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Characteristics of *Escherichia coli* isolated from livestock and related materials in the Philippines

(フィリピンにおいて家畜及び関連材料から分離された大腸菌の特徴)

Lawrence P. Belotindos

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ABBREVIATIONS

AMC	Amoxicillin-clavulanic acid
AMP	Ampicillin
AMR	Antimicrobial resistance
ARG	Antimicrobial resistance gene
CAZ	Ceftazidime
CC	Clonal complex
CHL	Chloramphenicol
CIP	Ciprofloxacin
CLSI	Clinical and Laboratory Standards Institute
CST	Colistin
CTX	Cefotaxime
dNTP	Deoxynucleoside triphosphate
ESBL	Extended beta-lactamase
EDTA	Ethylenediaminetetraacetic acid
EAST1	Enteroaggregative heat-stable enterotoxin 1
FEP	Cefepime
FOX	Cefoxitin
GEN	Gentamicin
HGT	Horizontal gene transfer
ICAMR	Inter-Agency Committee on Anti-Microbial Resistance
IPM	Imipenem
KAN	Kanamycin
MDR	Multidrug-resistant
MEM	Meropenem
MGE	Mobile genetic element
MIC	Minimum inhibitory concentration
MLST	Multilocus sequence typing
NAL	Nalidixic acid
PCR	Polymerase chain reaction
PFGE	Pulsed-field gel electrophoresis
PMQR	Plasmid-mediated quinolone resistance
QRDR	Quinolone-resistance determining regions

S1-PFGE	S1-nuclease pulsed-field gel electrophoresis
SPSS	Statistical Package for Social Sciences
ST	Sequence type
STR	Streptomycin
STX	Sulfamethoxazole-trimethoprim
TET	Tetracycline
UPGMA	Unweighted Pair-Group Method with Arithmetic mean
WHO	World Health Organization

PREFACE

Antimicrobial resistance (AMR) is defined as the ability of microbes, such as bacteria, viruses, parasites or fungi to grow despite the presence of antimicrobials that would normally kill them. The development of drug resistance can be due to the inherent resistant characteristics of microorganisms or through the acquisition of genes from other organisms that can be passed both horizontally and vertically to their progeny [1]. However, AMR development was aggravated by many human factors largely through the misuse and abuse of antibiotics leading to the loss of antimicrobial efficacy and the spread of drug resistant pathogens in the community [2].

AMR is a serious concern in public and animal health, and the emergence of multiple-antibiotic-resistant bacteria constitutes a global problem that needs to be addressed [3]. Livestock are considered as one of the major natural reservoirs for AMR. Antimicrobial-resistant microorganisms in livestock may transfer their AMR genes to humans microflora via food animals and environmental contact [4] [5–7]. Additionally, foodborne diseases associated with food-producing animals are an important issue in developing countries where poor sanitation is maintained during collection and processing [8,9] and empirical treatment were common that could promote AMR development.

Major foodborne pathogens of great concern around the globe that causes outbreaks were *Salmonella*, *Campylobacter* and *Escherichia coli* [10]. According to WHO report, the estimated burden of foodborne diseases caused by 31 agents (bacteria, viruses, parasites, toxins and chemicals) each year was as many as 600 million, or almost 1 in 10 people in the world, fall ill after consuming contaminated food. Of these, 420,000 people die, including 125,000 children under the age of 5 years. The South-East Asia Region has the second highest burden of foodborne diseases per population, after the African Region. However, in terms of absolute numbers, more people fall ill and die from foodborne diseases every year than in any other WHO Region, with more than 150 million cases and 175,000 deaths in a year [4].

Documented foodborne disease outbreaks in the Philippines were caused by *Salmonella*, *Vibrio*, *Aeromonas* and *E. coli*. The top three food vehicles were meat-based dishes and processed meat products, fish and other sea dishes and bakery and confectionary products. According to the study, majority of morbidity cases were shown to be mainly outbreaks occurring outside the home, particularly in workplaces and schools [11]. Major contributory factors to the occurrence of outbreaks were improper storage temperature and poor hygienic

practices [12]. In total, 115 food and water borne outbreaks were reported and verified from 2012-2016. During this period, a total of 17,246 cases and 143 deaths were reported [13].

In 2017, the WHO published a list of leading global pathogens and their antimicrobial resistance [14]. The resistance of leading global pathogens to quinolones were among the top priorities. Quinolones are considered a first choice in the treatment of intestinal bacterial infections in humans and animals. They were developed in the 1960s and 1980s. They were completely synthetic compound and has a bactericidal effect on most Enterobacteriaceae [15,16]. Nalidixic acid is one of the first-generation quinolones and effective against urinary tract infections [16]. Ciprofloxacin, norfloxacin, oxofloxacin, pefloxacin, and enrofloxacin belong to the second generation (fluoroquinolones), showing greater potency and a broader spectrum [23,24]. They are particularly effective against Gram-negative bacteria and several Gram-posib

tive and intracellular bacteria. Fluoroquinolones had an additional fluorine atom at the C-6 position and a piperazinyl or related ring at the position C-7 on the quinolone molecule [16,17]. Soon after quinolone introduction, resistant isolates emerged, which seems unlikely because it is a fully synthetic drug [15,16]. Then, resistance towards quinolones has become widespread among Enterobacteriaceae in the decades, hampering its effectiveness towards stubborn bacterial pathogens [18].

