing AmpC β-lactamases often render clinical resistance to all β-lactams, or β-lactam/β-lactamase-inhibitor combinations, with the exception of cefepime or cefpirome and is considered as a critical clinical concern, because such bacteria usually exhibit MDR phenotypes (Koga et al., 2019; Robatjazi et al., 2021). These were initially thought to be chromosome-mediated and occurred mostly in *Enterobacter* spp., *Serratia* spp., *Providencia* spp., *Morganella morganii*, *Citrobacter freundii*, and *Hafnia alvei*. However, plasmid-borne AmpC β-lactamases have also been reported in several species of Gram-negative bacteria such as *Escherichia coli*, *Salmonella* spp., and *Klebsiella* spp. (Burgess et al., 2021; El-aziz et al., 2022; Koga et al., 2019; Robatjazi et al., 2021; Tran et al., 2021).

In Japan, penicillin, tetracycline, sulfonamides, and aminoglycosides are used as veterinary drugs and feed additives in animal husbandry (Watanabe et al., 2023). Nonetheless,

there are concerns regarding the selective pressure from antibiotics that accelerates the evolution of resistant pathogens. Notably, such resistant pathogens with the production of ESBLs/AmpC β-lactamases have been reported in food, food-producing animals, and companion animals in Japan (Nakayama et al., 2022; Norizuki et al., 2018; Xedzro et al., 2023; Yasugi et al., 2021). These studies mainly focused on *E. coli* excluding other groups of gram-negative bacteria. Moreover, robust AMR data in the field is still limited; thus, periodic antimicrobial surveillance along the food chain remains underexplored in certain parts of Japan. This warrants major consideration, as other Gram-negative bacteria that are becoming more deadly are excluded without particular interest. Systematic AMR monitoring at the local and national levels is an integral part of the control strategy to curb the national and international spread of emerging resistance traits in humans and animals. Although AMR surveillance programs have shown an increase in overall *Enterobacteriaceae* resistance over time (Cooper et al., 2020; Huang et al., 2022), resistance rates vary between geographical regions and also among different species. Such a correlation among AMR, geographical region, and bacterial species of interest is extremely important for assessing the prevalence trends and impact of AMR on human health. It is important to mention that annual data on AMR for future monitoring of AMR trends in the current region of study are unknown, which makes it impossible to determine whether existing policies to curb AMR are effective (Xedzro et al., 2023).

Therefore, under the One Health approach, it is crucial to undertake surveillance studies to identify the genetic elements associated with drug resistance among Gram-negative bacteria to control the transmission of such bacteria to humans. Accordingly, this study characterized the prevalence of AMR determinants in a collection of Gram-negative bacterial isolates from retail meat. Comparative profiling was also conducted to assess the changes in AMR occurring among Gram-negative bacteria from two separate years of study in

Higashihiroshima, Japan.

3. Materials and methods

3.1 Study design, sampling plan, and bacterial isolates

A detailed description of the study location, sampling procedures, and bacterial isolation technique has been described previously (Xedzro et al., 2023). A total of 880 bacterial isolates were obtained over an 8-month period (May to December 2021) on MacConkey agar (Eiken Chemical Co., Ltd., Tochigi, Japan) containing ampicillin (100 µg/mL), streptomycin (50 µg/mL), meropenem (2 µg/mL), or colistin (2 µg/mL). This study also included 303 gram-negative bacterial isolates recovered from meat samples collected in 2009 from the same region.

3.2 Molecular detection of AMR genes

Extended-spectrum β-lactamase-encoding genes (*bla*$_{SHV}$, and *bla*$_{CTX-M}$), other β-lactamases (*bla*$_{TEM}$, *bla*$_{OXA-1}$, *bla*$_{OXA-2}$, *bla*$_{OXA-5}$, *bla*$_{OXA-9}$, and AmpC β-lactamases), integrons (class 1, 2, class 3), and aminoglycoside acetyltransferase gene, *aac(6′)-Ib* were previously amplified (Xedzro et al., 2023) using eicosaplex PCR (Soliman et al., 2020). Mobile colistin resistance genes (*mcr-1* to *-9*) (for all 2009 and 2021 isolates selected on MacConkey agar containing colistin) and acquired carbapenemases were also amplified. We then selected all AmpC β-lactamase producers for further characterization. Additionally, all bacterial isolates other than *E. coli* that were sequenced, but not reported in our previous study, were selected and included in subsequent analyses.

3.3 Genetic determination of plasmid-mediated AmpC β-lactamases (octaplex PCR)

Bacterial isolates that showed ~374 bp fragments as a result of eicosaplex PCR were selected and further characterized using octaplex PCR (Soliman et al., 2023) for the molecular detection of plasmid-mediated AmpC β-lactamases (pAmpC). The PCR system detected six different pAmpC families (DHA, CMY/LAT, ACT/MIR, MOX, FOX, and ACC), and intrinsic ADC and PDC in *Acinetobacter* and *Pseudomonas*, respectively. Positive isolates were selected and confirmed using single PCR. The thermal cycling conditions were the same (Xedzro et al., 2023), except for the annealing temperature, which was modified to 57.5 ºC (Soliman et al., 2023).

3.4 Molecular amplification of integron cassettes

The variable regions of class 1 and 2 integrons within Enterobacterales (except *E. coli*) and non-Enterobacterales were amplified using primers complementary to the 5′ and 3′ conserved segments of class 1 integrons (5′ CS and 3′ CS) (Table S1) and class 2 integrons (hep74 and hep51) (Table S1). PCR amplification conditions were as follows: initial denaturation at 98 ºC for 1 min, 30 cycles of denaturation at 98 ºC for 10 s, annealing at 50 ºC for 15 s, extension at 68 ºC for 2 min, and final extension at 68 ºC for 15 s.

3.5 DNA analysis and sequencing of PCR products

All PCR products were run at 100 V for 50 min on a 3% agarose gel (Fast Gene NE-A02 agarose gel) and stained with ethidium bromide. The bands embedded in the agarose gel were visualized under ultraviolet light to determine the quality of the amplified DNA. The PCR products were purified using ExoSAP-IT (Thermo Fisher Scientific, Japan) and sequenced by Eurofins, Japan.

3.6 16S rRNA gene sequencing

For this, 1500 bp fragments of selected AmpC β-lactamase-producers (those that showed resistance to at least one β-lactam antibiotic) were amplified using the full-length 27F and 14192R primer sets. The PCR products were purified using ExoSAP-IT and processed for sequencing to identify the bacteria at the species level. A total of 168 isolates, including those that were previously sequenced (except *E. coli*) and harbored at least one AMR gene, were obtained.

3.7 Phenotypic antimicrobial susceptibility

Eighty-nine isolates were selected and subjected to phenotypic antimicrobial susceptibility testing using the Kirby-Bauer method, according to the interpretative criteria described by the Clinical Laboratory Standards Institute (CLSI, 2020). The minimum inhibitory concentration (MIC) of colistin for *mcr*-positive strains was also assessed following the EUCAST recommendations (EUCAST, 2023). A single isolated colony of each bacterium was suspended in sterile normal saline (0.85% w/v NaCl), and the turbidity was adjusted to 0.5 McFarland standards. The surfaces of the Mueller-Hinton agar plates were then uniformly inoculated with the suspension using a sterile cotton swab. All isolates except AmpC-producers were tested against fifteen antimicrobial agents from twelve classes as follows: ampicillin (10 µg), cefotaxime (30 µg), ceftazidime (30 µg), cefepime (30 µg), cefoxitin (30 µg), aztreonam (30 µg), meropenem (10 µg), amikacin (10 µg), kanamycin (30 µg), tetracycline (30 µg), chloramphenicol (30 µg), ciprofloxacin (5 µg), sulfamethoxazole-trimethoprim (23.75/1.25 µg), colistin (30 µg), and fosfomycin (50 µg). Specific integron cassettes within 10 isolates could not be determined, hence they were eliminated from susceptibility testing. AmpC β-lactamase-producers (198 isolates from both 2009 and 2021) were screened for resistance only against ampicillin, cefotaxime, ceftazidime, and aztreonam,

and 69 resistant strains were selected for analysis. Antibiotic disks (Eiken Chemical Co., Ltd., Tochigi, Japan) were aseptically applied to the surfaces of inoculated Mueller-Hinton agar plates. After incubation at 37ºC for 16–18 h, the diameter of inhibition zones was measured and interpreted as sensitive, intermediate, or resistant based on the interpretative chart supplied with the antimicrobial agent. *E. coli* ATCC 25922 was used as the quality control strain. Multidrug resistance was defined as resistance to at least one antimicrobial agent belonging to three or more classes (Magiorakos et al., 2012). Isolates displaying intermediate resistance were considered as resistant to antimicrobial agents.

3.8 Plasmid analysis and PCR-based replicon typing of ESBL-producing *E. coli*

Seventeen well-characterized ESBL-producing *E. coli* strains from our previous study were assayed for plasmid replicon type. Plasmids were extracted using the alkaline lysis method, as previously described (Green and Sambrook, 2016). Briefly, 1.5 mL of overnight LB broth culture was centrifuged and resuspended in alkaline lysis buffers I, II, and III. The solution was vortexed and allowed to stand for 3–5 min after the addition of each buffer. The bacterial lysate was centrifuged, and the supernatant was mixed with an equal volume of isopropanol. The precipitated nucleic acids were collected and dissolved in 1 mL of 70% ethanol. The DNA was recovered as pellets by centrifugation and finally dissolved in 50 μL of TE (pH 8.0) containing 20 μg/mL DNase-free RNase A. Plasmid incompatibility (Inc) groups were determined using PCR-based replicon-typing, as previously described (Carattoli et al., 2005; Villa et al., 2010) by using the plasmid DNA as template for amplification. Eighteen primer pairs were used to amplify the target regions of the major incompatibility groups among *Enterobacterales* including HI1, HI2, I1, X, L/M, N, FIA, FIB, W, Y, P, FIC, A/C, T, FIIs, FrepB, K/B, and B/O in five multiplex and three simplex PCR assays.

3.9 BLAST search and statistical analysis

All sequenced data were subjected to a similarity search using the Basic Local Alignment Search Tool (BLAST) program available in the National Center for Biotechnology Information (NCBI) database. Pearson's chi-square test or Fisher's exact test was used to test for significant differences when comparisons were made. A 95% confidence interval was inferred, and *p*-values<0.05 were considered as statistically significant. Statistical analyses were performed using IBM SPSS version 29.0 (SPSS Corp., Armonk, NY, USA). Finally, the results were expressed as percentages and are presented in tables.

4. Results

4.1 Sample distribution and positive number

A total of 185 meat samples (raw chicken, n=87; beef, n=47; pork, n=48; and wild meat, n=3) were analyzed in this study (Table 6). Of the samples, 95 were collected in 2009 and 90 were obtained in 2021. The results of the PCR analysis indicated the presence of diverse Gram-negative bacterial species carrying AMR determinants in 62.1 (59/95) and 61.1% (55/90) of the 2009 and 2021 meat samples, respectively. Statistical analysis revealed an insignificant difference (*p*-value >0.05) in the total number of positive samples among the two years of our study. Similarly, we observed no significant difference in positive numbers of domestic and imported samples between 2009 and 2021.

4.2 Diversity of Gram-negative bacteria isolated from retail meat samples

The bacterial genera and their prevalence in the two isolation years are shown in Table 7. I identified 245 Gram-negative bacterial isolates from diverse genera carrying at least one AMR gene. Among the Enterobacterales, *E. coli* was predominant, accounting for

31.4% (77/245) of the total positive isolates. These *E. coli* isolates were genetically variable and were characterized in our previous study (Xedzro et al., 2023). Additionally, we found that other strains were highly diverse; for instance, *Rhanella* spp. (n=19) was prevalent in 2021, whereas none of these species were isolated in 2009. *Klebsiella* spp. (n=18), *Salmonella* spp. (n=13), and *Enterobacter* spp. (n=14) were predominant in 2009, and a few species of *Kluyvera* (n=2*)*, *Proteus* (n=2), *Providencia* (n=1), and *Pantoea* (n=1) were also identified. Apart from *E. coli* and *Rhanella* spp. that were detected as the prevalent species in 2021, we additionally found that *Hafnia* spp. (n=9), *Serratia* spp. (n=9), and *Morganella morganii* (n=7) were high in number. A high proportion of the isolates belonged to the order Enterobacterales. *Acinetobacter* spp. (n=15), *Pseudomonas* spp. (n=12), and *Aeromonas* spp. (n=16) were among the most prevalent non-Enterobacterales identified in this study (Table 7).

Table 6. Sample numbers and distribution of positive samples according to meat category and source

Isolation year	Meat category	Number of samples					
		Domestic	Positive samples (%) *	Imported	Positive samples (%) *	Total	Total positive samples (%) *
2009	Chicken	29	20 (69.0)	8	6 (75.0)	37	26 (70.3)
	Beef	18	13 (72.2)	10	7 (70.0)	28	20 (71.4)
	Pork	18	8 (44.4)	11	4 (36.4)	29	12 (41.4)
	Wild meat	0	0 (0.0)	1	1 (100)	1	1 (100)
	Total	**65**	**41 (63.1)**	**30**	**18 (60.0)**	**95**	**59 (62.1)**
2021	Chicken	37	24 (64.9)	13	10 (76.9)	50	34 (68)
	Beef	10	6 (60.0)	9	7 (77.8)	19	13 (68.4)
	Pork	9	3 (33.3)	10	5 (50.0)	19	8 (42.1)
	Wild meat	2	0 (0.0)	0	0 (0.0)	2	0 (0.0)
	Total	**58**	**33 (56.9)**	**32**	**22 (68.8)**	**90**	**55 (61.1)**

* Indicates no significant difference (p>0.05) in total positive number between 2009 and 2021

Table 7. Different Gram-negative bacteria of various genera isolated from meat samples

Group	Bacterial species	Isolation year (No. of isolates)		
		2009 (n=120)	2021 (n=125)	*p*-value
Enterobacterales	*Escherichia coli*	33 (27.5%)	44 (35.2%)	0.194
	Rhanella spp.	0 (0.0%)	19 (15.2%)	0.000
	Klebsiella spp.	18 (15.0%)	1 (0.8%)	0.000
	Salmonella spp.	13 (10.8%)	0 (0.0%)	0.000
	Enterobacter spp.	13 (10.8%)	1 (0.8%)	0.001
	Hafnia spp.	6 (5.0%)	8 (6.4%)	0.423
	Serratia spp.	2 (1.7%)	10 (8.0%)	0.020
	Citrobacter spp.	7 (5.8%)	1 (0.8%)	0.027
	Morganella morganii	4 (3.3%)	7 (5.6%)	0.392
	Buttiauxella spp.	0 (0.0%)	5 (4.0%)	0.027
	Kluyvera spp.	2 (1.7%)	0 (0.0%)	0.147
	Proteus mirabilis	2 (1.7%)	1 (0.8%)	0.537
	Pantoea spp.	1 (0.8%)	2 (1.6%)	0.585
	Providencia spp.	1 (0.8%)	0 (0.0%)	0.306
Non-Enterobacterales	*Acinetobacter* spp.	15 (12.5%)	0 (0.0%)	0.000
	Pseudomonas spp.	0 (0.0%)	12 (9.6%)	0.001
	Aeromonas spp.	3 (2.5%)	13 (10.4%)	0.012
	Shewanella spp.	0 (0.0%)	1 (0.8%)	0.326

4.3 Overall prevalence of AMR genes

Among the 120 isolates identified in 2009 to harbor AMR genes, 9.2, 13.3, and 7.5% carried the β-lactamase $bla_{\text{CTX-M}}$, bla_{SHV}, and bla_{TEM} resistance genotypes, respectively, including ESBL types. Additionally, we observed a high frequency (50/120, 41.7%) of AmpC

β-lactamases and class 1 integrons (29/120, 24.2%) within 2009 isolates, with only a few carrying class 2 integrases (n=8) (Table 8).

Concurrently, the most common resistance genotypes among the 2021 isolates were *bla*$_{\text{CTX-M}}$ (29.6%), *bla*$_{\text{TEM}}$ (22.8%), and AmpC (15.2%), and none of the isolates were positive for *bla*$_{\text{SHV}}$ and class 2 integrons. Carbapenemase-encoding genes were not detected in 2009; however, a single strain identified as *Shewanella xiamenesis* isolated in 2021 carried *bla*$_{\text{OXA-199,}}$ a carbapenemase-encoding gene. Regardless of the source, the most frequent resistance genes in beef and pork isolates were minor ESBLs (22/125, 17.6%), including *bla*$_{\text{RHAN}}$-type genotypes from the CTX-M group. Notably, all *bla*$_{\text{RHAN}}$ genes were detected in 2021, and included *bla*$_{\text{RHAN-1}}$ (n=13) and *bla*$_{\text{RHAN-2}}$ (n=7). We also found that a majority of AMR genes originated from chicken isolates derived from the two isolation years. Finally, the *mcr* gene which confers clinical resistance to colistin, a frontline therapeutic treatment of choice was detected in 4 and 2 isolates in 2009 and 2021, respectively (Table 8). Specific *mcr* variants included *mcr-3* in *Aeromonas hydrophila*, *mcr-4* in *Acinetobacter baumannii*, *mcr-5* in *E. coli*, *mcr-9* in *Enterobacter kobei*, and two *Serratia liquefaciens*.

Table 8. Prevalence and distribution of AMR genes among meat samples

Isolation year	Meat category (No. of positive isolates)	β-lactamases (%)				Integrons (%)		Carbapene-mases (%)	Colistin resistance genes (%)
		bla$_{CTX-M}$	*bla*$_{SHV}$	*bla*$_{TEM}$	AmpC	Class 1	Class 2		
2009	Chicken (66) [a]	11 (16.7)	10 (15.2)	8 (12.1)	20 (30.3)	13 (19.7)	7 (10.6)	0 (0.0)	2 (3.0)
	Beef (37) [a]	0 (0.0)	4 (10.8)	0 (0.0)	18 (48.6)	15 (40.5)	1 (2.7)	0 (0.0)	1 (2.7)
	Pork (15)	0 (0.0)	2 (13.3)	1 (6.7)	10 (66.7)	1 (6.7)	0 (0.0)	0 (0.0)	1 (6.7)
	Wild meat (2)	0 (0.0)	0 (0.0)	0 (0.0)	2 (100)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
	Total (120)	**11 (9.2)** [c]	**16 (13.3)** [e]	**9 (7.5)** [g]	**50 (41.7)** [i]	**29 (24.2)**	**8 (6.7)** [k]	**0 (0.0)**	**4 (3.3)**
2021	Chicken (88) [a]	15 (17.0)	0 (0.0)	35 (39.8)	14 (15.9)	36 (40.9)	0 (0.0)	1 (1.1)	2 (2.3)
	Beef (19) [b]	12 (63.2)	0 (0.0)	0 (0.0)	1 (5.3)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
	Pork (18)	10 (55.6)	0 (0.0)	1 (5.6)	4 (22.2)	3 (16.7)	0 (0.0)	0 (0.0)	0 (0.0)
	Wild meat (0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
	Total (125)	**37 (29.6)** [d]	**0 (0.0)** [f]	**36 (22.8)** [h]	**19 (15.2)** [j]	**39 (31.2)**	**0 (0.0)** [l]	**1 (0.8)**	**2 (1.6)**

Prevalence of AMR genes was expressed as percentage of total positive isolates.

Different superscripts in the same column represent significant difference (p<0.05).

[a] Some isolates have more than one AMR genes.

[b] Specific genes were not detected in some isolates.