Quinolones primarily inhibit the action of type II topoisomerases including DNA gyrase and topoisomerase IV. Type II topoisomerases are enzymes that mediate the relaxed-supercoiled and catenated-decatenated DNA and are crucial for several DNA-associated processes, such as replication and transcription [19]. DNA gyrase and topoisomerase IV are both heterotetrameric enzymes composed of two pairs of identical subunits, $GyrA_2GyrB_2$ and $ParC_2ParE_2$, respectively [20]. DNA gyrase was the primary target for Gram-negative bacteria, whereas topoisomerase IV in the Gram-positives bacteria [21]. Quinolones have been shown to bind to the DNA gyrase/topoisomerase IV–DNA complex. The intercalation of quinolone into DNA gyrase/topoisomerase IV-DNA complex is responsible for the inhibition of DNA replication and transcription, caused double-strand break [19,22].

Currently, the acquisition of quinolone resistance was associated with chromosomal mutations that alter the target sites and alter membrane permeability that reduced drug accumulation by efflux/influx pumps. Another quinolone resistance mechanism is by acquiring plasmid-mediated quinolone resistance (PMQR) genes [20].

Specific point mutations in DNA gyrase and topoisomerase IV, mostly confer high-level resistance are often found in a region termed the quinolone resistance determining region

(QRDR) [19,20]. Mutations in the QRDRs of these genes result in amino acid substitutions that architecturally altered the target protein, and subsequently, the drug-binding affinity of the enzyme. Mutations in QRDRs of GyrA and ParC are commonly found and less frequent in those of GyrB and ParE [23]. Resistance-conferring mutations outside the traditional QRDR have also been identified [19].

Recently, PMQR genes have been identified as an emerging clinical problem that usually leads to low-level resistance [19,20,23]. Currently, there are three clinically relevant genes related to plasmid-mediated quinolone resistance: (i) *qnr*, which encodes proteins belonging to the pentapeptide repeat proteins that protect target site; (ii) *aac(6')-Ib-cr*, acetylates the unsubstituted nitrogen of the C7 piperazine ring of norfloxacin and ciprofloxacin, which decreases drug activity; (iii) *qepA* and *oqxAB*, which mediates antibiotic efflux. Nonetheless, many PMQR determinants have been identify recently since the first PMQR was discover.

As with the 2019 Philippine AMR surveillance program report, clinical *E. coli* rates of resistance against fluoroquinolones and third generation cephalosporins have been increasing for the past 10 years [24]. The resistance rates against ciprofloxacin was 41.3% to 46.6% and ceftriaxone was 24.2% to 39.9%. Emerging resistance to carbapenem was also reported in 2019 with resistance rates of 1.1% to 6.4% for meropenem and slight decrease in imipenem from 10.6% to 6.0%, but not statically significant. Of all *E. coli* tested for extended-spectrum β – lactamase (ESBL) production, 53.2% were positive. *Salmonella enterica serovar* Typhi isolates have remained susceptible to first line antibiotics. Resistance rates of *S.* Typhi against ampicillin, co-trimoxazole, ceftriaxone and chloramphenicol still remained at less than 5% for the past 10 years. There has been an increase in resistance in *S.* Typhi to ciprofloxacin for the past two years, although not statistically significant. In non-typhoidal *Salmonella,* increasing resistance to levofloxacin was noted with resistance rates of 20.9%, which was higher than the rate of 5% reported in 2018 [24]. The resistance of non-typhoidal *Salmonella* against ciprofloxacin was seen to be decreasing from 14.3% to 9.8%. Among the confirmed non-typhoidal *Salmonella*, the most common identified were *S. enterica serovar* Typhimurium and *S. enterica serovar* Enteritidis, which were also the most common for the past five years. All of these data were mainly obtained through the antimicrobial resistance surveillance and monitoring system of Research Institute for Tropical Medicine of the Department of Health, the Philippines.

Unlike in human health sector, AMR surveillance system in animal health sector in the Philippines is still under development. The AMR surveillance system on animal health sector will prioritized zoonotic pathogen species (*Salmonella spp..*, and *Campylobacter spp.*)

and commensal bacteria (*E. coli* and *Enterococcus spp.*). According to Philippine Action Plan to Combat AMR 2019-2023, it is reported that limited studies were conducted in AMR related to livestock animals [25–30]. Surveillance of AMR within the entire agricultural sector is not yet unified, as some organizations conduct their own surveillance activities. Moreover, researches were difficult to access, and data were not yet disseminated to inform/share to both animal and human health stakeholders [2].