4.4 Overall AMR profiles of selected Gram-negative bacteria recovered from retail meat

Antimicrobial susceptibility was profiled to provide an overall picture of resistance among Gram-negative bacteria in the isolation region. A total of 166 isolates including 77 *E. coli* isolates, were profiled against 15 antimicrobials. All the isolates from 2009 and 2021 showed clinical susceptibility to meropenem and amikacin, respectively. For the two years of our study, we found a decreased rate of resistance to antimicrobials between 2009 and 2021 as follows (significance indicated by *p*-value in bracket): ceftazidime, 11.8 vs 5.6%; cefepime, 9.2 vs 3.3%; cefoxitin, 17.1 vs 7.8%; aztreonam, 17.1 vs 5.6% (0.017); amikacin, 1.3 vs 0.0%; chloramphenicol, 23.7 vs 18.9%; colistin, 7.9 vs 2.2%; and fosfomycin, 29.0 vs 17.8%% (Table 9). Notably, all the *mcr*-carrying isolates showed the colistin resistance phenotype with MIC ranging from 8 µg/mL to >64 µg/mL.

The antimicrobial resistance profiles of the two groups of Gram-negative bacteria are presented in Table 10. The Enterobacterales (excluding *E. coli*) isolated in 2009 showed predominant resistance to ampicillin (63.4%, n=27), cefoxitin (14.3%, n=6), tetracycline (21.4%, n=9), chloramphenicol/sulfamethoxazole-trimethoprim (11.9%, n=5), and fosfomycin (40.5%, n=17). Except for ampicillin (85.3%, n=29) and cefotaxime (35.3 %, n = 12) resistance, which were high, there were generally lower levels of antimicrobial resistance among Enterobacterales (excluding *E. coli*) isolates in 2021 than in 2009 (Table 10). All *Rhanella* spp. carrying the *bla*$_{\text{RHAN}}$ genotypes showed 100% resistance to ampicillin. Furthermore, the majority of these isolates showed intermediate resistance, with few conferring clinical resistances against extended-spectrum cephalosporins, particularly cefotaxime. MDR occurred in 11 and 15 of the 2009 and 2021 isolates, respectively, excluding *E. coli* strains and AmpC producers. Despite being the major carriers of class 1 integrons (*aadA1*/*aadA2*), only 2 of 13 *Salmonella enterica* strains were resistant to at least one antibiotic.

Table 9. Overall AMR profiles of selected Gram-negative bacteria isolated from retail meat

Antimicrobials	Disk content	Resistance profile		
		2009 (n=76)	2021 (n=90)	*p*-value
Ampicillin	10 µg	65.8%	87.8%	0.001
Cefotaxime	30 µg	26.3%	27.7%	0.833
Ceftazidime	30 µg	11.8%	5.6%	0.146
Cefepime	30 µg	9.2%	3.3%	0.113
Cefoxitin	30 µg	17.1%	7.8%	0.066
Aztreonam	30 µg	17.1%	5.6%	0.017
Meropenem	10 µg	0.0%	1.1%	0.357
Amikacin	30 µg	1.3%	0.0%	0.275
Kanamycin	30 µg	19.7%	38.9%	0.007
Tetracycline	30 µg	44.7%	51.1%	0.413
Chloramphenicol	30 µg	23.7%	18.9%	0.450
Ciprofloxacin	5 µg	6.6%	10.0%	0.429
Sulfamethoxazole/trimethoprim	23.75/1.25 µg	18.4%	30.0%	0.030
Colistin	10 µg	7.9%	2.2%	0.089
Fosfomycin	50 µg	28.9%	17.8%	0.088

E. coli was included in computing overall resistance patterns.

Table 10. Antimicrobial resistance profiles of Enterobacterales and non-Enterobacterales isolated from retail meat

Antimicrobials	Disk content	Enterobacterales				Non-Enterobacterales			
		2009 (n=42)		2021 (n=34)		2009 (n=1)		2021 (n=12)	
		R %	S %	R %	S %	R %	S %	R %	S %
Ampicillin	10 µg	64.3	35.7	85.3	14.7	0.0	100	91.7	8.3
Cefotaxime	30 µg	7.1	92.9	35.3	64.7	100	0.0	33.3	66.7
Ceftazidime	30 µg	4.8	95.2	2.9	97.1	100	0.0	8.3	91.7
Cefepime	30 µg	4.8	95.2	2.9	97.1	100	0.0	0.0	100
Cefoxitin	30 µg	14.3	85.7	2.9	97.1	0.0	100	33.3	66.7
Aztreonam	30 µg	2.4	97.6	2.9	97.1	100	0.0	0.0	100
Meropenem	10 µg	0.0	100	2.9	97.1	0.0	100	0.0	100
Amikacin	30 µg	2.4	97.6	0.0	100	0.0	100	0.0	100
Kanamycin	30 µg	7.1	92.9	8.8	91.2	0.0	100	16.7	83.3
Tetracycline	30 µg	21.4	78.6	11.8	88.2	0.0	100	58.3	41.7
Chloramphenicol	30 µg	11.9	88.1	2.9	97.1	100	0.0	50.0	50.0
Ciprofloxacin	5 µg	4.8	95.2	2.9	97.1	0.0	100	8.3	91.7
Sulfamethoxazole/trimethoprim	23.75/1.25 µg	11.9	88.1	2.9	97.1	0.0	100	58.3	41.7
Colistin	10 µg	9.5	90.5	8.8	91.2	100	0.0	0.0	100
Fosfomycin	50 µg	40.5	59.5	20.6	79.4	100	0.0	25.0	66.7

AmpC-producers were excluded from the table since they were not test against all the antimicrobials.

All *E. coli* strains were also excluded from the table.

4.5 Plasmid incompatibility groups detected in ESBL-producing *E. coli* isolates

We then characterized the presence of plasmids in ESBL-producing *E. coli* which may be associated with the horizontal gene transfer of ESBL genes. PCR-based replicon typing revealed that the ESBL-producing *E. coli* isolates carried plasmids belonging to more than one incompatibility group (Inc): IncI1, IncN, IncFIB, IncFrepB, IncK/B, IncB/O, IncHI1, and IncHI2 (Table 11). The commonly detected plasmid replicon types were the IncFIB, IncI1, and IncFrepB, suggesting that these plasmids may be the potential carriers of AMR genes, including CTX-M enzymes.

4.6 Genetic characterization of plasmid-mediated AmpC β-lactamases

The detection rates of plasmid-mediated AmpC β-lactamases in meat samples are shown in Table 12. Fifty out of 120 isolates from 2009 were AmpC β-lactamase-producers. The detected genes included bla_{DHA} (n=4), bla_{FOX} (n=2), bla_{MOX} (n=2), bla_{ACC} (n=6), bla_{CMY} (n=5) bla_{ADC} (n=18), and bla_{ACT} (n=13), which were present in different alleles (Table 12). Susceptibility testing indicated that these β-lactamases conferred clinical resistance to β-lactam antibiotics. For instance, *Acinetobacter* spp. with the bla_{ADC} gene commonly showed resistance to ampicillin, cefotaxime, and aztreonam, whereas *Enterobacter* spp. and *E. coli* showed resistance to all four antibiotics, including ceftazidime. In contrast, only 19 of the 125 isolates collected in 2021 carried the following AmpC genes: bla_{DHA} (n=6), bla_{FOX} (n=6), and bla_{ACC} (n=7). Ampicillin resistance is common among all 2021 AmpC-producers. Additionally, *Aeromonas* spp. and *Hafnia* spp. carrying bla_{FOX} and bla_{ACC}, respectively, showed resistance to cefotaxime and ceftazidime (Table 12).

Table 11. Plasmids detected in ESBL-producing *E. coli* isolated from meat sources

Isolate	Origin/Year	Genotype	Incompatibility groups
B1-A009	Japan/2021	*bla*$_{\text{TEM-1}}$, *bla*$_{\text{CTX-M-1}}$	IncI1, IncN, IncFIB, IncFrepB
BH1-S088	Brazil/2021	*bla*$_{\text{CTX-M-15}}$, *bla*$_{\text{TEM-1}}$, *aadA1*	IncI1, IncN, IncFrepB
H3-A056	Japan/2021	*bla*$_{\text{TEM-1}}$, *bla*$_{\text{CTX-M-14}}$	IncFIB, IncFrepB, IncK/B, IncB/O
K3-A121	Brazil/2021	*bla*$_{\text{CTX-M-15}}$, *bla*$_{\text{TEM-1}}$	IncI1, IncN, IncFrepB
HT1-S097	Japan/2021	*bla*$_{\text{CTX-M-2}}$	IncFrepB
HT3-S094	Brazil/2021	*bla*$_{\text{CTX-M-2}}$, *aadA1*	Untypable
MT1-A128	Japan/2021	*bla*$_{\text{CTX-M-2}}$	IncFIB, IncFrepB, IncB/O
E3-A018	Japan/2021	*bla*$_{\text{CTX-M-2}}$	IncHI1, IncHI2, IncB/O
J1-A076	Japan/2021	*bla*$_{\text{CTX-M-like}}$	IncFIB, IncFrepB
138-2H-3	Japan/2009	*bla*$_{\text{CTX-M-2}}$	IncI1, IncFrepB
139-2H-4	Japan/2009	*bla*$_{\text{CTX-M-2}}$	IncFIB, IncFrepB
174-2H-1X	Japan/2009	*bla*$_{\text{CTX-M-2}}$	IncI1, IncFrepB
267-34J1-2	Brazil/2009	*bla*$_{\text{CTX-M-2}}$, *aadA1*	IncFrepB, IncK/B
269-34J2-2	Brazil/2009	*bla*$_{\text{CTX-M-2}}$, *aadA1*	IncFIB, IncFrepB
286-36J2-1	Brazil/2009	*bla*$_{\text{CTX-M-2}}$, *aadA1*	IncFIB, IncFrepB
301-36J2-1X	Brazil/2009	*bla*$_{\text{CTX-M-2}}$, *aadA1*	IncFIB, IncFrepB
160-11H-1	Japan/2009	*bla*$_{\text{SHV-12}}$, *mcr-5.1*	IncI1, IncFIB, IncFrepB

Table 12. Phenotypic and genotypic profiles of AmpC β-lactamase producers

Year (No. of isolates)	Identification	Genotype (number of isolates)	Common resistance	Number of isolates from each meat category (%)				Total
				Chicken	Pork	Beef	Wild meat	
2009 (n=50)	*Morganella*	*bla*$_{DHA-1}$ (2) *bla*$_{DHA-13}$ (1) *bla*$_{DHA-18}$ (1)	AMP	2 (4.0)	1 (2.0)	1 (2.0)	0 (0.0)	4 (8.0)
	Aeromonas	*bla*$_{FOX-5}$ (1) *bla*$_{FOX-13}$ (1) *bla*$_{MOX-5}$ (1)	AMP	0 (0.0)	2 (4.0)	1 (2.0)	0 (0.0)	3 (6.0)
	Hafnia	*bla*$_{ACC-1}$ (2) *bla*$_{ACC-4}$ (3) *bla*$_{ACC-6}$ (1)	AMP	1 (2.0)	4 (8.0)	1 (2.0)	0 (0.0)	6 (12.0)
	Citrobacter	*bla*$_{CMY-137}$ (1) *bla*$_{CMY-74}$ (1) *bla*$_{CMY-79}$ (1)	AMP	1 (2.0)	1 (2.0)	1 (2.0)	0 (0.0)	3 (6.0)
	Acinetobacter	*bla*$_{ADC-32}$ (2) *bla*$_{ADC-30}$ (1) *bla*$_{ADC-133}$ (3) *bla*$_{ADC-216}$ (1) *bla*$_{ADC-235}$ (1) *bla*$_{ADC-50}$ (3) *bla*$_{ADC-239}$ (1) *bla*$_{ADC-26}$ (2) *bla*$_{ADC-120}$ (1)	AMP CTX ATM	10 (20.0)	0 (0.0)	3 (6.0)	2 (4.0)	15 (30.0)
	K. pneumoniae	*bla*$_{ACT-4}$ (1)	AMP	0 (0.0)	0 (0.0)	1 (2.0)	0 (0.0)	1 (2.0)

Tale 12. continued

	Enterobacter	*bla*$_{ACT-4}$ (4) *bla*$_{ACT-12}$ (2) *bla*$_{ACT-54}$ (2) *bla*$_{ACT-103}$ (1) *bla*$_{ACT-73}$ (1) *bla*$_{MOX-15}$ (1)	AMP CTX CAZ ATM	1 (2.0)	1 (2.0)	9 (18.0)	0 (0.0)	11 (22.0)
	Escherichia coli	*bla*$_{CMY-4}$ (2) *bla*$_{ACT-48}$ (1) *bla*$_{ACT-51}$ (1) *bla*$_{ADC-26}$ (1) *bla*$_{ADC-32}$ (2)	AMP CTX CAZ ATM	5 (10.0)	1 (2.0)	1 (2.0)	0 (0.0)	7 (14.0)
2021 (n=19)	*Morganella morganii*	*bla*$_{DHA-1}$ (1) *bla*$_{DHA-2}$ (1) *bla*$_{DHA-4}$ (1) *bla*$_{DHA-5}$ (1) *bla*$_{DHA-10}$ (1) *bla*$_{DHA-22}$ (1) *bla*$_{FOX-8}$ (1)	AMP	7 (36.8)	0 (0.0)	0 (0.0)	0 (0.0)	7 (36.8)
	Aeromonas	*bla*$_{FOX-8}$ (2) *bla*$_{FOX-16}$ (1) *bla*$_{FOX-18}$ (1) *bla*$_{FOX-19}$ (1)	AMP CTX	5 (26.3)	0 (0.0)	0 (0.0)	0 (0.0)	5 (26.3)
	Hafnia	*bla*$_{ACC-1}$ (7)	AMP CTX CAZ	2 (10.5)	4 (21.1)	1 (5.3)	0 (0.0)	7 (36.8)

4.7 Phenotypic confirmation of AmpC production against three classes of antimicrobials

As shown in Table 13, a major proportion of AmpC β-lactamase-producers obtained from 2009 were resistant to three classes of antibiotics. Penicillin resistance among the isolates was high (90.0%), with two or more isolates harboring one of the seven detected resistance genotypes. Cephalosporin resistance was observed in 25 isolates and was commonly associated with bla_{ADC} and bla_{ACT} genotypes. Monobactam resistance was observed in 12 (24.0%) isolates. All the isolates obtained in 2021 were resistant to ampicillin, but none conferred clinical resistance to monobactam. However, a single strain carrying bla_{FOX} and bla_{ACC} from 2021 was found to hydrolyze a cephalosporin antibiotic (cefotaxime). The results suggest that plasmid-mediated AmpC β-lactamase may be a clinical challenge due to the increased patterns of resistance they confer against recommend treatment options.

Table 13. Phenotypic detection rates of AmpC β-lactamases and their resistance patterns against three classes of antimicrobials

Isolation year	Resistance group	AmpC-type β-lactamases							Total resistance
		bla$_{DHA}$	*bla*$_{FOX}$	*bla*$_{ACC}$	*bla*$_{CMY}$	*bla*$_{ADC}$	*bla*$_{ACT}$	*bla*$_{MOX}$	
2009 (n=50)	Penicillin resistance	4 (8.0%)	2 (4.0%)	6 (12.0%)	5 (10.0%)	13 (26.0%)	13 (26.0%)	2 (4.0%)	**45 (90.0%)**
	Cephalosporin resistance	0 (0.0%)	0 (0.0%)	0 (0.0%)	2 (4.0%)	17 (34.0%)	6 (12.0%)	0 (0.0%)	**25 (50%)**
	Monobactam resistance	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	8 (16.0%)	4 (8.0%)	0 (0.0%)	**12 (24.0%)**
2021 (n=19)	Penicillin resistance	7 (36.8%)	5 (26.3%)	7 (36.8%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	**19 (100%)**
	Cephalosporin resistance	0 (0.0%)	1 (5.3%)	1 (5.3%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	**2 (10.5%)**
	Monobactam resistance	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	**0 (0.0%)**

5. Discussion

Meat consumption continues to increase in many countries, including Japan, as meat is important for human nutrition. Nevertheless, retail meat products have been established as potential hazards to the human population, owing to the increasing incidence of drug-resistant bacteria isolated from them (Cooper et al., 2020; Wang et al., 2022). Little is known about AMR surveillance in certain parts of Japan, including Higashihiroshima. I previously reported the first comparative data on MDR *E. coli* in the same region (Xedzro et al., 2023), and further confirmed the lack of surveillance data for future monitoring of AMR trends. Therefore, I investigated the prevalence of drug-resistant Enterobacterales and non-Enterobacterales, and the plasmids harboring ESBLs, among a collection of ESBL-producers.

This study shows that retail meat products are potential reservoirs of drug-resistant bacteria with AMR determinants. More than 60% of the samples investigated in the two isolation years were contaminated with these bacteria. Various factors such as the location and method of animal rearing, contaminated processing water, equipment, and climate change contribute to the contamination of meat samples. I found that there was no significant difference (p-value=0.889) in the contamination rates of samples (62.1 vs. 61.1%) between the two isolation years; however, such a high prevalence should be considered as a potential health risk to consumers. In view of the small sample size and lack of data on antimicrobial use, no conclusions can be drawn as to why these insignificant differences existed.

Furthermore, a variety of Enterobacterial and non-Enterobacterial species were detected in retail meat samples. Overall, Enterobacterales were highly prevalent, with *E. coli* being the dominant species in both 2009 (27.5%) and 2021 (35.2%) (Table 7). Several other bacterial species were identified; however, their prevalence depended on the year of isolation. For instance, *Klebsiella* spp., *Salmonella* spp., *Enterobacter* spp., and *Acinetobacter* spp. were predominantly isolated in 2009, whereas *Rhanella* spp., *Serratia* spp., *Pseudomonas*

spp., and *Aeromonas* spp. were mostly detected in 2021 samples. The sources of such Gram-negative bacteria in our study may have been food handlers, improper storage, poor meat hygiene, and/or unstrict stainless cleaning conditions, which may have caused serious cross-contamination in retail meat products. Furthermore, compromised process conditions, such as irregular temperature records in advanced cooling systems, may contribute to the rapid multiplication of bacterial species and their subsequent transfer via improper handling. Meat vendors and handlers may contribute to microbiological quality, especially if they are not fully knowledgeable about the significance of good manufacturing practices (Dib et al., 2018). Previous studies detected a wide variety of Enterobacterales in Japanese retail meat. Odoi et al. (2021) detected *Serratia* spp., *Hafnia* spp., *Proteus* spp., *Cedecea* spp., *Providencia* spp., and *Morganella* spp. in 81.3% of retail meat samples, which was relatively higher than the 61–62% positive samples in our study. Although there was a varying degree of prevalence, our study is consistent with previous studies that have isolated Enterobacterial and non-Enterobacterial species from retail meat in other countries. Studies from the United States have reported the presence of *Acinetobacter*, *Aeromonas*, *Buttiauxella*, *Citrobacter*, *Enterobacter*, *Hafnia*, *Klebsiella*, *Proteus*, among others, in retail meat samples (Kilonzo-Nthenge et al., 2013). Lee et al. (2023) documented 47.5% *E. coli* strains from retail meat in the United States. Furthermore, a recent study in Nigeria found a variety of bacterial species from seven genera, including *E. coli*, *Enterobacter*, *Klebsiella*, *Salmonella*, *Serratia*, *Citrobacter*, and *Proteus* in retail meat samples (Uzeh et al., 2021), suggesting that retail meat products are reservoirs of different groups of Gram-negative bacteria. The variations in the results may have been due to the different regions of study, isolation protocols, sample sizes, and seasonal changes (Xedzro et al., 2023).