Likewise, the detection of quinolone/fluoroquinolone-resistant bacteria has been increasing in animal and their food products in the Philippines. For instance, the prevalence rates of nalidixic acid and ciprofloxacin resistance in strains isolated from food-producing animals and their food products were found from 10% to 97.5% and 5% to 88.4%, respectively [25,27,30–32]. In environmental samples from soil and agricultural irrigation water, the detected resistance rate against nalidixic acid and ciprofloxacin was up to 35.4% and 6.8%, respectively [28,29]. Previous studies focused on the phenotypic characterization of the resistance determinants of bacteria to some extent. However, those studies mostly scrutinized the ESBL resistance mechanism of bacteria [31,32].

Despite on molecular research advancement, study on genetic diversity and antimicrobial resistance genes (ARGs) profile of foodborne bacterial isolates was still limited in the Philippines. Molecular data on foodborne bacteria can provide better epidemiological view for tracing foodborne infection. Hence, I carried out a research including surveillance and characterization of antimicrobial resistant *E. coli* in the Philippines. This thesis aimed to provide overview of the AMR status in food-producing animals and animal-derived food and clonal distribution of quinolone non-susceptible *E. coli* in the Philippines. In addition, the generated molecular data on this study would fill up the AMR information/data gap in animal health sector in the Philippines. In chapter I, the prevalence of *E. coli* and quinolone resistant determinant were studied from various food-producing animals and animal-derived food. In chapter II, characterization of plasmids among *qnr*-harboring isolates were further investigated.

CHAPTER I

Prevalence and Characterization of Quinolone Resistance Determinants in *Escherichia coli* Isolated from Food-producing Animals and Animal-derived Food in the Philippines

1. Introduction

Antimicrobials are necessary tools to fight diseases that create an economic burden, while at the same time contributing to health, welfare, food safety, and food security for both animals and humans [33]. The overuse of antimicrobials has led to the emergence of antimicrobial-resistant microorganisms in food-producing animals and those products derived from them such as meat, eggs, and milk. Consuming or being in contact with food containing antimicrobial-resistant microorganisms can cause the development of foodborne diseases that are difficult to treat [34]. In 2010 alone, over 400,000 people died due to foodborne diseases, which were caused by microorganisms such as bacteria, with over one-third of these deaths being children under the age of five years [35]. Therefore, foodborne diseases and the emergence of antimicrobial resistance are both public health concerns that need to be addressed on a global scale.

Quinolones are essential antimicrobials to treat bacterial infections in both animals and humans. Due to the rapid development of and increase in quinolone-resistant strains, the WHO has recommended reducing their use in livestock [36–38]. Quinolones prevent the activity of DNA gyrase and topoisomerase IV, which results in chromosomal fragmentation and death of bacteria [23]. Quinolone resistance in bacteria is acquired by the presence of one or more target-site mutations at quinolone-binding sites known as QRDRs in genes encoding DNA topoisomerases (*gyrA*, *gyrB*, *parC*, and *parE*). The development of mutations alter the drug-binding affinity with target enzymes [20]. Although they confer a low-level resistance to quinolones such as *qnr*, *acc(6')-lb-cr*, and *qepA*, the recent discovery of PMQR genes has aggravated the concern of health organizations. Indeed, for example, gene *qnr* encodes the pentapeptide-repeat protein competing with quinolones, *acc(6')-lb-cr* encodes the mutated aminoglycoside acetyltransferase (which can modify ciprofloxacin), and *qepA* encodes an efflux pump protein. Moreover, these genes can be spread horizontally across Enterobacteriaceae and positively contribute to the development of chromosome-encoded quinolone resistance mechanisms [17,39,40].

Elucidating the mechanism underlying the acquisition of antimicrobial-resistant genes would enable a deeper understanding of transmission that is crucial for effective infection

control. The data on the quinolone-resistance acquisition mechanism of *E. coli* in food-producing animals and products derived from them are limited, despite *E. coli* being listed as a priority pathogen in the Philippines by the WHO. Therefore, the present study aimed to investigate the quinolone-resistance determinants in *E. coli* isolated from food-producing animals and their food products in the Philippines. Furthermore, to elucidate the dissemination of high-risk clones, in the present work, the relationship between quinolone-resistant isolates was analyzed.