I profiled the prevalence of AMR determinants in the two years of our study, according to the positive isolates investigated. DNA sequencing revealed that AmpCs were

the common β-lactamase enzymes found in 2009 and were mostly detected in chicken meat (Table 8). Other prevalent β-lactamase genes were *bla*$_{SHV}$, *bla*$_{CTX-M}$ and *bla*$_{TEM}$. Similarly, I found that *bla*$_{CTX-M}$, *bla*$_{TEM}$, and AmpC β-lactamases also dominated in the 2021 isolates, and were frequently detected in chicken meat isolates. These results suggest that chicken meat products are mostly carriers of these resistance determinants. These results are consistent with those of previous studies reporting the common detection of *bla*$_{CTX-M}$ and *bla*$_{SHV}$ in retail chicken meat in Japan (Hayashi et al., 2018; Kawamura et al., 2014; Norizuki et al., 2018). Various studies in Egypt (Badr et al., 2022), Canada (Cormier et al., 2019), China (Jiang et al., 2023; Liao et al., 2023), and Singapore (Guo et al., 2021) have also frequently detected *bla*$_{CTX-M}$-types in chicken meat, but uncommon detection of *bla*$_{SHV}$ or *bla*$_{TEM}$. CTX-M genes are common in chickens, possibly because of the off-label use of ceftiofur to treat infections in the chicken industry (Casella et al., 2017). None of the beef or pork isolates in 2009 carried *bla*$_{CTX-M}$; however, the *bla*$_{RHAN-1/2}$-type β-lactamases of the CTX-M group were predominantly found in beef and pork in 2021. An inverse observation occurred for *bla*$_{SHV}$ genes, where none of these genotypes were detected in 2021, but were found in 2009. Furthermore, a comparative analysis between 2009 and 2021 indicated that there was a 15% increase (p-value=0.000) in the prevalence of *bla*$_{TEM}$ (*bla*$_{TEM-1}$) among the total positive isolates presented (Table 8). This indicates that the acquisition or transmission of TEM genes is progressively increasing and warrants the continuous implementation of AMR policies in the current study region. Collectively, these results confirmed that the season of isolation and/or region may influence the isolation of specific bacteria carrying a particular resistance gene of interest. Although TEM-1 is less efficient in degrading later generations of cephalosporins (such as ceftazidime and cefotaxime) and monobactams, different variants including TEM-20 (Muhammad et al., 2014) with the ability to hydrolyze majority of β-lactams have evolved owing to extensive β-lactam use in clinical settings. Single nucleotide

polymorphisms (SNPs) in the TEM-1 gene result in amino acid substitutions in the enzyme, which eventually intensify the hydrolytic performance of the enzyme, and subsequently, the selection for extended-spectrum β-lactamases (Muhammad et al., 2014; Zhao et al., 2022). Integrons are site-specific recombination genetic systems that can capture and insert gene cassettes into bacterial genomes (Qi et al., 2023). They confer antimicrobial resistance, particularly against aminoglycosides, trimethoprim, quaternary ammonium ions, and chloramphenicol. The prevalent integron type detected in this study was class 1. These occurred in 24.2 (29/120) and 31.2% (39/125) of the 2009 and 2021 isolates, respectively (Table 8). The most common integron cassettes detected in this study were *aadA1*, *aadA2*, *aadA5*, *aadA12*, and *dfrA17*. Previous studies have described integrons and reported the frequent detection of such a group of integrase genes in retail meat (Krüger et al., 2023; Liu et al., 2022). They have also been confirmed to be the most prevalent integrase genes and are linked to antimicrobial resistance, especially among Enterobacterales (Otero-Olarra et al., 2020). The worldwide use of trimethoprim and aminoglycosides may be a direct cause for the persistence of these genes in food-producing animals (Krüger et al., 2023). The detection rate of mobile colistin resistance in this study was generally low, although a high prevalence of colistin resistance has been reported in Japanese retail meat (Odoi et al., 2021).

Enterobacterial and non-Enterobacterial species displayed diverse rates of resistance to different classes of antibiotics. Meropenem and amikacin demonstrated excellent activity, with 0% or approximately 1% resistance in both isolation years. However, maximal ampicillin and tetracycline resistance was observed between 2009 and 2021 (p-value=0.001 vs 0.413, respectively). Kanamycin and sulfamethoxazole/trimethoprim resistance was also high, especially in the 2021 isolates. The National Action Plan (NAP) implemented in Japan in 2016 outlined the targets for antimicrobial usage, including those applied in animal husbandry (The government of Japan, 2020). Therefore, I expected to observe decreased

changes in AMR, which was true for some resistance patterns, such as ceftazidime, cefepime, cefoxitin, aztreonam, meropenem, amikacin, and chloramphenicol (Table 9). Members of Enterobacterales have been reported to be the main cause of human infections (Abd El-Aziz et al., 2021; Uzeh et al., 2021). The data show that Enterobacterales resistance to antibiotics was more predominant in 2009 than in 2021 isolates, except for ampicillin and third-generation cefotaxime (Table 10). Therefore, my data confirm that NAPs may play a key role in the future reduction of AMR in Higashihiroshima, Japan, and worldwide. However, it is worth mentioning that we were unable to trace antibiotic usage on farms or consider the small sample size used in the current study, which may have biased the conclusions drawn in this study. The high third-generation cefotaxime resistance observed in 2021 was mainly attributed to the frequent detection of *bla*$_{\text{RHAN}}$-type β-lactamases in *Rhanella* spp. in beef and pork. A decreasing trend in cephalosporin resistance among *E. coli* strains was previously reported by NAOR (2020) and Xedzro et al. (2023) in Japan. This was attributed to voluntary restrictions on the use of third-generation cephalosporins in the poultry industry in 2012 (Hayashi et al., 2018; Hiki et al., 2015). However, the data suggest that cephalosporin resistance among other Enterobacterales may continue to increase if NAPs are not fully activated. Furthermore, our findings contradict those of the NAOR, which reported a 0% prevalence of cefotaxime resistance in cattle and pigs. Although the observed increase in resistance patterns was low and may vary depending on the year of bacterial isolation, annual surveillance data are needed to provide a clear picture as to whether AMR in the current study region is affected by NAPs.

Various plasmid incompatibility groups have been reported to play key roles in the spread of AMR among Enterobacterales including *E. coli* (Gawish et al., 2021; Norizuki et al., 2018). ESBL-producing *E. coli* strains in this study carried multiple plasmid replicon types (Table 6). IncFIB, IncI1, and IncFrepB were the most common plasmids detected in the

ESBL-producers (*bla*$_{CTX-M}$ or *bla*$_{SHV}$). This is consistent with Hayashi et al. (2018), who frequently identified FIB and I1 plasmids in *bla*$_{CTX-M}$-carrying *E. coli* strains in Japan. Despite their varied occurrence, N-and B/O-type plasmids, which are rarely reported in Japan, were found in the three *E. coli* isolates. Although we could not further investigate the plasmid carrying the ESBL gene, PCR amplification using plasmid DNA as a template was used to confirm the presence of *bla*$_{CTX-M}$ or *bla*$_{SHV}$, suggesting that one such plasmid might be responsible for carrying the ESBL genes. A previous report has indicated that CTX-M-type enzymes are typically encoded by plasmids (Nakano et al., 2023). Notably, horizontal gene transfer of mobile genetic elements is highly feasible if these plasmids are mobile.

The present study also demonstrates the public health significance associated with AmpC β-lactamase production among Enterobacterales. AmpC is a cephalosporinase-encoding gene that promotes the resistance to broad-spectrum cephalosporins (Sakai and Maesaki, 2022). However, the prevalence of AmpC in Japanese food sources remains underexplored. Several groups of AmpC β-lactamases including *bla*$_{DHA}$, *bla*$_{FOX}$, *bla*$_{MOX}$, *bla*$_{ACC}$, *bla*$_{CMY}$, *bla*$_{ADC}$, and *bla*$_{ACT}$ were detected and distributed across the various meat categories, but predominantly in chicken meat samples (Table 12 and 13). The most common type of AmpC gene frequently found in Japan is CMY, and to a lesser extent, DHA, but has mainly been reported in clinical isolates of *E. coli* and *K. pneumoniae* (Matsumura et al., 2012; Yamasaki et al., 2010). Other studies have detected CMY-2 and DHA-1 in horses and/or companion animals in Japan (Harada et al., 2016; Sukmawinata et al., 2020). The overall prevalence of AmpC production in this study (28.2%) (Tables 8 and 12) was higher than that previously reported in Japan (0.3 or 2.8%) (Sukmawinata et al., 2020; Yamasaki et al., 2010), Egypt (15.2%); (Nossair et al., 2022), Iran (16.7% or 21.7%) (Rizi et al., 2020; Robatjazi et al., 2021), and the United States (2.3%) (Moon et al., 2022). For the first time in Hiroshima Prefecture, this study reports a collection of AmpC genes in different variants of

meat origin, which must be considered as a public health risk because they can be transmitted to humans. To the best of my knowledge, this report is the first detection of DHA-13, DHA-18, FOX-5, FOX-13, ACC-4, and ACC-6 variants in Japan, suggesting further large-scale research, since these genes display clinical resistance against cephalosporins, which are the current recommended treatment options. Moreover, it is tempting to speculate that the majority of the detected AmpC variants (Table 12) in this study have not yet been identified in Japan, because these variants have not been reported. Although the AmpC-producing isolates were not tested against many antibiotics, I showed that they were capable of hydrolyzing later generations of cephalosporins, which represent a public health concern. A high incidence of cephalosporin resistance was observed in 2009 (50%), which has decreased to 10.5% in 2021 (Table 12). Interestingly, monobactam resistance was not detected in AmpC-producers in 2021, but was found in 24.0% of the 2009 isolates. The observed resistance phenomenon in the isolates may have emerged through plasmid-mediated gene transfer mechanisms between bacterial isolates. It is important to note that plasmid-mediated AmpC mobilization can facilitate the induction of multiple acquired resistances to antimicrobial agents (Sakai and Maesaki, 2022). To ensure efficient treatment of infections that might arise due to AmpC-producers, a large-scale survey is urgently required to assess their occurrence and choose antimicrobial agents that are appropriate or effective against them.

6. Conclusion

This study investigated the genetic determinants of AMR in Enterobacterales and non-Enterobacterales isolates recovered from retail meat samples. I also characterized the phenotypic AMR profiles and presented evidence of resistance to the tested antibiotics. The identification of different plasmid replicon types in ESBL-producing *E. coli* strains represents

a public health concern, as they can act as transmission vectors for AMR genes. Furthermore, molecular profiling of plasmid-mediated AmpC β-lactamases was confirmed for the first time in the Hiroshima Prefecture. A large-scale and yearly investigation of resistance genes in the study region or in Japan as a whole would help understand the impact of drug-resistant microbial contamination in food-producing animals and track the transmission of emerging or newly evolved resistance genes circulating in the food production continuum.

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Chapter 4

Predominance of multidrug-resistant Gram-negative bacteria isolated from supermarket retail seafood in Japan

1. Summary

Reports have documented antimicrobial usage in aquaculture, and the aquatic ecosystem can be considered a genetic storage site for antibiotic-resistant bacteria. This study assessed the prevalence of antimicrobial resistance among Gram-negative bacteria recovered from retail seafood in Hiroshima, Japan. A total of 412 bacteria were isolated and screened for the presence of β-lactamases, acquired carbapenemases, and mobile colistin-resistance (*mcr*) genes. Forty-five isolates were dominated by *Morganella* (28%), *Proteus* (22%), *Aeromonas* (14%), *Citrobacter* (8%), and *Escherichia* (8%) and carried antimicrobial resistance (AMR) genes. The identified AMR genes included integrons (19), *aac(6′)-Ib* (11), $bla_{\text{TEM-1}}$ (7), $bla_{\text{CTX-M-like}}$ (12), $bla_{\text{CTX-M-65}}$ (2), $bla_{\text{SHV-12}}$ (1), $bla_{\text{SHV-27}}$ (1), $bla_{\text{OXA-10}}$ (1), $bla_{\text{OXA-2}}$ (1), and *mcr* (2). The most common clinical resistances were against ampicillin, colistin, sulfamethoxazole/trimethoprim, tetracycline, and ciprofloxacin. Multidrug resistance (MDR) occurred in 27 (60%) isolates, and multiple antibiotic resistance indices ranged from 0.2–0.8. A conjugation experiment showed that 10 of the 11 selected MDR strains harbored conjugable plasmids, although PCR-based replicon typing described seven strains as untypable. IncF replicon was identified in MDR extended-spectrum β-lactamase-producing *Escherichia coli* of pathogenic B2 phylogroup. The findings suggest that retail seafood harbors MDR bacteria of human interest that require strict resistance surveillance in the seafood production continuum.

Key words: antimicrobial resistance, retail seafood, plasmid, surveillance, Japan

2. Introduction

Seafood has great nutritional benefits and economic importance and serves as a rich source of animal proteins, vitamins, minerals, and essential fatty acids, which make seafood essential to fight malnutrition, especially in low- and middle-income countries (Issifu et al., 2022; Thilsted et al., 2016). Consequently, seafood consumption has increased over the years with most seafood products originating from aquaculture-based systems (Issifu et al., 2022). Aquaculture remains an important food sector and is recognized as a pillar of economic growth (Silvester et al., 2022). For instance, the total aquaculture production reached 122.6 million tons in 2020 and provided approximately 58.5 million jobs worldwide (FAOSTAT, 2022).

Globally, aquaculture production has been associated with negative impacts such as the emergence of fish diseases and loss of aquatic life, compelling farmers to apply antibiotics in intensive culture systems (Nadella et al., 2021). The increasing demand for seafood products has driven farmers to use antimicrobials as growth enhancers. Several antimicrobials including tetracyclines, quinolones, and penicillin are critical in human medicine and have been approved by the World Health Organization for use in aquaculture (Marijani, 2022). In Japan, antibiotics such as tetracyclines, sulfonamides, macrolides, quinolones, and penicillin are used in seawater aquaculture (Ido, 2023). These antimicrobials have significant applications in the Asian aquaculture industry for the continuous production of seafood products (Shakerian et al., 2017). Excessive use of antibiotics and other chemotherapeutics in aquaculture has resulted in the emergence and dissemination of antimicrobial resistance across diverse bacterial species in aquatic environments (Nadella et al., 2021; Stratev et al., 2023). Compared to antimicrobial applications in terrestrial animal production, the use of antimicrobials in aquaculture seems to provide a wider exposure pathway for drug distribution within water, which facilitates the accumulation of

antimicrobial residues with potential ecosystem health implications.

Furthermore, aquaculture environments that use antimicrobials may serve as genetic reservoirs for antimicrobial resistance, providing direct pathways for human exposure to antimicrobial-resistant bacteria through the consumption of contaminated seafood (Schar et al., 2020; Tate et al., 2022). In addition, there is indirect or horizontal gene transfer of mobile genetic elements (including plasmids, transposable elements, and superintegrons) from aquatic bacteria to human-related pathogens (Mancini et al., 2023; Shakerian et al., 2017). Antimicrobial resistance (AMR) among seafood-borne bacteria can potentially decrease the effectiveness of antimicrobial therapy in humans and lead to human death, financial burden, and a major reduction in aquaculture production. Estimates suggest that approximately 10 million lives and USD 100 trillion will be lost by 2050 because of AMR if existing control measures to prevent the spread of AMR are compromised (Agyarkwa et al., 2022; Xedzro et al., 2023).

A variety of fresh fish and seafood products are served raw in the Japanese cuisine. For instance, sashimi, thinly sliced seafood prepared from raw fish, is commonly used in Japanese cuisine (Hammad et al., 2014). Although processed under special and strict hygienic conditions, the possibility of human exposure to critical-priority bacteria exists. Considering that the use of antimicrobials is a common feature in the Japanese aquaculture industry (Matsuura et al., 2019), seafood surveillance for antimicrobial resistance is crucial for monitoring the occurrence and status of resistance traits in seafood.

In 2016, Japan implemented a National Action Plan (NAP) for AMR by outlining specific targets for antimicrobial usage, including those applied in aquaculture (The government of Japan, 2020). However, the prevalence of AMR in seafood following NAP implementation remains unknown. In addition, reportedly, there are no updated data on seafood surveillance for the future monitoring of AMR trends. Following the principles

outlined in the NAP, strengthening the continuous approach for collecting and reporting AMR data is necessary. Such data evaluated at the local, regional, and national levels play a pivotal role in policymaking and justify the effectiveness of the AMR policy. As antimicrobial resistance surveillance is an important aspect of implementing effective control programs, the current study primarily assessed the prevalence of antimicrobial-resistant foodborne bacteria in retail seafood and examined the conjugal transfer mechanisms that might play a key role in the dissemination of AMR genes.

3. Materials and Methods

3.1 Sample collection and pre-processing

Fifty seafood samples were collected from 21 retail supermarkets in Hiroshima, Japan, between December 2022 and August 2023. Samples included fish (n = 15), shrimp (n = 13), oysters (n = 4), seaweed (n = 1), squid (n = 16), and clams (n = 1). All samples were transported in thermally insulated bags (at 4 °C) to the laboratory and processed within 24 h of collection. Equal portions from the head, abdominal, and tail regions of fish were pooled and mixed before taking the required portion for analysis. The clam samples were broken using a sterile hammer and scalpel, and meat was collected from two to three specimens.

3.2 Microbiological analyses and bacterial isolation

Bacterial isolation was performed as previously described (Xedzro et al., 2023). Briefly, a 25 g portion of each sample was weighed into a sterile stomacher bag and homogenized in 225 mL buffered peptone water (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan) containing 2% NaCl. The resulting homogenate was incubated at 37 °C for 6–8 h and plated on MacConkey agar containing ampicillin (100 µg/mL), meropenem (2 µg/mL),

streptomycin (50 µg/mL), or colistin (2 µg/mL). Further, a 1000 µL of each suspension was transferred into 9 mL Luria-Bertani (LB) broth (Nacalai Tesque, Inc., Kyoto, Japan) with or without ampicillin and streptomycin. To detect β-lactamase producers, ampicillin broth cultures were plated on MacConkey agar containing ampicillin (100 µg/mL) or meropenem (2 µg/mL). In case of selecting aminoglycoside-resistant strains, streptomycin broth cultures were plated on MacConkey agar containing streptomycin (50 µg/mL). Colistin in broth culture was not performed, instead antibiotic free LB cultures were plated on MacConkey agar containing colistin (2 µg/mL) and incubated under experimental conditions. Subsequently, 3–5 morphological distinct colonies were selected and re-cultured on individual antibiotic-containing plates. Finally, 412 Gram-negative bacterial isolates were recovered and purified on LB agar without antibiotics.

3.3 Molecular screening to detect AMR determinants

Total genomic DNA was extracted from the boiled lysates. Molecular screening of the 412 isolates was performed to identify extended-spectrum β-lactamase (ESBL) -encoding genes (*bla*$_{SHV}$ and *bla*$_{CTX-M}$), other β-lactamases (*bla*$_{TEM}$, *bla*$_{OXA-1}$, *bla*$_{OXA-2}$, *bla*$_{OXA-5}$, *bla*$_{OXA-9}$, AmpC), integrons (classes 1, 2, 3), and aminoglycoside acetyltransferase gene, *aac(6′)-Ib* as previously described (Soliman et al., 2023; Xedzro et al., 2023). PCR detection of mobile colistin-resistance genes (*mcr-1–10*) (Borowiak et al., 2020; Jousset et al., 2019; Wang et al., 2020) and acquired carbapenemases (KPC, NDM, OXA-48, VIM, and IMP) (Poirel et al., 2011) was performed. The variable regions of classes 1 and 2 integrons within the integron positive isolates were amplified using primers complementary to the 5′ and 3′ conserved segments of class 1 integrons (5′ CS and 3′ CS) and class 2 integrons (hep74 and hep51) (Table S1). PCR amplification conditions were as follows: initial denaturation at 98 °C for 1 min, 30 cycles of denaturation at 98 °C for 10 s, annealing at 50 °C for 15 s, extension at

68 °C for 2 min, and final extension at 68 °C for 15 s (Xedzro et al., 2023). PCR products were purified using ExoSAP-IT (Thermo Fisher Scientific) and processed for Sanger sequencing using Eurofins Genomics, Japan. Bacteria were identified by amplifying the full-length (1,500 bp) 16S rRNA fragment using 27F (AGAGTTTGATCMTGGCTCAG) and 1492R (CGGYTACCTTGTTACGACTT) primer sets.