2. Materials and Methods

2.1. Sample Collection, Bacterial Enrichment and Isolation

A total of 601 samples (beef, chicken, and pork samples from supermarkets, open-air markets, and abattoirs, milk samples from dairy buffalo farms, cloacal swabs and eggs from poultry farms, rectal swabs from pig farms, and environmental swabs from abattoirs and poultry and swine farms) were collected in the Philippines from November 2017 to July 2018. The samples were collected using a convenience sampling approach. Bacterial isolation was conducted using a culture-based method. Briefly, 25 g or an equal volume of each sample was suspended 1:10 in Brain Heart Infusion broth (Nissu Pharm Co. Ltd., Tokyo, Japan) and homogenized using a sample blender (Bag Homogenizer BH-W, AS ONE Corp., Osaka, Japan). Next, the homogenates were incubated for 18–22 h at 44 °C. After incubation, the homogenates were streaked on plates with MacConkey agar (Becton Dickinson Co., Ltd., Franklin Lakes, NJ, USA) and incubated for 18–22 h at 37°C. Five presumptive *E. coli* colonies (color: brick-red) were selected and identified using a standard biochemical test as previously described [41]. Confirmed *E. coli* isolates were stored at −20 °C in Luria–Bertani broth (Merck, Darmstadt, Germany) containing glycerol 50% *v*/*v* (Difco Laboratories, Inc., Detroit, MI, USA).

2.2. Antimicrobial Susceptibility Testing

All confirmed *E. coli* isolates were subjected to antimicrobial susceptibility testing using the Kirby–Bauer disc diffusion method according to the CLSI [42] standard protocol, using commercially available antibiotic discs (Becton Dickinson Co., Ltd.). Seventeen antimicrobial agents were used in the present study: kanamycin (30 µg), gentamicin (10 µg), streptomycin (10 µg), cefoxitin (30 µg), cefotaxime (30 µg), cefepime (30 µg), ceftazidime (30 µg), imipenem (10 µg), meropenem (10 µg), nalidixic acid (30 µg), ciprofloxacin (5 µg), ampicillin (10 µg), amoxicillin/clavulanic acid (20 µg/10 µg), chloramphenicol (30 µg), tetracycline (30 µg), sulfamethoxazole/trimethoprim (23.75 µg/1.25 µg), and colistin (10 µg). The results were classified as susceptible, intermediate, or resistant as per the diameter of the zone of inhibition, using Clinical and Laboratory Standards Institute (CLSI) breakpoints [42]. An isolate was considered multidrug-resistant (MDR) if it was resistant to at least one agent from three or more antimicrobial categories. In addition, the minimum inhibitory concentrations (MICs) of nalidixic acid and ciprofloxacin were determined using broth microdilution [43] for isolates harboring mutations in QRDR and/or PMQR genes, and the results interpreted according to the Clinical and Laboratory Standards Institute breakpoints [44]. *E. coli* ATCC

25922 was used as the control strain.

2.3. Detection of Quinolone Resistance Determinants

Using the disc diffusion method, a total of 141 *E. coli* isolates not susceptible to quinolones, intermediate and/or resistant to nalidixic acid and/or ciprofloxacin, were selected by screening for quinolone-resistance determinants. Genomic DNA was extracted using the boiling method. Briefly, the bacterial colonies were suspended in 500 µL of TE buffer [(Tris-HCl (10 mM), Ethylenediaminetetraacetic acid (EDTA) (1 mM)] in microcentrifuge tubes and subjected to 15 min of boiling. Immediately after boiling, the microcentrifuge tubes were centrifuged for 10 min at 12,000× *g* at room temperature. The supernatant containing DNA (100 µL) was transferred to new sterile microcentrifuge tubes and used for the PCR analysis. PMQR determinants (*qnrA*, *qnrB*, *qnrS*, *qepA*, and *acc(6')-lb-cr*) and amino-acid substitutions in the QRDRs of GyrA, GyrB, ParC, and ParE were determined by polymerase chain reaction (PCR) and sequencing as previously described [15,45–50]. The PCR mixture (20 µL) contained 1.25 U of Taq DNA polymerase (Promega, Madison, WI, USA), 1× PCR buffer (Promega), 0.2 mM of each Deoxynucleoside triphosphate (dNTP) (Takara Bio Inc., Shiga, Japan), 2.5 mM $MgCl_2$ (Promega), 1 µM of each primer, and 1 µL of DNA template. The PCR conditions for QRDR were as follows: for *gyrA* and *gyrB*, denaturation at 96°C for 1 min followed by 35 cycles of denaturation at 96°C for 10 s, annealing at 52°C for 10 s, an extension at 72°C for 30 s, and a final extension at 72°C for 5 min; for *parC* and *parE*, denaturation at 94°C for 3 min followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s, an extension at 72°C for 30 s, and a final extension at 72°C for 5 min. The PCR conditions for PMQR were as follows: for *qnrB*, denaturation at 95°C for 5 min followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 56°C for 40 s, an extension at 72°C for 1 min, and a final extension at 72°C for 10 min; denaturation at 95°C for 5 min followed by 35 cycles of denaturation at 94°C for 45 s, annealing at 51°C (for *qepA*), 53°C (for *qnrA* and *qnrS*), 55°C (for *acc(6')-lb-cr*) for 45 s, an extension at 72°C for 1 min, and a final extension at 72°C for 5 min.