3.4 Phenotypic antimicrobial susceptibility testing

The susceptibility to 15 antimicrobial agents (from 12 different classes) was investigated using the Kirby–Bauer disk diffusion assay method in compliance with the Clinical and Laboratory Standard Institute (CLSI, 2020) (Hiki et al., 2015). The antibiotic panel included ampicillin (10 µg), cefotaxime (30 µg), ceftazidime (30 µg), cefepime (30 µg), cefoxitin (30 µg), aztreonam (30 µg), meropenem (10 µg), amikacin (30 µg), kanamycin (30 µg), tetracycline (30 µg), chloramphenicol (30 µg), ciprofloxacin (5 µg), sulfamethoxazole-trimethoprim (23.75/1.25 µg), and fosfomycin (50 µg). Bacterial cultures (single colonies) from each isolate were suspended in sterile normal saline (0.85% NaCl, w/v) and the optical density was adjusted to 0.5 MacFarland standard. The resulting suspension was surface-spread on Muller–Hinton agar using a sterile cotton swab. Each antibiotic-treated disk was carefully placed on an inoculated agar plate. The plates were incubated at 37 °C for 16–18 h and the results were interpreted based on the interpretative chart supplied with the antimicrobial agent. The minimum inhibitory concentration (MIC) of colistin was determined using the broth microdilution method in cation-adjusted Mueller-Hinton broth, in accordance with the 2021 recommendations of the European Committee on Antimicrobial Susceptibility Testing (EUCAST, 2021). *E. coli* ATCC 25922 was used as the quality control strain. Isolates displaying intermediate resistance were considered resistant to antimicrobial agents. Multidrug resistance is defined as the resistance to three or more antimicrobial classes

(Magiorakos et al., 2012). The multiple-antibiotic resistance (MAR) index of the isolates was established as previously described (Titilawo et al., 2015a). MAR index is defined as the ratio of *a* and *b*, where "*a"* is the number of antibiotics to which an isolate was resistant, whereas "*b"* is the number of antibiotics to which the same isolate was exposed. An MAR index higher than 0.2 means that the tested isolates originated from a high-risk contamination source where antibiotics are frequently used.

3.5 Detection of *E. coli* phylogenetic group

Quadruplex PCR was performed to detect *E. coli* phylogroups as previously described (Clermont et al., 2013). PCR amplification targets and techniques for assigning phylogroups were as follows: *arpA*|*chuA*|*yjaA*|TspE4.C2. *E. coli* ATCC 25922 was used as a quality control strain for phylogroup B2, and nuclease-free water served as a negative control.

3.6 Conjugation assay

A conjugation experiment was conducted using a filter-mating conjugation assay as previously described (Sadek et al., 2021). Exponential-phase lysogeny broth cultures of donor bacteria (11 strains selected based on their genotype and phenotype) and azide-resistant *E. coli* J53 recipient strains were used. Both the donor and recipient strains were mixed in a ratio of 1:9 (100 µL donor:900 µL recipient) and centrifuged for 3 min at 6,000 rpm. The supernatant was removed, and the pellets were resuspended in 200 µL LB broth. The resulting suspension was then plated on a conjugation filter (0.22 µm pore size) on LB agar and incubated for 3–5 h at 37 °C. The filter was removed and placed in 3 mL fresh LB medium and incubated for 1 h at 37 °C. Transconjugants were selected on LB agar containing 100 µg/mL sodium azide and 100 µg/mL ampicillin. Transconjugants were confirmed using PCR targeting the resistance genes and antimicrobial susceptibility testing (ampicillin alone).

3.7 Plasmid isolation and PCR-based replicon typing (PBRT)

Plasmids were isolated from the 11 selected strains as well as the *E. coli* J53 transconjugants. The alkaline lysis method was used for plasmid preparation as described previously (Green and Sambrook, 2016) with slight modifications. Briefly, alkaline lysis (lysis buffers I, II, and III) was performed on overnight LB broth cultures for 3–5 min. Supernatants were obtained after centrifugation and mixed with an equal volume of isopropanol. The precipitated nucleic acids were collected and dissolved in 1 mL 70% ethanol. Plasmid DNA was recovered as pellets and finally dissolved in 50 µL of Tris-EDTA (pH 8.0) buffer containing 20 µg/mL DNase-free RNase A. PBRT was then performed to identify the plasmid incompatibility (Inc/rep) groups using 18 primer sets targeting HI1, HI2, I1, X, L/M, N, FIA, FIB, W, Y, P, FIC, A/C, T, FIIs, FrepB, K/B, and B/O, as previously described (Carattoli et al., 2005). The assay was conducted using five multiplex and three simplex PCR assays.

3.8 Blast search and statistical analyses

All sequenced data were subjected to a similarity search using the Basic Local Alignment Search Tool (BLAST) available in the National Center for Biotechnology Information (NCBI) database. Phenotypic resistance data were recorded in Microsoft Excel 365, and the results are presented as percentages. Statistical analyses were performed using IBM SPSS version 29.0 (SPSS Corp., Armonk, NY, USA). Pearson's chi-square or Fisher's exact tests were used to analyze significant differences in the prevalence of AMR determinants among samples or antibiotic resistance among different bacterial species. The results were considered not statistically significant when p-values were >0.05.

4. Results

4.1 Isolation and identification of bacterial species

Among the 50 samples analyzed, 22 (44.0%) were contaminated with gram-negative bacteria carrying antimicrobial resistance determinants. The contaminated samples included 8/15 (53.3%) fish, 6/13 (46.2%) shrimp, 7/16 (43.8%) oysters, 1/4 (25.0%) oysters, and 0/1 (0.0%) seaweed and clams (Figure 9).

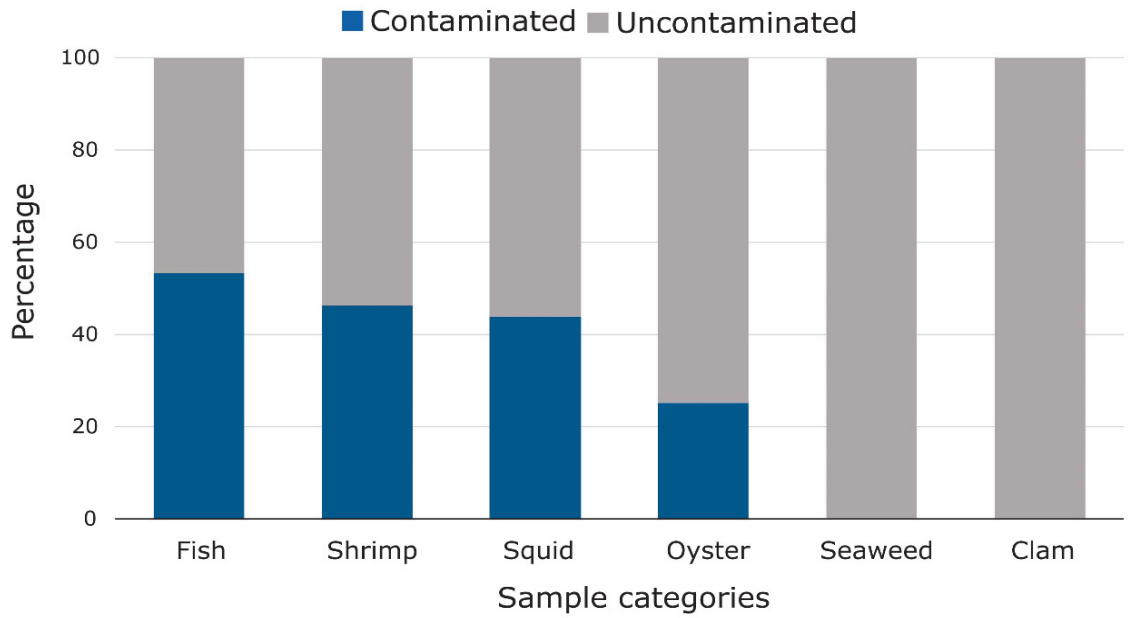

Fig 9. Bar graph showing the percentage of seafood samples contaminated in each sample category

A total of 45 non duplicate Gram-negative bacteria carrying AMR genes were recovered from the contaminated seafood products. The most prevalent bacterial species isolated were *Morganella morganii* (28.0%), *Proteus* spp. (22.0%), *Aeromonas* spp. (14.0%), *Citrobacter* spp. (8.0%), and *E. coli* (8.0%) (Table 14). Other identified species were *Enterobacter* spp. (6.0%), *Klebsiella pneumoniae* (2.0%), and *Pseudomonas putida* (2.0%). Fish and squid samples were predominantly contaminated with *Morganella*, *Proteus*, and *Aeromonas*, although none of the squid samples tested positive for *Aeromonas* spp. (Table 14).

Four *E. coli* strains belonging to the pathogenic B2, commensal A, and B1 strains were isolated from a single sample of fish, shrimp, oysters, or squid. The results show that seafood is a potential carrier of diverse bacteria carrying AMR genes, which is a risk factor for consumer safety.

Table 14. Occurrence of different Gram-negative bacteria isolated from retail seafood in Hiroshima

Bacterial species	Prevalence (%)						
	Fish (n=15)	Shrimp (n=13)	Oyster (n=4)	Seaweed (n=1)	Squid (n=16)	Clam (n=1)	Total (N=50)
Morganella morganii	5 (33.3)	3 (23.1)	0 (0.0)	0 (0.0)	6 (37.5)	0 (0.0)	14 (28.0)
Proteus spp.	4 (26.7)	2 (15.4)	0 (0.0)	0 (0.0)	5 (31.3)	0 (0.0)	11 (22.0)
Aeromonas spp.	4 (26.7)	3 (23.1)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	7 (14.0)
Citrobacter spp.	1 (6.7)	2 (15.4)	0 (0.0)	0 (0.0)	1 (6.3)	0 (0.0)	4 (8.0)
Escherichia coli	1 (6.7)	1 (7.7)	1 (25.0)	0 (0.0)	1 (6.3)	0 (0.0)	4 (8.0)
Enterobacter spp.	1 (6.7)	0 (0.0)	0 (0.0)	0 (0.0)	2 (12.5)	0 (0.0)	3 (6.0)
Klebsiella spp.	0 (0.0)	1 (7.7)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	1 (2.0)
Pseudomonas spp.	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	1 (6.3)	0 (0.0)	1 (2.0)

N: total number of samples, n= number of samples for each seafood category

4.2 Prevalence of β-lactamase-encoding genes and other AMR determinants from seafood samples

Of the 45 isolates identified as carrying AMR genes, 31.1 and 15.6% carried the *bla*$_{\text{CTX-M}}$ and *bla*$_{\text{TEM}}$ resistance genotypes, respectively (Table 15). These genes were predominantly found in squid isolates. The *bla*$_{\text{CTX-M}}$ type was identified in 25.0% and 33.3% of the fish and shrimp isolates, respectively ($p > 0.05$). Only two isolates carrying *bla*$_{\text{SHV}}$ or *bla*$_{\text{OXA}}$ were found in fish, shrimp, or squid. The most prevalent AMR genes detected in this study were integrons (42.2%). They also confer resistance to trimethoprim, quaternary ammonium compounds, aminoglycosides, and chloramphenicol. The aminoglycoside acetyl transferase gene, *aac(6′)-Ib*, which also confers resistance to aminoglycosides was prevalent in 11 (24.4%) of the total isolates recovered from seafood. Although none of the isolates tested positive for carbapenemase-encoding genes, we found two (4.4%) that harbored mobile colistin resistance (*mcr*) gene (Table 15). Generally, no significant differences ($p > 0.05$) were observed in the occurrence rates of AMR determinants among the contaminated samples of different categories.

4.3 Antibiogram profiles of isolated Gram-negative bacteria

The isolates (n=45) were tested against 15 antimicrobial agents as illustrated in Figure 10. All isolates were resistant to at least one antimicrobial agent. Overall, the highest resistance rate was observed for ampicillin (93.3%), followed by colistin (62.2%), sulfamethoxazole/trimethoprim (48.9%), tetracycline (40.0%), ciprofloxacin (35.6%), and cefoxitin (33.3%) (Figure 10). Meropenem resistance was observed in three (6.7%) isolates, while seven (15.6%) isolates also conferred resistance to cephalosporins. The percentage resistance of the most abundant species is listed in Table 16. Ampicillin resistance (100%) was observed in all isolates except *Proteus* spp. (72.7%), although the differences were not

statistically significant ($p > 0.05$). Cephalosporin (third and fourth generation)-resistant phenotypes were found in *M. morganii* 1/14 (7.1%), *P. mirabilis* 1/11 (9.1), *E. coli* 2/4 (50.0%), and *E. cloacae* 3/3 (100%) isolates. Notably, some of these isolates do not carry extended-spectrum β-lactamase-encoding genes responsible for hydrolyzing this drug class. Twenty-five isolates of *Morganella* and *Proteus*, which are known to have some intrinsic polymyxin resistance (Olaitan et al., 2014), were all resistant to colistin [minimum inhibitory concentration (MIC); 8 µg/mL to >128 µg/mL]. Colistin resistance was also identified in one strain of C. freundii and in 3/6 (42.9%) of the *Aeromonas* spp. It is worth mentioning that the *mcr-3.2* and *mcr-10.1* identified in *A. hydrophila* and *E. cloacae*, respectively, were susceptible to colistin (MIC; 1 µg/mL).

4.4 Multiple antibiotic resistance (MAR) indices among MDR isolates

Twenty-seven of the 45 (60.0%) strains exhibited an MDR phenotype (Table 17). MDR is defined as non-susceptibility to three or more antimicrobial classes (Magiorakos et al., 2012). MAR index ranged from 0.20–0.8 with the highest MAR index originating from *mcr-10.1*-carrying E. cloacae (Table 17). Of the 27 isolates that showed MDR phenotypes, 12 (44.4%) had MAR index of 0.2–0.27, 11 (40.7%) showed MAR index of 0.33–0.47, and the remaining isolates had MAR index of 0.53–0.8, suggesting that the isolates originate from a high-risk contaminated source.

Table 15. Distribution of AMR genes among retail seafood samples

Sample type	No. of isolates	*β-lactamase types (%)				*Others (%)			
		bla$_{SHV}$	*bla*$_{CTX-M}$	*bla*$_{TEM}$	*bla*$_{OXA}$	*aac (6′)-Ib*	Integrons	*mcr*	Carbapenemase
Fish	16	1 (6.3)	4 (25.0)	1 (6.3)	1 (6.3)	5 (31.3)	7 (43.8)	1 (6.3)	0 (0.0)
Shrimp	12	1 (8.3)	4 (33.3)	2 (16.7)	0 (0.0)	1 (8.3)	4 (33.3)	1 (8.3)	0 (0.0)
Oyster	1	0 (0.0)	1 (100)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Seaweed	0	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Squid	16	0 (0.0)	5 (31.3)	4 (25.0)	1 (6.3)	5 (31.3)	8 (50.0)	0 (0.0)	0 (0.0)
Clam	0	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Total (%)	45	2 (4.4)	14 (31.1)	7 (15.6)	2 (4.4)	11 (24.4)	19 (42.2)	2 (4.4)	0 (0.0)

*Indicates no significant difference (p>0.05) in the occurrence of AMR genes among samples.

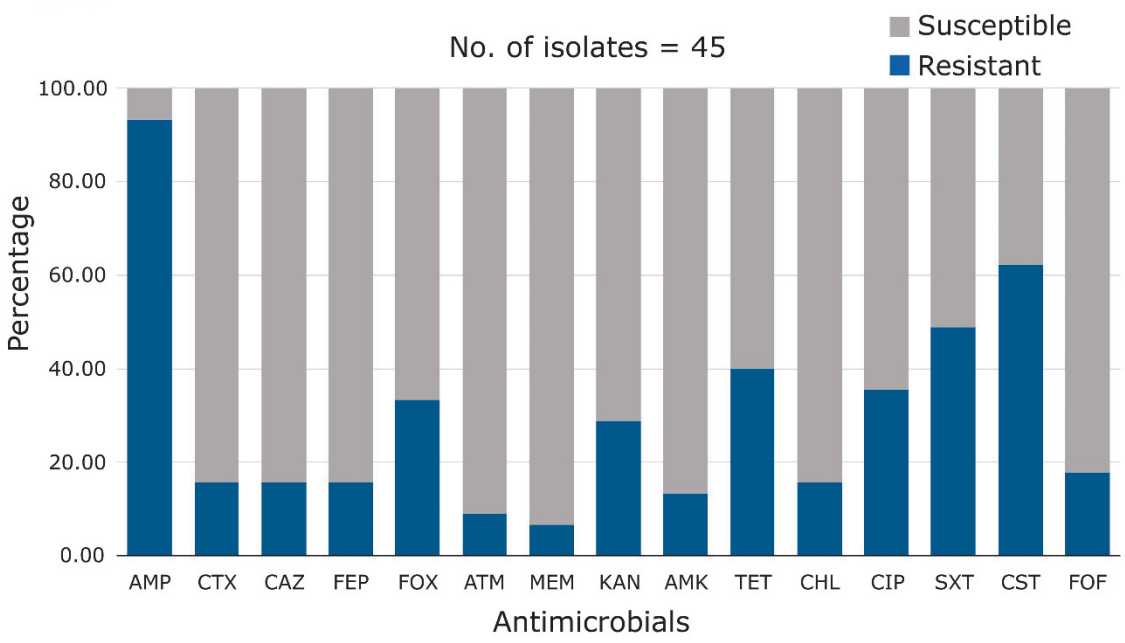

Fig 10. Overall antimicrobial resistance profiles of Gram-negative bacteria isolated from retail seafood. AMP: ampicillin, CTX: cefotaxime, CAZ: ceftazidime, FEP: cefepime, FOX: cefoxitin, ATM: aztreonam, MEM: meropenem, KAN: kanamycin, AMK: amikacin, TET: tetracycline, CHL: chloramphenicol, CIP: ciprofloxacin, SXT: sulfamethoxazole/trimethoprim, CST: colistin, FOF: fosfomycin

4.5 *E. coli* phylogroup, plasmid transferability, and PBRT

The four *E. coli* strains identified in our study were assigned to phylogroups based on the Clermont classification (Clermont et al., 2013) . Two strains carrying $bla_{\text{CTX-M-65}}$ were assigned to the pathogenic B2 phylogroup, whereas the other two strains carrying $bla_{\text{TEM-1}}$ belonged to avirulent A or B1 phylogroups. To assess the involvement of some isolates in the spread of antimicrobial resistance, 11 ampicillin-resistant strains exhibiting MDR phenotypes were selected and used as donors in the conjugation experiments. Ampicillin-resistant *mcr-3*-carrying *A. hydrophila* was also included, although conjugation was unsuccessful—possibly because *mcr-3* is located on the chromosome. All other isolates successfully transferred their resistance plasmids and ampicillin resistance traits to *E. coli* J53 (conjugation efficiency: 2.8×10^{-7} to 8.6×10^{-6}). Two or five transconjugants obtained from the two mating pairs were

analyzed for resistance gene acquisition. All the selected transconjugants carried the resistance determinants identified in the original strains. Although the strains carried conjugable plasmids, PBRT revealed that most of them carried untypable plasmids. Nonetheless, we found that three *E. coli* isolates harbored the IncFIA, IncFIB, IncFIC, or IncFrepB plasmid replicon types. The same plasmids were identified in *E. coli* J53 transconjugants (Table 18).