PCR products were purified using ExoSAP® IT (Thermo Fisher Scientific Co., Ltd., MA, USA) and subjected for sequencing using a BigDye® ver. 3.1 Terminator Cycle Sequencing Kit (Thermo Fisher Scientific Co., Ltd.) in an ABI 3500 xL Genetic Analyzer (Thermo Fisher Scientific Co., Ltd.). The obtained sequences were confirmed using data from the National Center for Biotechnology Information website (www.ncbi.nlm.nih.gov/BLAST/ (accessed on 28 February 2021)). Furthermore, inferred amino-acid sequences of QRDR-encoding genes were aligned with the corresponding regions

of *E. coli* K-12 (GenBank accession no. AL513382.1) as a reference strain using the ClustalW program by MEGA v7.0.21.

2.4. Multilocus Sequence Typing Analysis

Genotyping of all *E. coli* isolates harboring mutations in QRDR and/or PMQR genes was conducted as per the multilocus sequence typing protocol for *E. coli* [51]. Seven housekeeping genes, namely, *adk*, *fumC*, *gyrB*, *icd*, *mdh*, *purA*, and *recA*, were amplified using the recommended primers. The PCR mixture was the same as that mentioned in Section 2.3. The PCR conditions were as follows: denaturation at 95°C for 5 min, followed by 30 cycles consisting of denaturation of 95°C for 1 min, annealing at 54°C (for *adk*, *fumC*, *icd*, and *purA*), 58°C (for recA), 60°C (for *mdh*) for 1 min, an extension at 72°C for 2 min, and a final extension at 72°C for 5 min. The amplified products were purified using ExoSAP® IT (Thermo Fisher Scientific Co., Ltd.) and subjected to bidirectional sequencing using the BigDye® ver. 3.1 Terminator Cycle Sequencing Kit (Thermo Fisher Scientific Co., Ltd.) in the ABI 3500 xL Genetic Analyzer (Thermo Fisher Scientific Co., Ltd.). To determine the respective alleles, sequence types (STs), colonal complexes (CCs), and singleton assignments, the obtained sequences were submitted to the MLST database (https://pubmlst.org/escherichia/andenterobase.warwick.ac.uk). A minimum spanning tree/UPGMA was generated following the cluster analysis of the MLST allelic profiles of the isolates using BioNumerics 6.6 software (Applied Maths, Sint-Martens-Latem, Belgium).

2.5. Detection of Virulence Genes

Major virulence determinants associated with major *E. coli* pathotypes were determined in all *E. coli* isolates harboring mutations in QRDR and/or PMQR genes. Pathotypes were identified according to the presence of specific virulence genes (VGs): Shiga toxin-producing *E. coli* (*stx1*, *stx2* and *eaeA*), typical/atypical Enteropathogenic *E. coli* (*eaeA*, and *bfpA*), Enterotoxigenic *E. coli* (*elt*, *STp*, *STh* and *astA*), Enteroinvasive *E. coli* (*invE*), and Enteroaggregative *E. coli* (*astA* and *aggR*), as described previously [52]. The PCR mixture was the same as that mentioned in Section 2.3. The PCR conditions were as follows: denaturation at 94°C for 2 min, followed by 30 cycles consisting of denaturation of 94°C for 1 min, annealing at 52°C (for *eae*), 55°C (for *aggR*, *elt*, *STp*, *STh*, *invE*, *astA*, and *recA*), 56°C (for *stx2*), and 58°C (for *stx1*) for 1 min, an extension at 72°C for 2 min, and a final extension at 72°C for 10 min. PCR amplicons were visualized on 1.5% agarose gels stained with GelRed (Biotium, Inc., CA, USA). After the gel electrophoresis, images of the PCR amplicons were captured using

Printgraph Classic (ATTO Corp., Tokyo, Japan). Control DNA (*E. coli* O157:H7 for *stx1* and *stx2*, *E. coli* O125:H45 for *eae* and *bfpA*, *E. coli* O6:H16 for *elt*, *STh*, *E. coli* O169:H41 for *STp*, *E. coli* O11: H30 for *aggR*, *E. coli* O25:HNM for *elt*, and *Shigella flexneri* 2a for *invE*) kindly provided by the Division of Microbiology, the Osaka Institute of Public Health, Japan was used in each PCR experiment.

2.6. Phylogenetic Group Analysis

All *E. coli* isolates harboring mutations in QRDR and/or PMQR genes were assigned to phylogenetic groups (A, B1, B2, or D) on the basis of the presence or absence of genes *chuA* and *yjaA* and the DNA fragment tspE4 C2 by triplex-PCR, as previously described [53]. The PCR mixture was the same as that mentioned in Section 2.3. The PCR conditions were as follows: denaturation at 94°C for 4 min, followed by 30 cycles of denaturation at 94°C for 5 s and annealing at 59°C for 10 s, and a final extension at 72°C for 5 min. PCR amplicons were visualized using 1.5% agarose gels stained with GelRed (Biotium, Inc., CA, USA), and their images were captured using Printgraph Classic (ATTO Corp.).

2.7. Data Analysis

The data were descriptively analyzed using SPSS software version 22.0 (IBM Corp., Armonk, NY, USA). Differences in proportions were compared using a χ^2 test or Fisher's exact test. All the tests were analyzed with a 95% confidence interval. A p-value less than 0.05 was considered statistically significant.

3. Results

3.1. Prevalence of *E. coli* in samples

Of the 601 samples collected, 339 (56.4%) were positive for *E. coli*, ranging from 47.8% to 87.2% in meat samples. The isolation rates of *E. coli* were 53.3% in cloacal swab samples, 64.4% in rectal swab samples, and 73.3% in environmental swab samples (4/4 from abattoirs, 23/31 from swine farms, and 6/10 from poultry farms). In contrast, only one egg sample (2%, 1/50) was positive for *E. coli*, and no *E. coli* isolate was found in milk samples (Table 1).

3.2. Antimicrobial Susceptibility

In total, 791 *E. coli* isolates were detected in samples. The antimicrobial susceptibility of isolates is shown in Table 2 . Of the tested antimicrobials, resistance rates to tetracycline, ampicillin, sulfamethoxazole-trimethoprim, chloramphenicol, streptomycin, nalidixic acid, kanamycin, and ciprofloxacin were 57.5%, 56.6%, 43.7%, 35.9%, 30.0%, 25.3%, 14.7%, and 11.6%, respectively. Resistance to colistin, carbapenems (imipenem and meropenem), and cephems (cefoxitin, cefotaxime, ceftazidime, and cefepime) was detected in less than 10% of the isolates (Table 2). Overall, 21.5% of the isolates were pan-susceptible, 78.6% were resistant to at least one antimicrobial agent, and 55.4% were pan-MDR (i.e., resistance to at least one agent in three or more antimicrobial categories) (Table 3). The multidrug-resistant rates were 27.2%, 48.4%, and 67.4% in beef, pork, and chicken samples, respectively. Notably, the MDR rate of isolates in chicken samples was significantly higher than that in other meat samples ($p < 0.05$). By contrast, while multidrug-resistant rates in swab samples were high, no significant differences were observed (Table 3).

3.3. QRDR and PMQR Determinant Analysis

In 141 isolates not susceptible to quinolones, 46.8% had an amino-acid substitution in the QRDR of GyrA, GyrB, ParC, and ParE; 19.8% harbored PMQR genes, whereas 14.9% had both of them. The predominant amino-acid substitution in the QRDR of GyrA was serine to leucine at codon 83 (Ser83Leu) (95.4%, 84/87) and aspartic acid to asparagine (Asp87Asn) (67.8%, 59/87) or tyrosine (Asp87Tyr) (3.4%, 3/87) at codon 87. In GyrB, serine to aspartic acid at codon 492 (Ser492Asn) (11.5%, 10/87) was the predominant amino-acid substitution. In ParC, the most frequent substitutions were serine to isoleucine (Ser80Ile) (70.1%, 61/87) or arginine (Ser80 Arg) (1.1%, 1/87) at codon 80 and glutamic acid to glycine at codon 84 (Glu84

Gly) (10.3%, 9/87). In ParE, the predominant amino-acid substitutions were serine to alanine at codon 458 (Ser452 Ala) (18.4%, 16/87) and isoleucine to phenylalanine at codon 464 (Ile464 Phe) (2.3%, 2/87). Amino-acid substitutions at codon 83 and 87 in GyrA, along with substitutions at codon 80 in ParC, were the most frequent substitution patterns (31/141) (Figure 1). Isolates carrying double amino-acid substitutions in GyrA plus a single or double amino-acid substitution in other QRDR genes demonstrated a high-level quinolone resistance (Table 4).

PMQR and *qnr*-family genes were detected in 49 (34.8%) isolates. The most frequent *qnr* was *qnrS1* found in 31 isolates (63.2%). In one of these isolates, *qnrS1* coexisted with *qnrA1*, while three isolates had additional amino-acid substitutions in *gyrA*. The second most frequent *qnr* was *qnrB4*, which was detected in 18 isolates (36.7%). All *qnrB4*-positive isolates had an additional amino-acid substitution in QRDR, with the exception of ParE (Figure 1). All PMQR-positive isolates displayed a variable resistance to quinolones (MIC of CIP <0.03 to >32 μg/mL and MIC of NAL 8 to >128 μg/mL). Notably, the presence of both of *qnrA1* and *qnrS1* exhibited a high-level quinolone resistance (MIC of CIP >32 μg/mL and MIC of NAL >128 μg/mL). The other PMQR genes, namely, *qepA* and *acc(6')-Ib-cr*, were not detected in any of the tested isolates (Figure 1 and Table 4). In total, 115 (81.6%) isolates had quinolone-resistance determinants, whereas, in 26 (18.4%), no quinolone-resistant determinants were observed.