Table 16. Percent resistance of the most abundant Gram-negative bacteria against various antimicrobials

Species	No. of isolates	*AMP	CTX	CAZ	FEP	FOX	*ATM	*MEM	KAN	AMK	*TET	*CHL	CIP	*SXT	CST	FOF
Morganella morganii	14	100	7.1	7.1	7.1	21.4	7.1	0.0	21.4	0.0	42.9	14.3	21.4	50.0	100	35.7
Proteus spp.	11	72.7	9.1	9.1	9.1	0.0	9.1	9.1	18.2	9.1	45.5	18.2	36.4	36.4	100	0.0
Aeromonas spp.	7	100	0.0	0.0	0.0	57.1	0.0	0.0	14.3	14.3	14.3	0.0	0.0	28.6	42.9	0.0
Citrobacter spp.	4	100	0.0	0.0	0.0	100	0.0	0.0	25.0	0.0	50.0	25.0	50.0	100	25.0	0.0
Escherichia coli	4	100	50.0	50.0	50.0	0.0	25.0	0.0	75.0	50.0	50.0	25.0	75.0	25.0	0.0	0.0
Enterobacter spp.	3	100	100	100	100	100	33.3	0.0	100	66.7	33.3	0.0	100	100	0.0	0.0

*Indicates no significant difference (p>0.05) in antibiotic resistance among bacterial species. AMP: ampicillin, CTX: cefotaxime, CAZ: ceftazidime, FEP: cefepime, FOX: cefoxitin, ATM: aztreonam, MEM: meropenem, KAN: kanamycin, AMK: amikacin, TET: tetracycline, CHL: chloramphenicol, CIP: ciprofloxacin, SXT: sulfamethoxazole/trimethoprim, CST: colistin, FOF: fosfomycin

Table 17. Multidrug resistance phenotypes and multiple antibiotic resistance (MAR) indices of Gram-negative bacteria isolated from retail seafood

Isolate	Sample	Identification	Genotypes	MDR profiles	MAR Index
D2-C042	Squid	*Proteus mirabilis*	$bla_{\text{CTX-M-9}}$, $bla_{\text{OXA-2}}$	CTX, CAZ, FEP, ATM, KAN, AMK, CIP, SXT, CST	0.60
B2-S021	Shrimp	*Klebsiella pneumoniae*	$bla_{\text{SHV-27}}$	AMP, MEM, CIP, FOF	0.27
B2-S020	Shrimp	*Citrobacter freundii*	$bla_{\text{TEM-1B}}$, *dfrA14*	AMP, FOX, CIP, SXT	0.27
D2-S025	Squid	*Proteus vulgaris*	*dfrA1-catB2-aadA1*	AMP, MEM, TET, CIP, SXT, CST	0.40
A2-A044	Oyster	*Escherichia coli*	$bla_{\text{CTX-M-65}}$	AMP, CTX, CAZ, FEP, ATM, KAN, CIP	0.47
A3-A049	Fish	*Proteus mirabilis*	$bla_{\text{CTX-M-like}}$	AMP, TET, CST	0.20
A3-A053	Fish	*Escherichia coli*	$bla_{\text{CTX-M-65}}$	AMP, CTX, CAZ, FEP, KAN, AMK, CIP	0.47
C3-A056	Shrimp	*Escherichia coli*	$bla_{\text{TEM-1}}$	AMP, TET, CHL, CIP, SXT	0.33
B3-A054	Squid	*Morganella morganii*	Class 1 integron	AMP, FOX, CST, FOF	0.27
B3-M090	Squid	*Pseudomonas putida*	Class 1 integron	AMP, MEM, CHL, SXT, FOF	0.33
D6-A190	Shrimp	*Aeromonas hydrophila*	$bla_{\text{CTX-M-like}}$	AMP, FOX, CST	0.20
C6-S206	Fish	*Citrobacter freundii*	$bla_{\text{OXA-10}}$, *aadA1-*$bla_{\text{CARB-2}}$	AMP, FOX TET, CHL, CIP, SXT, CST	0.47
C6-S212	Fish	*Morganella morganii*	*aac (6')-Ib3, aadA-aadB,* bla_{DHA}	AMP, FOX, TET, CIP, SXT, CST	0.40
C6-A180	Fish	*Proteus cibarius*	$bla_{\text{CARB-2}}$	AMP, TET, CHL, CIP, SXT, CST	0.40
B7-S201	Fish	*Aeromonas allosaccharophila*	*aadA2*, bla_{FOX}	AMP, FOX, SXT	0.20
B7-S203	Fish	*Aeromonas sobria*	*aadA1-catB8*	AMP, FOX, SXT, CST	0.27
A9-A238	Squid	*Enterobacter cloacae*	$bla_{\text{TEM-1}}$, *dfrA17-aadA5*	AMP, CTX, CAZ, FEP, FOX, KAN, AMK, CIP, SXT	0.60
B14-A346	Squid	*Citrobacter braakii*	$bla_{\text{TEM-1}}$	AMP, FOX, KAN, TET, SXT	0.33
B12-S377	Fish	*Enterobacter cloacae*	$bla_{\text{TEM-1}}$, $bla_{\text{SHV-12}}$, *mcr-10.1*	AMP, CTX, CAZ, FEP, FOX, ATM, KAN, AMK, TET, CIP, SXT, FOF	0.80
D7-C395	Squid	*Escherichia coli*	$bla_{\text{TEM-1}}$	AMP, KAN, AMK, TET	0.27
B14-C405	Squid	*Proteus alimentorum*	$bla_{\text{CTX-M-like}}$	AMP, KAN, CST	0.20
B10-C300	Squid	*Morganella morganii*	*aacA4*	AMP, CIP, CST	0.20
A8-C303	Fish	*Morganella morganii*	*aacA4*	AMP, CST, FOF	0.20
A10-C310	Fish	*Morganella morganii*	*aacA4*	AMP, CST, FOF	0.20
C10-C315	Squid	*Morganella morganii*	*aacA4*	AMP, CTX, CAZ, FEP, FOX, ATM, SXT, CST	0.53
B14-S360	Squid	*Morganella morganii*	*aacA4*, *dfrA12-aadA2*	AMP, KAN, TET, CIP, SXT CST	0.40
B13-M388	Shrimp	*Morganella morganii*	*aadA1-aadB-1a-cmlA6*	AMP, TET, CHL, SXT, CST, FOF	0.40

Table 18. Results of conjugation and plasmid replicon typing of selected MDR bacteria isolated from retail seafood

Isolate	Species	Resistance genes	Replicon type	Plasmid transfer	Replicon type (TC)	Resistance genes (TC)*	Ampicillin resistance (TC)
A2-A044	*E. coli*	*bla*$_{\text{CTX-M-65}}$	IncFIA, IncFIB	Transferred	IncFIA	*bla*$_{\text{CTX-M}}$	Resistant
A3-A053	*E. coli*	*bla*$_{\text{CTX-M-65}}$	IncFIB, IncFIC, IncFrepB	Transferred	IncFIB, IncFIC, IncFrepB	*bla*$_{\text{CTX-M}}$	Resistant
C3-A056	*E. coli*	*bla*$_{\text{TEM-1}}$	IncFIA, IncFIB	Transferred	IncFIA, IncFIB	*bla*$_{\text{TEM}}$	Resistant
C6206	*C. freundii*	*bla*$_{\text{OXA-10}}$, *aadA1-bla*$_{\text{CARB-2}}$	Untypeable	Transferred	Untypeable	Class 1 integron, *bla*$_{\text{OXA-5}}$	Resistant
D6-A192	*A. hydrophila*	*mcr-3.2*	ND	Not transferred	ND	ND	Resistant
C6-A180	*P. cibarius*	*bla*$_{\text{CARB-2}}$	Untypeable	Transferred	Untypeable	Class 1 integron	Resistant
B13-M388	*M. morganii*	*aadA1-aadB-1a-cmlA6*	Untypeable	Transferred	Untypeable	ND	Resistant
C6-S212	*M. morganii*	*aac (6')-Ib3*, *aadA-aadB*, *bla*$_{\text{DHA}}$	Untypeable	Transferred	Untypeable	*aac(6')-Ib*	Resistant
B12-S377	*E. cloacae*	*bla*$_{\text{TEM-1}}$, *bla*$_{\text{SHV-12}}$, *mcr-10.1*	Untypeable	Transferred	Untypeable	*bla*$_{\text{CTX-M}}$, *bla*$_{\text{SHV}}$	Resistant
C10-C315	*M. morganii*	*aacA4*	Untypeable	Transferred	Untypeable	*aac(6')-Ib*	Resistant
A9-A238	*E. cloacae*	*bla*$_{\text{TEM-1}}$, *aadA5-dfrA17*	Untypeable	Transferred	Untypeable	*bla*$_{\text{TEM}}$	Resistant

TC: Transconjugant.

ND: Not determined.

*Resistance genes in the transconjugants were not sequenced for the specific alleles.

Note: *bla*$_{\text{CARB-2}}$ is a β-lactamase gene which was part of integron cassette

5. Discussions

Thus, the food chain may be a direct source of antimicrobial-resistant bacteria that affect humans. Various bacterial pathogens have been isolated from seafood (Abdulhakeem et al., 2023; Castello et al., 2022), ready-to-eat (RTE) raw seafood (Hammad et al., 2014, 2012; Harada et al., 2018), and seafood processing water (Agyarkwa et al., 2022). In Japan, the Ministry of Health, Labor, and Welfare has issued and strengthened hygiene management criteria, including proper refrigeration storage temperature (10 °C or lower) and disinfection procedures for proper sanitary processing of seafood or RTE raw seafood and pickled vegetables (Harada et al., 2018; Taguchi et al., 2017). However, to the best of my knowledge, data on the incidence of antimicrobial-resistant pathogens in seafood and the potential health risks that might arise from foodborne pathogens are scarce. Therefore, studying the antimicrobial resistance of bacteria isolated from seafood is essential.

In the present study, 45 bacterial species isolated from various seafood products were characterized along with their resistance attributes. More than 40% of the seafood products were contaminated with different resistant bacteria belonging to eight genera: *Morganella*, *Proteus*, *Aeromonas*, *Citrobacter*, *Escherichia*, *Enterobacter*, *Klebsiella*, and *Pseudomonas*. These bacterial species have been frequently identified in fish, shrimp, and squid samples and have also been detected in seafood samples in other studies in Japan and France (Delannoy et al., 2022; Harada et al., 2018). Additionally, several other studies have reported the presence of *Aeromonas*, *Proteus*, *Enterobacter*, *E. coli*, *Vibrio*, *Yersinia*, and *Acinetobacter* in the internal organs and muscles of fish (Novoslavskij et al., 2016; Raharjo et al., 2023). Given that these seafood products are widely consumed in Japan, it would certainly be of high priority to continuously examine them in surveillance programs. Furthermore, the presence of coliform bacteria such as *E. coli*, *Klebsiella*, *Citrobacter*, and *Enterobacter* is an indication of fecal or environmental contamination, although some coliforms can also be found in aquatic

environments (Chitanand et al., 2010). The contamination rates of samples differ depending on the geographical location, seasonal factors, method of investigation, and sample size. In view of our small sample size, we are unable to comment on this further, although the contamination rate (44%) found in the current study was consistent with or lower than that reported in previous studies (45–96.7%) (Hammad et al., 2014; Harada et al., 2018; Marijani 2022). Periodic surveillance with small sample sizes may be effective for AMR surveillance because it provides fast and frequent data for monitoring AMR trends.

PCR and sequence analyses revealed the presence of various AMR genes in the recovered isolates. The commonly encountered AMR genes included class 1 integrons (*aadA1*, *aadA2*, *aadA5*, *aadB-1a*, *dfrA14*, *dfrA12,* or *dfrA17*) (42.2%), CTX-M ($bla_{\text{CTX-M-65}}$, $bla_{\text{CTX-M-9}}$ or $bla_{\text{CTX-M-like}}$) (31.1%), *aac(6′)-Ib* genes (*aacA4* or *aac(6′)-Ib3*) (24.4%) as well as TEM genes ($bla_{\text{TEM-1}}$) (15.6%). The detection of these genes was not surprising, as they have been reported in seafood products in Japan (Ahmed et al., 2015) and other countries (Changsen et al., 2023; Kamala and Sivaperumal, 2023; Singh et al., 2020). Recently, the molecular characterization of ESBL-producing Enterobacterales with a wide range of drug resistance was retrieved from marine fish (Kamala and Sivaperumal, 2023), chicken, and chicken meat (Nakayama et al., 2022; Tran et al., 2021; Xedzro et al., 2023), suggesting a wide distribution of ESBLs. These genes were commonly identified, possibly because of plasmid transfer mechanisms, which play an important role in the rapid dissemination of mobile genetic elements, not only in humans and animals but also within the aquatic environment. Seaweed, oysters, and clams were not proportionally sampled, which reduced the probability of detecting AMR genes. We identified this as a limitation of our study that requires further consideration in future surveillance programs.

Several studies have reported antimicrobial use in aquaculture (Ido, 2023; Nadella et al., 2021; Tate et al., 2022; Troella et al., 2007). The irrational use of antimicrobials has

resulted in the emergence of resistant pathogens, including multidrug-resistant strains (Nadella et al., 2021). Similarly, antibiotic resistance was identified in the isolates recovered in this study. Most of the isolates were resistant to ampicillin and colistin, followed by sulfamethoxazole/trimethoprim, tetracycline, and ciprofloxacin (Figure 10 and Table 16). Resistance to some of these antimicrobials has been reported in isolates isolated from seafood products in Japan (Ahmed et al., 2015; Harada et al., 2018). These antimicrobials, except colistin, have been frequently used in Japanese culture farms for several years (Ido, 2023). The common use of such antimicrobials may be a cause of the high resistance conferred by the foodborne bacteria isolated in this study. Colistin resistance was also high and was mainly mediated by *Morganella* and *Proteus*, which have been reported to have an intrinsic resistance to polymyxins, including colistin (Olaitan et al., 2014). I also found three isolates of *Aeromonas* that conferred colistin resistance, which was not surprising because some *Aeromonas* species tend to have inducible resistance to colistin (Gonzalez-Avila et al., 2021). Cephalosporins are currently among the recommended treatment options for patients with severe multidrug-drug resistant infections (Laird et al., 2021; Xedzro et al., 2023). In this context, it is important to mention the third and fourth generation cephalosporin resistance observed in this study. Seven (15.6%) isolates that conferred clinical resistance to cefotaxime and ceftazidime also hydrolyzed cefepime, a fourth generation cephalosporin drug with a wide spectrum of activity against many gram-negative bacterial pathogens (Chong et al., 2010) (Figure 10). Of the cephalosporin-resistant isolates, two *E. coli*, one *E. cloacae*, and one *P. mirabilis* strains carried $bla_{\text{CTX-M-65}}$, $bla_{\text{SHV-12}}$, and $bla_{\text{CTX-M-9}}$, respectively, whereas the remaining strains did not carry ESBL genes responsible for hydrolyzing cephalosporins. Surprisingly, the *P. mirabilis* strain harboring the $bla_{\text{CTX-M-9}}$ genotype was susceptible to ampicillin—suggesting an unknown phenotype. MDR was observed in 27 (60%) isolates (Table 17), which was alarming. Considering that the consumption of seafood is a part of

Japanese culture, the prevalence of MDR constitutes a potential consumer health risk. Among the MDR isolates, the MAR index ranged from 0.2–0.8, with approximately 55% having a MAR index greater than 0.3, indicating that these isolates originated from high-risk contamination source (Titilawo et al., 2015a). The high incidence of MDR can be attributed to multiple resistance mechanisms, such as overexpression of multidrug efflux pumps and effective enzymatic hydrolysis of drugs (Titilawo et al., 2015b).

Specific plasmids associated with resistance gene dissemination have been identified in both clinical settings and food chains. The frequent detection of some plasmid types indicates their role in spreading drug resistance traits, especially among *Enterobacteriaceae*. In the present study, we investigated the transferability of plasmids associated with the spread of resistance traits. Even though seven out of the 11 selected strains with conjugable plasmids were negative (untypable) for all the target replicons, IncF replicons were detected in three *E. coli* strains. It is possible that the untypable plasmids were divergent or novel, and PBRT could not detect them because it targets classic incompatibility groups (Carattoli, 2009). The detection of IncF replicons in this study is consistent with a previous report that IncF plasmids are common in *Enterobacteriaceae*, especially *E. coli* (Carattoli, 2009). Evidence suggests that IncF is the most commonly described plasmid type identified in animals and humans, with the most frequently detected resistance genes being ESBLs (Rozwandowicz et al., 2018), suggesting that the plasmids identified in seafood may have originated from human or animal sources. IncFIA, IncFIB, IncFIC, and IncFrepB in ESBL-producing *E. coli* strains belonging to pathogenic B2 or commensal A phylogroups were also detected. Pathogenic B2 and commensal A phylogroups were recently reported in a collection of MDR ESBL-producing *E. coli* (Xedzro et al., 2023). In a recent report, IncF-positive commensal MDR *E. coli* strains were recovered from clams and sediments in Italy (Citterio et al., 2020). In Japan, clinical isolates of MDR *E. coli* were found to carry IncFrepB, IncFIA, and IncFIB

replicons (Yamochi et al., 2022), further suggesting that contamination of seafood products originated from human or animal sources. The presence of these conjugative plasmids contributes to the spread of MDR traits in seafood.

6. Conclusions

In summary, this study revealed the presence of AMR determinants and resistant bacteria in supermarket retail seafood samples collected from Hiroshima, Japan. The multiple antibiotic-resistant phenotypes and indices evaluated in this study suggest an alarming incidence of AMR in seafood, which could pose serious health risks to consumers. The findings also indicate, to some extent, a high propensity for horizontal gene transfer of mobile genetic elements in seafood and provide baseline information on AMR in the current study region. Therefore, continuous surveillance programs are needed to monitor resistance patterns in seafood. In future studies, I will consider larger sample sizes and/or samples from aquaculture farms to draw definitive conclusions on the status of AMR/MDR originating from seafood in Japan.

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Chapter 5

Antimicrobial susceptibility and genetic profiles of foodborne bacteria carrying mobile colistin resistance (*mcr*) genes

1. Summary

Colistin is a last-resort antimicrobial agent recommended for the treatment of severe multidrug-resistant infections. However, bacterial resistance to colistin has raised major public health concerns due to the widespread of plasmid-mediated mobile colistin resistance (*mcr*) genes. Here, the impact of *mcr* genes on colistin resistance and the genetic attributes of *mcr*-carrying isolates were evaluated.

Eight *mcr*-carrying isolates were obtained from meat and seafood samples. Antimicrobial susceptibility was performed using the broth microdilution method. Six strains were selected for whole genome sequencing to further understand the mechanism of colistin resistance. The results show that *Acinetobacter baumannii* (*mcr-4*), *Escherichia coli* (*mcr-5*), *Enterobacter kobei* (*mcr-9*), *Aeromonas hydrophila* (*mcr-3*), and two *Serratia liquefaciens* (*mcr-9*) were resistant to colistin at minimum inhibitory concentration (MIC) of 8 to >64 μg/mL. Whole genome sequencing revealed that some *mcr* genes were encoded on the plasmid, suggesting high chance of horizontal gene transfer. It was found that all the meat isolates conferred resistance to colistin. However, the *A. hydrophila* (*mcr-3*), and *E. cloacae* (*mcr-10*) isolated from seafood samples were susceptible at MIC <1 μg/mL.