3.4. Multilocus Sequence Typing and Phylogenetic Group Analysis

In 115 isolates harboring mutations in QRDR and PMQR determinants, 46 unique STs were identified. Seventy-eight (67.8%) of the isolates were clustered into 13 CCs, while 37 (32.2%) isolates were singletons. The most common CCs were CC155 (n = 16), followed by CC101 (n = 15), CC469 (n = 10), CC10, and CC206 (n = 9). Overall, ST155 (n = 13, 11.3%) was the most frequent ST, followed by ST162 (n = 11, 9.6%), ST359 (n = 9, 7.8%), and ST354 (n = 8, 7%). Thirteen STs were found in more than one sample type (Figure 1 and Figure 2).

The majority of the isolates were assigned to phylogenetic group B1 (55.7%) ($p < 0.05$), followed by group A (28.7%) and group D (15.7%). None of the isolates belonged to phylogenetic group B2 (Figure 1 and 3). While isolates with amino-acid substitutions in QRDRs were significantly associated with phylogenetic group B1 ($p < 0.05$), isolates harboring PMQR genes were significantly associated with phylogenetic group A ($p < 0.05$). In contrast, isolates that harbored both QRDR and PMQR genes were distributed evenly among phylogenetic groups (A, B1, and D) (Figure 3).

3.5. Prevalence of virulence genes

Twenty-six *E. coli* isolates harboring mutations in QRDR and/or PMQR genes carried one virulence gene, *astA*, encoding the enteroaggregative heat-stable enterotoxin 1 (EAST1) of Enteroaggregative *E. coli*. The remaining 89 (77.4%) isolates carried no virulence genes (Figure 1).

4. Discussion

E. coli is a common bacterium, but some of its strains can cause diseases in both animals and humans. Indeed, pathogenic *E. coli* is usually a food contaminant that can cause severe public health problems. Furthermore, *E. coli* serves as a reservoir for drug-resistant genes that can be horizontally transferred to other pathogenic bacteria [10]. In the present study, 54.6% of the samples were contaminated with *E. coli*, with the highest prevalence being observed in chicken meat (70.6–82.7%). The high-level contamination of *E. coli* in chicken was consistent with that reported by previous studies conducted in the Philippines [27,54], China [45], and Bangladesh [55]. Contamination of meat samples with pathogenic *E. coli* usually indicates poor hygiene during slaughter and handling, and unsuitable storage after slaughter [45,54]. Another source of contamination is meat supplied from unauthorized abattoirs (e.g., no proper hygiene inspections by authorities), which is then sold at local retail meat shops. Contamination with pathogenic bacteria jeopardizes food safety and human health. For example, according to a Philippines foodborne disease outbreaks report (2005–2018), 14.4% of infection cases were associated with animal-derived food [11]. Therefore, monitoring the food production process and the implementation of food hygiene practices is essential and, hence, improved practices and management in slaughterhouses must be implemented.

Among the antimicrobials used in the present study, resistance to tetracycline, ampicillin, and sulfamethoxazole/trimethoprim was frequently observed. The resistance pattern detected in the present work was concordant with previous reports in the Philippines [25,27,30]. These antimicrobials are commonly used in livestock and poultry in the Philippines [56]. The extent and pattern of antimicrobial-resistant rates seen in *E. coli* isolated from meat samples were very similar to those in isolates from farms. Therefore, a possible link could be established to antimicrobial usage at farm level, which leads to a high number of resistant *E. coli* in food products. Furthermore, indirect antimicrobial ingestion through animal-derived food consumption is projected to increase due to an increased demand in animal products in the Philippines [57]. To meet the demand, to maintain the health of animals, and to increase their productivity, there has been a shift in production systems from backyards to intensive production systems that rely more on antimicrobials. Notably, a high resistance to chloramphenicol has been found despite the practice being prohibited [58], and even resistance to carbapenems, which is not commonly used in livestock animals in the Philippines, was observed in the present study. The resistance to these two antimicrobials may be due to cross- or co-resistance against the same or other antimicrobial classes, which were possibly used illegally [27]. Therefore, there is a need to fully enforce the laws regarding the usage of these

antimicrobials and to monitor the presence of resistant bacteria that may pose a risk to food safety and public health.

Overall, 55.4% of the *E. coli* isolates were multidrug-resistant. The *E. coli* MDR rates in animals and animal-derived food in this study were lower compared with those of previous studies in the Philippines (70–95%) [25,27,30] and neighboring countries such as Thailand and Cambodia (75.3%) [41]. Yet, it was slightly higher than in food samples from Myanmar (50%) [55] and in human clinical samples (46% in blood samples) [24] in the Philippines. These discrepancies may be explained by the fact that previous studies focused on specific resistance phenotypes [40], with differences in target samples, geographical dominant strains, and numbers of samples tested.