These *mcr*-producing strains from food origin may pose a huge food safety threat and highlight the importance of colistin resistance surveillance to ensure food safety and safeguard the public.

Keywords: Colistin, *mcr*, meat, seafood.

2. Introduction

Colistin (also called polymyxin E) is a cationic antimicrobial compound discovered in Japan in 1947 (Gogry et al., 2021). The compound was commercialized in the 1950s, and has been considered as a miracle antibiotic because of its bactericidal efficacy against Gram-negative bacteria, including carbapenem-resistant Enterobacterales (Baron et al., 2016; Hamel et al., 2021). However, the use of colistin was reconsidered owing to the fact that intravenous administration has been coupled with side effects such as renal and neurological disorders (Falagas and Kasiakou, 2006). As a result, colistin was replaced by antimicrobials with lower toxicity such as quinolones and β-lactams. Due to the lack of new antimicrobial agents, colistin was reintroduced in the 2000s as a frontline antimicrobial agent to fight the increasing incidence of carbapenem-resistance among Enterobacterales (Hamel et al., 2021; Olaitan et al., 2014). Since then, colistin has often been administered as a last-resort antimicrobial treatment option against severe infections caused by multidrug-resistant (MDR) and extensively drug-resistant Gram-negative bacteria (Anyanwu et al., 2020; Borowiak et al., 2020; Gogry et al., 2021; Luo et al., 2017). In veterinary medicine, colistin is frequently used for the following reasons; (1) as a growth enhancer, (2) to treat infections caused by Gram-negative bacteria, and (3) as prophylaxis to prevent the occurrence of enteric diseases (Borowiak et al., 2020; Valiakos and Kapna, 2021).

Bacterial resistance to colistin is due to chromosomal point mutations within two-component systems (TCSs), mainly PhoP/Q and PmrA/B, which are non-transferable through horizontal gene transfer (Aghapour et al., 2019; Borowiak et al., 2017). However, the emergence of plasmid-mediated mobile colistin resistance (*mcr*) genes has prompted the significance of routine surveillance, especially among Enterobacterales. To date, ten *mcr* genes—*mcr-1* (Liu et al., 2016), *mcr-2* (Xavier et al., 2016), *mcr-3* (Yin et al., 2017), *mcr-4* (Carattoli et al., 2017), *mcr-5* (Borowiak et al., 2017), *mcr-6* (AbuOun et al., 2017), *mcr-7*

(Yang et al., 2018), *mcr-8* (Wang et al., 2018), *mcr-9* (Carroll et al., 2019), and *mcr-10* (Wang et al., 2020) have been identified. Globally, *mcr-1* is the most commonly detected gene, followed by *mcr-3* and *mcr-5* (Fukuda et al., 2022; Valiakos and Kapna, 2021) and such trend has also been observed in Japan (Fukuda et al., 2022; 2018). This suggests that horizontal gene transfer of specific mobile genetic elements, including plasmids, are associated with the rapid dissemination of such *mcr* homologs. *mcr* encodes phosphoethanolamine transferase, which mediates colistin resistance by modifying the lipid A moiety of lipopolysaccharide, resulting in the reduction of negative charges across bacterial membrane (Baron et al., 2016; Sun et al., 2018; Wang et al., 2020).

MCR has been frequently identified in *Enterobacteriaceae* isolated from pigs (Fukuda et al., 2018), chicken meat (Schrauwen et al., 2017), and vegetables (Liu et al., 2019; Liu and Song, 2019; Oh et al., 2020; Xedzro et al., 2023). Moreover the prevalence of *mcr* genes among Gram-negative bacteria isolated from retail meat (including pork, chicken, beef), and diseased pigs was evaluated in Japan (Fukuda et al., 2022; Nishino et al., 2017; Odoi et al., 2021).

The Food Safety Commission of Japan (FSCJ) recommended that information on *mcr* genes be regularly reported to provide updated information for infection control strategies (FSCJ, 2017). Although the use of colistin as feed additive in livestock production has been abolished in Japan, its therapeutic administration to cattle has been allowed and is typically used to treat porcine diarrhoea in pigs (Fukuda et al., 2018; Odoi et al., 2021; Xedzro et al., 2023), which may trigger the evolution and persistence *mcr* genes as well as its resistance to colistin. Given the importance of *mcr* genes, and the uncertainties regarding their expression in different bacteria, it is of outmost importance to study *mcr*-carrying bacteria as well as plasmids harboring *mcr* to control the dissemination of antimicrobial resistance. This study aimed at investigating the genetic characteristics of *mcr*-carrying isolates isolated from meat

and seafood products.

3. Materials and methods

3.1 Bacterial strains and culture conditions

A total of eight *mcr*-carrying strains were obtained from the previous studies (Chapter 2, 3, and 4). These isolates were recovered from meat and seafood samples originating from different geographical regions. The isolates included *A. baumannii* (*mcr-4*), *E. coli* (*mcr-5*), *S. liquefaciens* (*mcr-9*) (two isolates), *E. kobei* (*mcr-9*), *A. hydrophila* (*mcr-3*) (two isolates), and *E. cloacae* (*mcr-10*). The isolates were initially identified by 16S rRNA gene sequencing and later confirmed by whole genome sequencing (WGS). Prior to susceptibility testing and molecular analyses, isolates were retrieved from the -80°C glycerol stock and cultured on LB agar without antibiotics. Subsequently, a single isolated colony from each strain was used for further analyses.

3.2 Antimicrobial susceptibility testing

The minimum inhibitory concentrations (MICs) of twelve antimicrobial agents were determined by using the standardized broth microdilution method according to the interpretative criteria described by the Clinical and Laboratory Standards Institute (CLSI, 2020). The breakpoint for colistin was determined according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST, 2021). Antimicrobial agents included ampicillin, cefotaxime, ceftazidime, cefoxitin, meropenem, kanamycin, gentamicin, chloramphenicol, ciprofloxacin, tetracycline, colistin, and fosfomycin. A stock solution of each antimicrobial agent was prepared and diluted according to the CLSI recommendations. Quality control for MIC analysis was performed using *E. coli* ATCC 25922 (American Type Culture Collection).

3.3 Plasmid isolation and PCR-based replicon typing (PBRT)

Plasmids were isolated from the *mcr*-carrying strains. The alkaline lysis method was used for plasmid preparation as described previously (Green and Sambrook, 2016). Briefly, 1.5 mL of overnight LB broth culture was centrifuged and resuspended in alkaline lysis buffers I, II, and III. The solution was vortexed and allowed to stand for 3–5 min after the addition of each buffer. Supernatants were obtained after centrifugation and mixed with an equal volume of isopropanol. The precipitated nucleic acids were collected and dissolved in 1 mL 70% ethanol. Plasmid DNA was recovered as pellets and finally dissolved in 50 µL of Tris-EDTA (pH 8.0) buffer containing 20 µg/mL DNase-free RNase A. PBRT was then performed to identify the plasmid incompatibility (Inc/rep) groups using 18 primer sets targeting HI1, HI2, I1, X, L/M, N, FIA, FIB, W, Y, P, FIC, A/C, T, FIIs, FrepB, K/B, and B/O, as previously described (Carattoli et al., 2005). The assay was conducted using five multiplex and three simplex PCR assays.

3.4 Whole genome sequencing, bioinformatic analysis, and conjugation experiments

The whole genome of the six *mcr*-carrying isolates recovered from meat was sequenced as previously described (Xedzro et al., 2023). A hybrid assembly of both the short reads and long reads was performed using Unicycler software v0.4.8 (https://github.com/rrwick/Unicycler). Genome annotation was performed using RAST (*Tk*) (Bretin et al., 2015) and the whole genome sequences were analyzed at the center for genomic epidemiology (http://www.genomicepidemiology.org/services/). Bacterial species were predicted using the kmerFinder algorithm v3.2 (https://cge.food.dtu.dk/services/KmerFinder/). Acquired antimicrobial resistance genes were identified using ResFinder v4.1 (https://cge.food.dtu.dk/services/ResFinder/). The multilocus locus sequence type was determined using MLST v2.0

(https://cge.food.dtu.dk/services/MLST/) and the plasmid repilicon types were predicted using the PlasmidFinder v2.1 (https://cge.food.dtu.dk/services/PlasmidFinder/). A filter-mating conjugation experiment was conducted in an attempt to evaluate the transfer of *mcr-5* and *-4* harboring plasmids using *E. coli* J53 as a recipient. Transconjugants were selected on LB agar containing 100/150 µg/mL sodium azide and 2/4 µg/mL colistin. Suspected positive transconjugants were confirmed by PCR targeting the *mcr-5* and *-4* gene.

4. Results

4.1 Origin of *mcr*-carrying strains

A total of eight *mcr*-positive strains were characterized in this study (Table 19). The isolates included *A. baumannii* (*mcr-4.3*), *E. coli* (*mcr-5.1*), *S. liquefaciens* (*mcr-9.1*), *E. kobei* (*mcr-9*), *A. hydrophila* (*mcr-3.25/9*), *S. liquefaciens* (*mcr-9*), *A. hydrophila* (*mcr-3.2*), and *E. cloacae* (*mcr-10.1*). These strains were isolated from meat and seafood products from different geographical regions. Four strains were isolated from chicken, beef, or pork in 2009, two from chicken in 2021, and the remaining two isolates were recovered from seafood products in 2023. Of all *mcr*-positive strains, three were isolated from meat products imported to Japan.

Table 19. Origin and genetic features and *mcr*-carrying Gram-negative bacteria

Isolate	Identification	Genotype	Source	Origin	Isolation year
266-34J1	*A. baumannii*	*mcr-4.3*, $bla_{\text{ADC-25}}$, $bla_{\text{OXA-67}}$	Chicken	Brazil	2009
160-11H1	*E. coli*	*mcr-5.1*, $bla_{\text{SHV-12}}$, *sul2*, *sitABCD*	Chicken	Japan	2009
35E-19E1	*S. liquefaciens*	*mcr-9.1*, *lsa(A)*, *ClpL*	Beef	Australia	2009
292-28A1	*E. kobei*	*mcr-9*, $bla_{\text{ACT-9}}$, *fosA*	pork	Denmark	2009
CST-042	*A. hydrophila*	*mcr-3.25*, *mcr-3.9*, *ampH*, *cphA1*, *aph(3")-Ib*, *aph(6')-Id*	Chicken	Japan	2021
CST-066	*S. liquefaciens*	*mcr-9*	Chicken	Japan	2021
D6-A192	*A. hydrophila*	*mcr-3.2*	Seafood	Japan	2023
B12-S377	*E. cloacae*	$bla_{\text{TEM-1}}$, $bla_{\text{SHV-12}}$, *mcr-10.1*	Seafood	Japan	2023

4.2 Antimicrobial susceptibility testing

To clarify whether the *mcr*-carrying strains might mediate colistin resistance or resistance to other antimicrobials, the activity of twelve antimicrobial agents against the isolates was evaluated. The MICs of the agents for the 2009 strains are shown in Table 20. Six isolates were found to show high colistin resistance (MIC 8 to >64 μg/mL). *A. baumannii* harboring the *mcr-4.3* was additionally resistant to ampicillin, cefotaxime, ceftazidime, cefoxitin, chloramphenicol, and fosfomycin. Whole genome sequencing revealed that this strain carried a chromosomal AmpC gene, $bla_{\text{ADC-25}}$, and a β-lactamase gene, $bla_{\text{OXA-67}}$. The *mcr-5*-carrying *E. coli* also harbored sulfonamide resistance determinant, *sul2* (two copies), metal ion transporter, *sitABCD*, and an extended-spectrum β-lactamase, $bla_{\text{SHV-12}}$ genotype. Susceptibility testing showed that it was additionally resistant to ampicillin, cefotaxime, and ceftazidime but remained susceptible to the remaining antibiotics tested. *S. liquefaciens* and *E. kobei* carry *mcr-9*, but they differ in their respective capacities in conferring resistance to the antimicrobial agents. Cefoxitin and fosfomycin resistance were identified in both isolates. Nonetheless, ampicillin, ceftazidime, or chloramphenicol resistance varied between the two

strains. Analyzing the genome data showed that *S. liquefaciens* does not carry a β-lactamase gene—this may explain why it was susceptible to ampicillin and cefotaxime. However, the isolate did confer high resistance to ceftazidime. On the other hand, *E. kobei* was found to carry $bla_{\text{ACT-9}}$ and *fosA* gene, and showed resistance to ampicillin and fosfomycin. Notably, all the isolates were susceptible to meropenem, kanamycin, gentamicin, ciprofloxacin, and tetracycline (Table 20).

The sensitivity and resistance profiles of 2021 and 2023 *mcr*-carrying isolates are presented in Table 21. While *mcr-3* and *mcr-9* isolates from meat sources conferred colistin resistance, the *mcr*-positive strains recovered from seafood samples were susceptible to colistin at MIC of <1 µg/mL when EUCAST breakpoint was taken into consideration. It was found that *A. hydrophila* isolated from seafood was resistant to only two antimicrobials (ampicillin and tetracycline). The *mcr-10*-carrying *E. cloacae* was highly resistant to antimicrobials and exhibited multiple drug resistance phenotypes. MIC values of antimicrobials for this strain were generally high (>32 µg/mL) except for intermediate resistance it displayed against ciprofloxacin (1 µg/mL). Generally, cefoxitin resistance was prevalent, occurring among 6 isolates with MIC ranging from 16–128 µg/mL. It was noted that *mcr-9*-carrying *S. liquefaciens* isolates demonstrated susceptibility to ampicillin and cefotaxime but were resistant to ceftazidime—an unknown phenotype which requires major investigation.

Table 20. MIC of various antimicrobials for *mcr*-positive isolates obtained in 2009

Agent	266-34J1 (*mcr-4.3*)	160-11H1 (*mcr-5.1*)	35E-19E1 (*mcr-9.1*)	292-28A1 (*mcr-9*)	*E. coli* ATCC 29522
	A. baumannii	*E. coli*	*S. liquefaciens*	*E. kobei*	
Ampicillin	**16**	**512**	8	**64**	1
Cefotaxime	**4**	**>32**	1	1	0.25
Ceftazidime	**8**	**>128**	**128**	0.25	0.25
Cefoxitin	**128**	8	**16**	**128**	4
Meropenem	0.25	<0.0625	1	0.125	<0.0625
Kanamycin	4	16	8	8	8
Gentamicin	8	4	1	4	4
Chloramphenicol	**32**	4	**16**	8	4
Ciprofloxacin	<0.125	<0.125	<0.125	<0.125	<0.125
Tetracycline	4	4	**8**	4	2
Fosfomycin	**128**	4	**128**	**128**	8
Colistin	**16**	**8**	**>64**	**>64**	0.5

Shaded regions indicate resistance.

Table 21. MIC of various antimicrobials for *mcr*-positive isolates obtained in 2021 and 2023

Agent	CST-042 (*mcr-3.2*)	CST-066 (*mcr-9.3*)	D6-A192 (*mcr-3.2*)	B12-S377 (*mcr-10.1*)	*E. coli* ATCC 29522
	A. hydrophila	*S. liquefaciens*	*A. hydrophila*	*E. cloacae*	
Ampicillin	**64**	8	**>512**	**>512**	1–2
Cefotaxime	0.5	1	0.5	**>32**	0.25
Ceftazidime	2	**>128**	0.25–0.5	**128**	0.25–1
Cefoxitin	**16–32**	**16**	8	**128**	4
Meropenem	1	<0.0625	0.125	<0.0625	<0.0625
Kanamycin	4	8	<0.125	0.25	4
Gentamicin	1	1	0.25	0.5	4
Chloramphenicol	2	8	0.5–1	8	2–4
Ciprofloxacin	<0.125	<0.125	<0.125	**2**	<0.125
Tetracycline	0.5	4	**32**	**64**	2
Fosfomycin	64	**128**	8-16	**>1024**	8
Colistin	**64**	**>64**	0.5–1	0.5–1	0.5

Shaded regions indicate resistance.

4.3 Antimicrobial resistance profiles and PBRT

All the isolates were resistant to at least one antimicrobial agent and conferred resistance to at least three antimicrobial classes except the *mcr-3*-carrying *A. hydrophila* isolated from seafood. Cephalosporin resistance was observed in *A. baumannii* (*mcr-4*), *E. coli* (*mcr-5*), and *E. cloacae* (*mcr-10*). These isolates carry extended-spectrum β-lactamase or cephalosporinase encoding gene responsible for hydrolyzing cephalosporins. Three isolates were resistant to six antimicrobial classes and two showed resistance to four antimicrobial classes. All the isolates exhibited multidrug resistance phenotypes except *mcr-3*-carrying *A. hydrophila* isolated from seafood product (Table 22). PCR-based replicon typing identified different incompatibility (Inc) groups among the strains, although some contradict the results of the predicted replicon types when the WGS data was used. The most identified Inc group (based on PBRT) was the IncFrepB replicon and was found in five isolates. Interestingly, three replicon types; IncI1, IncFIB, and IncFrepB were identified in *mcr-5*-carrying *E. coli*. Although the *mcr-10*-carrying *E. cloacae* strain carried conjugable plasmids, PBRT revealed that the plasmid was untypable.

Table 22. Antimicrobial resistance profiles and replicon types (PBRT) of *mcr*-carrying isolates from different origin

Isolate	Species	Resistance genes	Replicon type	Resistance profiles
266-34J1	*A. baumannii*	*mcr-4.3*, $bla_{\text{ADC-25}}$, $bla_{\text{OXA-67}}$	IncFrepB	AMP, CTX, CAZ, FOX, CHL, FOF, CST
160-11H1	*E. coli*	*mcr-5.1*, $bla_{\text{SHV-12}}$, *sul2* (2 copies), *sitABCD*	IncI1, IncFIB, IncFrepB	AMP, CTX, CAZ, CST
35E-19E1	*S. liquefaciens*	*mcr-9.1*, *lsa(A)*, c*lpL*	IncFIA, IncFIB, IncFrepB	CAZ, FOX, CHL, TET, FOF, CST
292-28A1	*E. kobei*	*mcr-9*, $bla_{\text{ACT-9}}$, *fosA*	Inc1I, IncFIB, IncFrepB	AMP, FOX, FOF, CST
CST-042	*A. hydrophila*	*mcr-3* (two copies), *ampH*, *cphA1*, *aph(3'')-Ib*, *aph(6')-Id*	IncFrepB, IncK/B	AMP, FOX, CST
CST-066	*S. liquefaciens*	*mcr-9.3*	-	CAZ, FOX, FOF, CST
D6-A192	*A. hydrophila*	*mcr-3.2*	-	AMP, TET
B12-S377	*E. cloacae*	$bla_{\text{TEM-1}}$, $bla_{\text{SHV-12}}$, *mcr-10.1*	Untypable	AMP, CTX, CAZ, FOX, CIP, TET, FOF

NB: The predicted replicon types were based on PBRT. AMP: ampicillin, CTX: cefotaxime, CAZ: ceftazidime, FOX: cefoxitin, TET: tetracycline, CHL: chloramphenicol, CIP: ciprofloxacin, CST: colistin, FOF: fosfomycin

4.4 Genomic features of chromosomes and AMR/mcr- carrying plasmids

The genomic features of chromosome and plasmids (carrying AMR gene) identified in each strain along side their sizes and average guanine-cytosine (GC) content are presented in Table 23. Species identification using the k-mer algorithm identified the strains as *A. hydrophila*, *A. baumanii*, *E. coli*, *S. liquefaciens* (CST-066), *S. liquefaciens* (35E-19E1), and *E. kobei*, as they shared significant template identity coverage (~80%) with the reference strain for *A. hydrophila* NUITM-VA1 (accession number NZ_AP025277.1), *A. baumannii* EC (NZ_CP038262.1), *E. coli* CFS3292 (NZ_CP026935.2), *S. liquefaciens* FG3 (NZ_CP033893.1), *S. liquefaciens* FDAARGOS_125 (NZ_CP014017.2), and *E. kobei* C16 (NZ_CP042578.1), respectively. The identified strains have variable chromosomal sizes with average GC content of >50%.

Figure 11 describes the genetic context of chromosomally encoded *mcr-3* (two copies adjacent to each other) located within the physical boundaries of multicopy transposons identified in *A. hydrophila* CST-042. One copy of *mcr-3* (most likely a new variant) shares ~85% sequence identity with original *mcr-3* identified in *E. coli* in China (Yin et al., 2017). Further *in silico* analysis revealed that the strain carried additional 1,626 bp *mcr-3*-like (lipid A phosphoethanolamine transferase) protein which was in an operon with uncharacterized MFS-type transporter upstream of an integrase gene.

mcr-4 in *A. baumannii* 266-34J1 was located on a 26, 838 bp plasmid (GC, 41%) of unknown identity (Fig 12A) and was bracketed by ParE and ParA like toxin proteins (Fig 12B). *mcr-4* was additionally flanked by a transposase gene which was located at its upstream boundary (Fig 12 and 13). Full sequence query against NCBI database showed that the plasmid was similar to a portion (99.99% sequence identity) of a non-transferable novel plasmid pAB18PR065 (accession number: MK360916) identified in China (Ma et al., 2019). A conjugation experiment showed that the *mcr-4* carrying plasmid was non-transferable to *E.*

coli J53 recipient. *In silico* analysis showed that the plasmid had no origin of transfer (oriT) and does not carry conjugation related proteins.

E. coli 160-11H1 harbored *mcr-5* on a 66,887 bp IncFII plasmid backbone (53% GC) content (Fig 13A), which was homologous to a plasmid pCoo (96.91 % sequence similarity) (Froehlich et al., 2005). The gene was found in the upstream vicinity of a Tn*3*-type family transposase (Fig 13B). Analyzing the genetic background of *mcr-5* revealed that it was encoded in an operon with ChrB domain protein and uncharacterized MFS-type transporter. WGS analysis further showed that strain harbored $bla_{\text{SHV-12}}$ on a 147,929 bp plasmid of IncFIA:FIB:FIC backbone (contig 2). Both *mcr-5* and $bla_{\text{SHV-12}}$-carrying plasmids were transferred to *E. coli* J53 recipient in a conjugation experiment.

Figure 14 describes the genetic context of chromosomally encoded *mcr-9* identified in *S. liquefaciens* and *E. kobei*. The genetic environments were characterized by the presence of a functional two component system, *qseB/C* responsible for inducing *mcr-9* expression among Enterobacterales. Furthermore, the *mcr-9* genes were enclosed by two intact insertion sequences which can facilitate direct mobilization and transposition events leading to insertion of these genes into bacterial genomes.

Table 23. Features of chromosomes and plasmids harboring AMR determinants identified in *mcr*-carrying isolates

Strain	Chromosome or plasmid	Length (bp)	GC (%)	MLST/pMLST	Replicon type	
A. hydrophila CST-042	Chromosome	5,541,409	60	ST1142	-	*mcr-3* (two copies), *ampH*, *cphA1*
	pCST-042_2	153,005	55	Unknown	ND	*aph(3″)-Ib*, *aph(6′)-Id*,
A. baumanni 266-34J1	Chromosome	3,842,281	39	ST747	-	$bla_{\text{ADC-25}}$, $bla_{\text{OXA-67}}$
	p266-34J1_5	26,838	41	Unknown	ND	*mcr-4.3*
E. coli 160-11H1	Chromosome	5,031,330	51	ST10	-	*sul2*, *sul2*
	p160-11H1_2	147,292	51	F18:A-:B8	IncFIA:FIB:FIC	$bla_{\text{SHV-12}}$, *sitABCD*
	p160-11H1_4	66,887	53	F14:A-:B-	IncFII (pCoo)	*mcr-5.1*
S. liquefaciens 35E-19E1	Chromosome	5,261,506	55	ND	-	*mcr-9*
	p35E-19E1_4	748,497	38	Unknown	ND	*lsa(A)*
	p35E-19E1_7	10,555	35	Unknown	ND	*clpL*
E. kobei 292-28A1	Chromosome	4,849,160	55	ST32	-	*mcr-9*, $bla_{\text{ACT-9}}$, *fosA*
S. liquefaciens CST-066	Chromosome	5,529,704	55	ND	-	*mcr-9*

NB: Only plasmids carrying AMR determinants were included in the Table were named according to the contig number.
ND: not detected

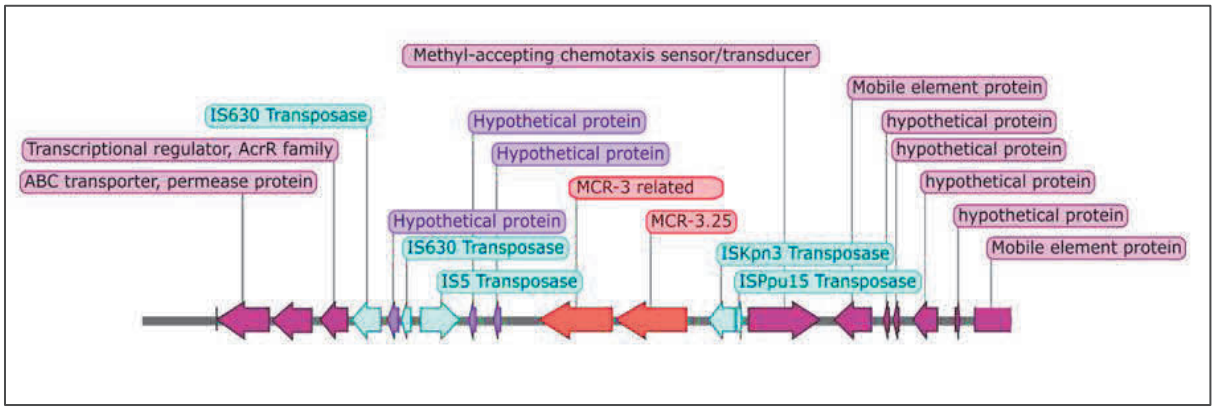

Figure 11: Genetic context of chromosomally encoded *mcr-3* (two copies) identified in *A. hydrophila*

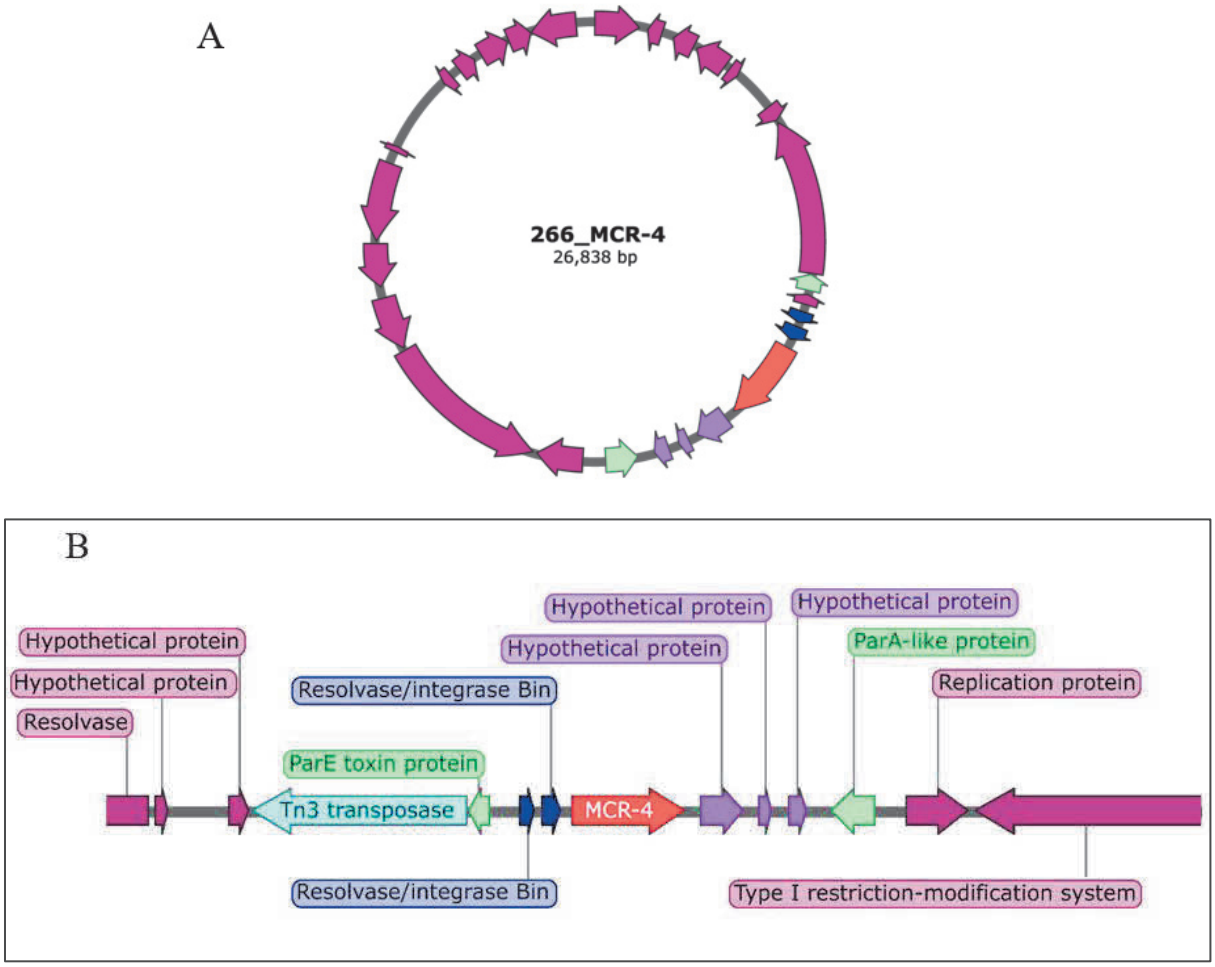

Figure 12. Structure of an unknown *mcr-4-carrying* plasmid (A) and the genetic context of *mcr-4* (B) identified in *A. baumannii*

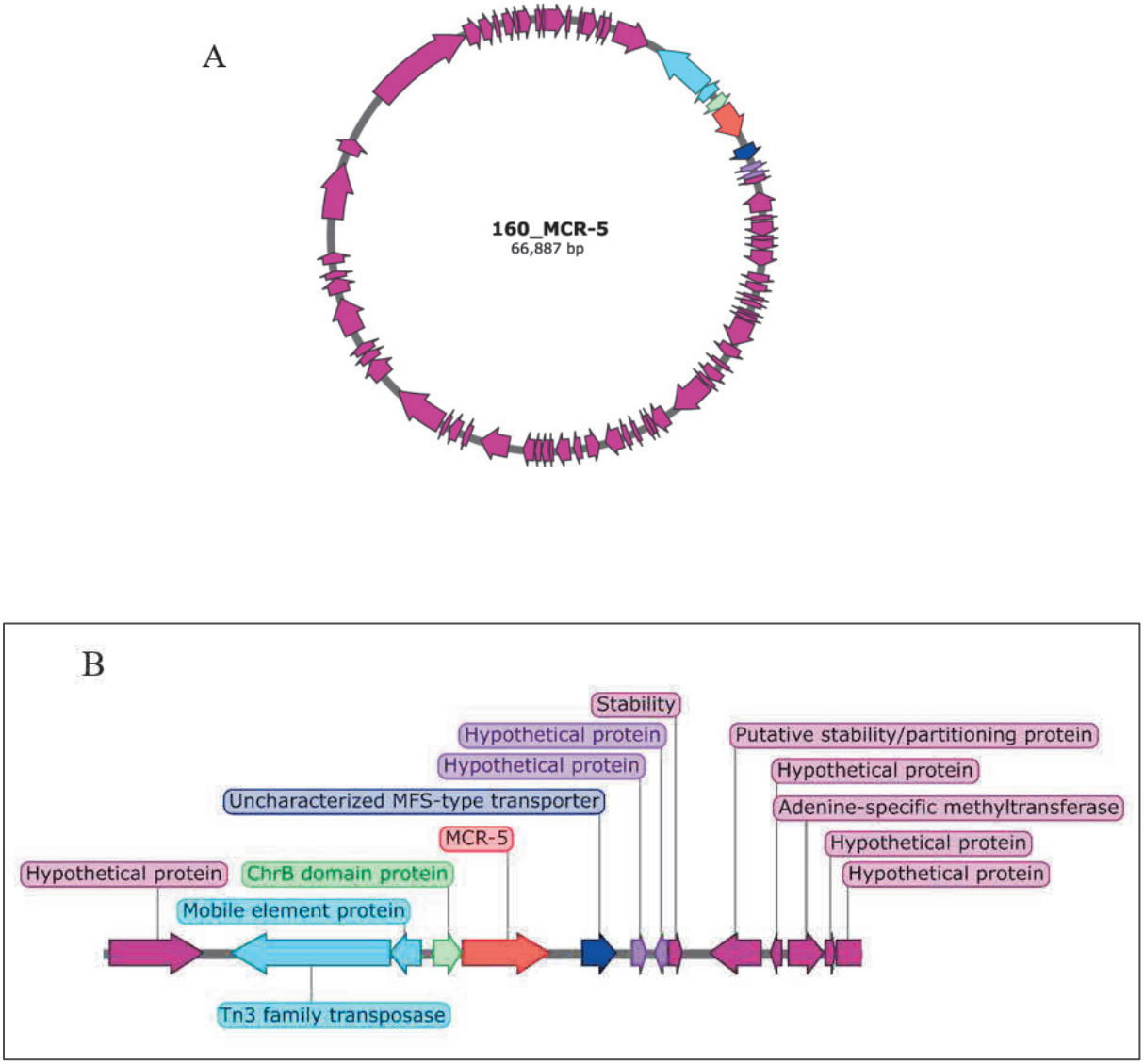

Figure 13. Structure of *mcr-5*-carrying IncFII plasmid backbone (A) and the corresponding genetic features of *mcr-5* (B) identified in *E coli*

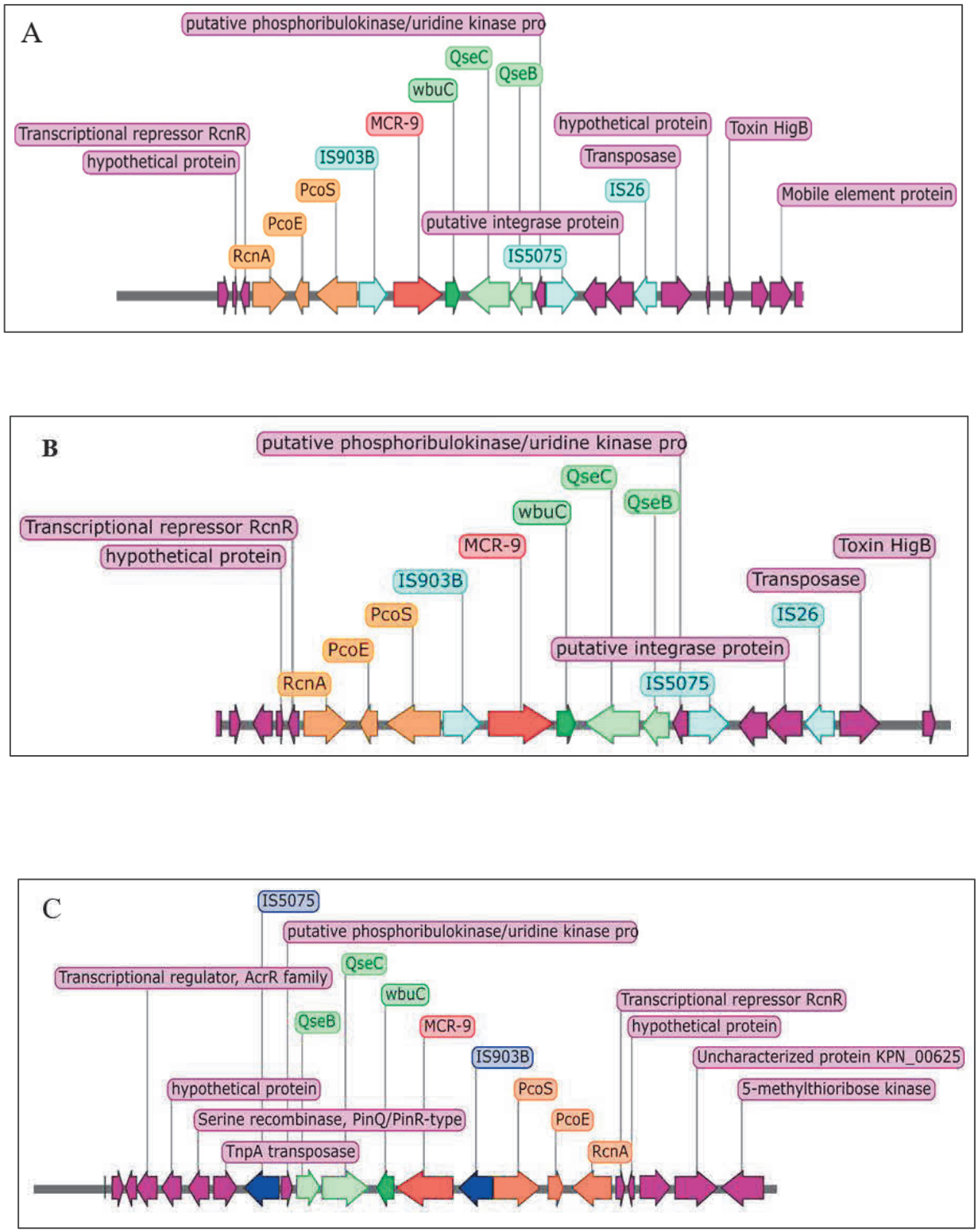

Fig 14. Genetic environment of chromosomally encoded *mcr-9* identified in *S. liquefaciens* (A and B) and *E. kobei* (C)

5. Discussion

Since the first report of mobilized colistin resistance (*mcr*) gene, several *mcr* homologs have been reported in clinical and food isolates from different regions. Among the *mcr* genes, *mcr-1*, *mcr-3*, and *mcr-5* have been frequently detected (Fukuda et al., 2022). *mcr-9* is also widely distributed and has been identified in Enterobacterales in over 40 countries across six continents (Ling et al., 2020). The mechanism by which *mcr* genes confer colistin resistance is variable and greatly depends on the *mcr* variant or bacterial species. Therefore, it is important to study the characteristics of *mcr*-harboring bacteria.

In this study, *mcr*-positive gram-negative bacteria isolated from different food sources were characterized. The current reports of *mcr* genes in Japan are mostly linked to *E. coli* and *Salmonella* isolated from livestock (Odoi et al., 2021). Although few isolates were characterized in this study, *mcr* was found in different bacteria species including *E. coli*. The *mcr-4* was detected in a multidrug-resistant *A. baumannii* isolate recovered from chicken imported to Japan. The epidemiology of *mcr-4* is currently unknown in Japan, and it is tempting to speculate that no study has reported such gene in Japan. The current information suggest that *mcr-4* has been only detected in South America, Europe, and China (Ling et al., 2020). Given that the identified strain was isolated from meat imported from one of these regions, international imports of meat can provide wide distribution pathways for *mcr* genes. WGS analyses revealed that the strain harbored plasmids of unknown identity which can facilitate horizontal gene transfer of AMR determinants including *mcr* variants. Although *mcr-4* was located on unidentified plasmid without a replication origin, WGS analysis revealed that it was in an operon with Tn*3* family transposase and two copies of resolvase (site-specific recombinase) which can aid mobilization events leading to the spread of this gene. Unhygienic practices in the meat processing or slaughterhouses could also result in cross contamination and silent dissemination of *mcr-4*. Additionally, *mcr-9* was identified in

E. kobei and two *S. liquefaciens* isolated from beef or pork imported to Japan which is also a grave concern to the public health since the mechanism of antibiotic resistance related to this gene is still unclear (Xedzro et al., 2023). The remaining isolates were recovered from meat and seafood samples in Japan. It should be noted that the presence of *mcr*-harboring bacteria can be attributed to contamination of meat and seafood products which may have arised from different factors, including poor meat hygiene, unstrict stainless cleaning conditions, intestinal flora of animals, and processing methods in slaughterhouses or processing centers.

MCR has been detected in *Enterobacteriaceae* isolated from food origin (Borowiak et al., 2017; Carattoli et al., 2017; Xedzro et al., 2023.) and clinical specimen (Kieffer et al., 2019; Umeda et al., 2021). The contamination of meat and continuous detection of such genes from food sources is a public health risk since critical priority bacteria can be transmitted to humans through the consumption of contaminated meat products. Furthermore, the identified species are some of the important pathogens that cause gastroenteritis and wound infections in humans. The identification of *mcr-5*-carrying *E. coli* and *mcr-3* carrying *A. hydrophila* in this study is in accordance with the food poisoning cases associated with *E. coli* and *Aeromonas* previously reported in Japan (Tanaka et al., 1992).

To ascertain the clinical relevance of the *mcr* harboring isolates, the strains were tested for resistance to various antimicrobial agents, and their molecular attributes such as potential plasmids that might carry the *mcr* alleles were also evaluated. Noteworthy, all the meat isolates showed resistance to colistin with MICs ranging from 8 to >64 μg/mL which were relatively higher compared to the MICs of antimicrobials observed for seafood isolates. Such high colistin resistance conferred by meat isolates seems very alarming and may be attributed to the worldwide use of colistin as veterinary drugs in animal production, which results in a selective pressure in veterinary practice (Carattoli et al., 2017; Sun et al., 2018; Valiakos and Kapna, 2021). Furthermore, overexpression of some *mcr* genes can actually

confer colistin resistance as previously reported (Fukuda et al., 2022). To comment on this further, expression of some *mcr* variants is governed by a two-component regulatory system. For instance, the presence of chromosomal or plasmid *qseC*/*qseB* genes have been reported to induce *mcr-9* expression leading to colistin resistance in many enterobacterial species (Kieffer et al., 2019). It is important to mention that the strains not only displayed colistin resistance but were also phenotypically resistant to ampicillin, cefotaxime, ceftazidime, cefoxitin, chloramphenicol, or fosfomycin depending on the bacterial species.

Specific plasmids have been described to play a key role in the rapid spread of *mcr* genes. For instance, *mcr-9* has been mainly detected on IncHI2 plasmids (Li et al., 2020; Xu et al., 2022), while *mcr-10* was frequently found on IncFIA/FIB/FII plasmid backbone (Liao et al., 2022; Wang et al., 2020). In this context, the presence of a single plasmid was investigated in an attempt to assess the possibility of horizontal gene transfer of mobile genetic elements. It was found that some strains harbor plasmids mainly IncFrepB, along with IncFIA, IncFIB, or IncI1 replicon. Reports suggest that IncF is the most commonly described plasmid type in humans and animals (Rozwandowicz et al., 2018), suggesting a common route since those IncF positive isolates were retrieved from animal sources. Some studies have also reported the identification of such IncF replicons among *mcr*-carrying strains (Carattoli et al., 2017; Liao et al., 2022). Although WGS revealed different contigs (other than chromosomes) within the *mcr*-harboring isolates, further analyses showed that most of these contigs are of unknown identity as detected by PlasmidFinder. It is likely that novel plasmids are emerging due to insertion of DNA fragments possibly by transposons into core bacterial genomes. Broad epidemiological surveillance would be necessary to evaluate the impact and extent of dissemination AMR plasmids including *mcr*-harboring plasmids among animal specimens.

6. Conclusions

In this chapter, the genetic features of *mcr*-carrying isolates from different geographical regions were investigated. The isolates showed high colistin resistance although those recovered from seafood products were susceptible to colistin. This study also provided new information regarding the detection of some *mcr* variants in certain bacterial species in Japan. The distribution of *mcr* genes has been reported in different environments and requires further evaluation in terms of resistance mechanisms and transmission routes. Continuous monitoring is required to assess the status and impact of *mcr* genes on human health.

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Chapter 6

1. General summary, Conclusions, and Recommendations

Antimicrobial resistance (AMR) is a major public health challenge in many countries including Japan. Estimates suggest that approximately 10 million lives and USD 100 trillion will be lost by 2050 because of AMR if existing control measures to prevent the spread of AMR are compromised. Literature claims suggest that, if left unattended, AMR will continue to increase beyond imagination and could facilitate the evolution of new resistance mechanisms in bacterial pathogens which may be much more fatal in the future than the existing superbugs.

Although several efforts to implement AMR surveillance programs along the food supply chain have experienced overall increase over decades, resistance rates vary between geographical regions and among different species. Also concerning, are human activities and urbanization, as well as acquisition of genetic elements by the so-called superbugs which are key players in the increasing rates of AMR around the globe. In this study, the genetic attributes associated with antimicrobial resistance in foodborne bacteria was investigated.

Chapter 2 compared antimicrobial resistance among several *Escherichia coli* isolates obtained from meat samples in 2009 and 2021. The occurrence of antimicrobial resistance determinants especially cephalosporinases (AmpC) or extended-spectrum β-lactamases (ESBL) was significantly higher (p-value = 0.037) among *E. coli* isolates obtained in 2009 than in 2021. The results of this study showed a 22% decrease in meat contamination by AmpC/ESBL-carriers over the two years of study. Interestingly, susceptibility testing revealed a decreased rate of resistance to critical antimicrobials including cefotaxime, ceftazidime, and cefepime between 2009 and 2021. Additionally, it was observed that more

than 75% of the *E. coli* isolates obtained from the two years of study exhibited multidrug resistance (MDR) phenotypes, although the differences were not statistically significant for comparisons. The *E. coli* isolates were also assigned to various phylogroups in order to determine their pathogenic potential. The determination of *E. coli* phylogenetic groups revealed that the majority are commensal strains belong to A, B1, and F phylogroups. Phylogroup A strains occurred more frequently in 2009, whereas the B1 phylogroup was prevalent in 2021. Some strains from both isolation years were also found to belong to pathogenic B2 phylogroup.

In chapter 3, the overall rate of antimicrobial resistance occurring among Gram-negative bacteria isolated from meat products was evaluated. Different Gram-negative bacteria were isolated and grouped into Enterobacterales and non-Enterobacterales. Members within the Enterobacterales group were more prevalent than the non-Enterobacterales isolated in both 2009 and 2021. Overall, the most common clinical resistance was observed against ampicillin, cefotaxime, cefoxitin, aztreonam, kanamycin, tetracycline, sulfamethoxazole/trimethoprim, chloramphenicol, and fosfomycin. Seventeen *E. coli* isolates characterized in chapter 2 were also included in this study. Specifically, these *E. coli* isolates were evaluated for the presence of a single plasmid that might carry extended-spectrum β-lactamases identified within these strains. Different plasmid incompatibility groups were found within these isolates. The commonly detected plasmid replicon types were the IncFIB, IncI1, and IncFrepB, suggesting that these plasmids might be the potential carriers of antimicrobial resistance genes, including extended-spectrum β-lactamases. The molecular identification of AmpC β-lactamases were also studied. These β-lactam resistance genes were dominantly found in Gram-negative bacteria recovered in 2009 than in 2021. They conferred clinical resistances mostly to penicillin and cephalosporin. In summary, this study showed that different resistant Gram-negative bacteria exist in meat products and calls for the routine

implementation of surveillance programs to track the spread of AMR in meat products.

In chapter 4, I performed epidemiological study to investigate the occurrence of antimicrobial resistance in supermarket retail seafood samples. Different seafood-borne bacteria carrying antimicrobial resistance determinants were recovered mostly from the fish, squid, and shrimp samples investigated. The isolates were found to show high ampicillin resistance, and some also conferred clinical resistances to cephalosporins, which are currently among the recommended treatment of choice against many severe infections. It was alarming to observed high colistin resistance, considering that colistin is a last-resort antimicrobial against MDR infections. This study also evaluated the possibility of plasmid transfer that might play a role in the dissemination of antimicrobial resistance especially within aquatic environments. Most of the selected MDR strains were found to transfer their resistance plasmid to a plasmid free *E. coli* J53 strain. Even though seven out of the 11 selected MDR strains have conjugable plasmids, they were negative (untypable) for all the target replicons, as investigated using PCR-based replicon typing (PBRT). It is possible that the untypable plasmids were divergent or novel, and PBRT could not detect them because it targets classic incompatibility groups. In summary, this study demonstrated that seafoods are potential carriers of antimicrobial-resistant bacteria which can be transferred to humans through the consumption of contaminated products. Continuous monitoring is required to assess the prevalence and distribution of AMR in the seafood supply chain.

Chapter 5 discussed the genetic and phenotypic characteristics of foodborne bacteria carrying mobile colistin resistance (*mcr*) genes. Colistin is a last-resort antimicrobial agent recommended for the treatment of MDR infections including carbapenem-resistant Enterobacterales. Thus, the identification of even a single strain carrying such resistance genotype from food is of grave concern and signifies potential health risk to consumers. In this study, eight *mcr*-carrying isolates retrieved from meat and seafood products in the above

chapters were characterized. Six isolates were highly resistant to colistin and showed additional resistance to other antimicrobial agents. It was observed that only the meat-derived isolates conferred clinical resistance to colistin. The other two *mcr*-carrying isolates recovered from seafood were susceptible to colistin although they did confer resistance to other antimicrobial agents. Whole genome sequencing (WGS) of six isolates revealed that some *mcr*-genes are encoded on the plasmid, which can be transferred to other bacterial species via horizontal gene transfer. Furthermore, WGS showed that some isolates harbored other antimicrobial resistance genes encoded on the plasmid or the chromosome. In summary, this study provided some genetic insights into *mcr*-carry isolates and highlights the importance of colistin resistance surveillance along the meat and seafood production chain.

Finally, to prevent the persistence of antimicrobial resistance, possible strategies such as limiting the use of antibiotics in farming practices can help reduce the selective pressure on resistant microorganisms. Additionally, proper sanitation processes in food production can prevent the risk of cross-contamination and the spread of AMR along the food supply chain. Continuous surveillance programs would be necessary to provide updated information on the status of antimicrobial resistance and evaluate the extent of dissemination at the local, regional, and national level.

APPENDICES

Table S1 Oligonucleotides used in this study

Primer	Primer sequence (5′-3′)	Target	Size	Reference
515F	GTGCCAGCMGCCGCGGTAA	16S rRNA	1001	(Soliman et al., 2023)
1492R	CGGYTACCTTGTTACGACTT			
β-lactamases				
OXA-1-F	ATGAAAAACAATACATATCAACTTC	*bla*$_{OXA-1}$	831	(Soliman et al., 2023)
OXA-1-R	TTATAAATTTAGTGTGTTTAGAATGGTG			
SHV-FW	GTGTATTATCTCCCTGTTAGCC	*bla*$_{SHV}$	722	(Soliman et al., 2023)
SHV-RV	GGCCAAGCAGGGCGACAAT			
TEM-FW	TGAGAGTTTTCGCCCCGAA	*bla*$_{TEM}$	602	(Soliman et al., 2023)
TEM-RV	ACGGGAGGGCTTACCATCTG			
CTXM-FW	TGCAGYACCAGTAARGTKATGGC	*bla*$_{CTX-M}$	509	(Soliman et al., 2023)
CTXM-RV	CCGCTGCCGGTYTTATC			
OXA2-FW	ATAGTTGTGGCAGACGAACG	*bla*$_{OXA-2}$	452	(Soliman et al., 2023)
OXA2-RV	TTGACCAAGCGCTGATGTTC			
OXA5-FW	GTATTTCAACAAATYGCCAGAGA	*bla*$_{OXA-5}$	312	(Soliman et al., 2023)
OXA5-RV	CCACCAWGCGACACCAGGA			
OXA9-FW	CAGTTCCGTGGCTTCTGATG	*bla*$_{OXA-9}$	211	(Soliman et al., 2023)
OXA9-RV	GTTGTATTCCGGCTTCAATTCC			
Aminoglycoside acetyltransferase				
AAC6-FW	TTGCGATGCTCTATGAGTGGCTA	*aac(6′)-Ib*	274	(Soliman et al., 2023)
AAC6-RV	AGTTGTGATGCATTCGCCAG			
AmpC β-lactamases				
DHA-SPF	ACCGCTGATGGCACAGCAG	*bla*$_{DHA}$	648	(Soliman et al., 2023)
DHA-SPR	CAGCGCAGCATATCTTTTGAG			
CMY/LAT-SPF	AAAACAGAACAACARATTGCCGATA	*bla*$_{CMY}$	529	(Soliman et al., 2023)
CMY/LAT-SPR	GGACGCGTCTGGTCATTGCC			
ACT/MIR-SPF	CTGGGYTCTATAAGTAAAACCTTCACCG	*bla*$_{ACT}$	428	(Soliman et al., 2023)
ACT/MIR-SPR	CGGTATCCCCAGGCGTAATG			

Table S1 continued

MOX-SPF MOX-SPR	GCCCCGTGGTGGATGCCAG GYCCACTGGCGGTAGTAGGC	*bla*$_{MOX}$	392	(Soliman et al., 2023)
FOX-SPF FOX-SPR	TGGTCACCGGTTTATCCGGC GCATCTCCCTGATACCCCATGTT	*bla*$_{FOX}$	323	(Soliman et al., 2023)
PDC-SPF PDC-SPR	GACCTGCTGCGCTTCGTC GGTCTTGTTCAGCAGGCGCT	*bla*$_{PDC}$	263	(Soliman et al., 2023)
ADC-SPF ADC-SPR	CTTTTTATTTTTAGTACCTCAATTTATG TGCTATTTACGGCTTTTTTATCTTGAAC	*bla*$_{ADC}$	202	(Soliman et al., 2023)
ACC-SPF ACC-SPR	GATGAGAGCAAAATTAAAGACACCG AGGCTGTTTTGCCGCTAACC	*bla*$_{ACC}$	141	(Soliman et al., 2023)
Integrons				
IntI1-FW IntI1-RV	AGCTTGGCACCCAGCCTG GACACCGCTCCGTGGATC	*intI1*	192	(Soliman et al., 2023)
IntI2-FW IntI2-RW	AAGGTTATGCGCTGAAAACTGAA TCTGCGTGTTTATGGCTACATG	*intI2*	159	(Soliman et al., 2023)
IntI3-FW IntI3-RV	CACCGAGAAGCAAGTGG AATCCGCTTGCGTTCTG	*intI3*	133	(Soliman et al., 2023)
5′ CS 3′ CS	GGCATCCAAGCAGCAAG AAGCAGACTTGACCTGA	Class 1 integron	-	Ahmed et al., 2007
hep74 hep51	CGGGATCCCGGACGGCATGCACGATTTGTA GATGCCATCGCAAGTACGAG	Class 2 integron	-	Ahmed et al., 2007
Carbapenemase-encoding genes				
KPC-Fw KPC-Rv	CGTCTAGTTCTGCTGTCTTG CTTGTCATCCTTGTTAGGCG	*bla*$_{KPC}$	798	Poirel et al., 2011
NDM-Fw NDM-Rv	GGTTTGGCGATCTGGTTTTC CGGAATGGCTCATCACGATC	*bla*$_{NDM}$	621	Poirel et al., 2011
OXA48-Fw OXA48-Rv	GCGTGGTTAAGGATGAACAC CATCAAGTTCAACCCAACCG	*bla*$_{OXA-48}$	438	Poirel et al., 2011
VIM-Fw VIM-Rv	GATGGTGTTTGGTCGCATA CGAATGCGCAGCACCAG	*bla*$_{VIM}$	390	Poirel et al., 2011

Table S1 continued

IMP-Fw	GGAATAGAGTGGCTTAAYTCTC	bla_{IMP}	232	Poirel et al., 2011
IMP-Rv	GGTTTAAYAAAACAACCACC			
Plasmid-mediated colistin resistance				
Multi mcr-1-/2-Fw	TATCGCTATGTGCTAAAGCCTG	*mcr-1*	1139	Jousset et al., 2019
Multi mcr-1-Rv	CGTCTGCAGCCACTGG			
Multi mcr-1-/2-Fw	TATCGCTATGTGCTAAAGCCTG	*mcr-2*	816	Jousset et al., 2019
Multi mcr-2-Rv	AAAATACTGCGTGGCAGGTAGC			
Multi mcr-3-Fw	CAATCGTTAGTTACACAATGATGAAG	*mcr-3*	676	Jousset et al., 2019
Multi mcr-3-Rv	AACACATCTAGCAGGCCCTC			
Multi mcr-4-Fw	ATCCTGCTGAAGCATTGATG	*mcr-4*	405	Jousset et al., 2019
Multi mcr-4-Rv	GCGCGCAGTTTCACC			
Multi mcr-5-Fw	GGTTGAGCGGCTATGAAC	*mcr-5*	207	Jousset et al., 2019
Multi mcr-5-RV	GAATGTTGACGTCACTACGG			
MCR-6-mp Fw	AGCTATGTCAATCCCGTGAT	*mcr-6*	252	Borowiak et al., 2020
MCR-6-mp Rv	ATTGGCTAGGTTGTCAATC			
MCR-7-Fw	GCCCTTCTTTTCGTTGTT	*mcr-7*	551	Borowiak et al., 2020
MCR-7-Rv	GGTTGGTCTCTTTCTCGT			
MCR-8Fw	TCAACAATTCTACAAAGCGTG	*mcr-8*	856	Borowiak et al., 2020
MCR-8RV	AATGCTGCGCGAATGAAG			
MCR-9F	TTCCCTTTGTTCTGGTTG	*mcr-9*	1011	Borowiak et al., 2020
MCR-9R	GCAGGTAATAAGTCGGTC			
MCR-10F	TGTTTGATCAGTCCATGATC	*mcr-10*	439	-
MCR-10R	CGTGCCGTTTCGCCAATTA			
Phylogenetic group				
chuA.1b	ATGGTACCGGACGAACCAAC	*chuA*	288	Clermont et al., 2013
chuA.2	TGCCGCCAGTACCAAAGACA			
yjaA.1b	CAAACGTGAAGTGTCAGGAG	*yjaA*	211	Clermont et al., 2013
yjaA.2b	AATGCGTTCCTCAACCTGTG			
TspE4C2.1b	CACTATTCGTAAGGTCATCC	TspE4.C2	152	Clermont et al., 2013
TspE4C2.2b	AGTTTATCGCTGCGGGTCGC			

Table S1 continued

AceK.f	AACGCTATTCGCCAGCTTGC	*arpA*	400	Clermont et al., 2013	
ArpA1.r	TCTCCCCATACCGTACGCTA				
ArpAgpE.f	GATTCCATCTTGTCAAAATATGCC	*arpA* (Group C)	301	Clermont et al., 2013	
ArpAgpE.r	GAAAAGAAAAAGAATTCCCAAGAG				
trpAgpC.1	AGTTTTATGCCCAGTGCGAG	*trpA* (Group E)	219	Clermont et al., 2013	
trpAgpC.2	TCTGCGCCGGTCACGCCC				