Quinolone and fluoroquinolones are the drugs of choice for human foodborne and other infections caused by *Salmonella* and *E. coli* [59,60]. They are also used as prophylactics and for treatment of chronic respiratory diseases, skin and soft tissue infections, urinary tract infections, enteritis, and mastitis in animals [61]. However, there is an increasing presence of resistance to quinolone and fluoroquinolone in bacteria that threaten their efficacy. They are, therefore, classified as high-priority, critical drugs, and it has been recommended to reduce their use in food animals [62]. Some countries (the United States of America (USA), Finland, the Netherlands, and Australia) have already reduced or banned altogether the usage of fluoroquinolones in food-producing animals [36,37,63]. In Southeast Asia, however, including the Philippines, they are still employed in animal production. In the present study, high numbers of *E. coli* not susceptible to quinolones/fluoroquinolones were observed in chicken, followed by swine-associated samples, which were similar to those detected by recent but unrelated reports in the Philippines [31,32]. These findings may correspond with the use of quinolone/fluoroquinolone in poultry and swine production in the Philippines. There are at least three to four quinolones/fluoroquinolones, primarily enrofloxacin, used in poultry and swine (backyard/commercial) farms as growth-promoting, therapeutic, or prophylactic agents [56]. Therefore, to minimize the development/acquisition of resistance to these antimicrobials, it is necessary to establish a proper monitoring of the usage of antimicrobials in the Philippines.

The prevalence of mutations in QRDR and PMQR genes in *E. coli* isolates from humans, animals, and the environment has been found in many countries [63–68]. However, limited data are available on the presence of chromosomal mutations in the QRDR and PMQR genes conferring resistance to quinolones in samples from food-producing animals in the Philippines. In the present study, we found that isolates not susceptible to quinolones had predominantly double amino-acid substitutions in *GyrA* (Ser83 to Leu; Asp87 to Asn) and an

amino-acid substitution in *ParC* (Ser80 to Ile) (Table 4). This QRDR mutation pattern has been frequently observed in fluoroquinolone-resistant *E. coli* clinical isolates in the Philippines and elsewhere [65]. In the previous studies, a single mutation of Ser83 in *gyrA* was enough to cause a high-level resistance to nalidixic acid and decreased susceptibility to fluoroquinolones. However, with an additional mutation in *gyrA* and *parC*, it is a stepwise event that caused a high-level resistance to nalidixic acid and ciprofloxacin [20,23,39,61]. This evidence correlates with our results showing the observed isolates having these mutations, as well as high resistance to nalidixic acid (MIC > 128 μg/mL) and full resistance to ciprofloxacin (MIC 4 to >32 μg/mL).

The *qnr* are known to protect DNA gyrase against the effect of quinolones [18,61,69]. We found that 34.8% of isolates not susceptible to quinolones harbored *qnr* in the present study. Most of the *qnr*-harboring isolates were moderately to fully resistant to nalidixic acid (MIC 8 to >128 μg/mL) and susceptible to less susceptible to ciprofloxacin (MIC 0.25 to 2 μg/mL). In addition, we observed a coexistence of *qnrA1* and *qnrS1* in an isolate with high MIC (MIC of ciprofloxacin >32 μg/mL and MIC of nalidixic acid >128 μg/mL). The identification of *qnrA1* in the present work is the first reported in the Philippines. [45,68]. The MICs of ciprofloxacin for *E. coli* harboring *qnrA1* were reported to be 0.12–0.25 μg/mL [45,68]. In the present study, isolates harboring *qnrS1* alone had 0.25–2 μg/mL MIC of ciprofloxacin (Table 4). These findings seem to indicate that the combination of *qnrA1* and *qnrS1* did not compete for binding in gyrases and had a synergistic or additive effect with the MIC, although the actual mechanism remains unclear. Coexistence of *qnrA* and *qnrS* has also been found in *Enterobacter cloacae* showing higher (2–8-fold) MICs of quinolones than strains harboring only *qnrA*, seemingly indicating that both genes had an additive effect when conferring quinolone resistance [69]. In contrast, past work showed the coexistence of *qnr* in one isolate, which tended to have the same resistance activity as that of a single one [18]. In the present study, we identified *qnr* (*qnrA1*, *qnrB4*, and *qnrS1*) in 28 isolates not susceptible to quinolones. Gene *qnrS1*, in particular, was observed most frequently, which is in agreement with work reported elsewhere [40,45,64,70]. Again, this result seems to indicate that *qnrS1* is the predominant gene in food-producing animals and in their food products. Within the qnr family, *qnrB* is the most commonly observed [16]. In the present study, however, *qnrB4* was the only one observed among *qnrB* alleles, but it was less predominant and mostly detected in chicken meat samples, similar to data reported in Korea [38]. Gene *qnrB4* was also found in extended-spectrum beta-lactamase-producing Enterobacteriaceae from clinical samples in the Philippines [71,72]. PMQR genes are often found in plasmids with other antibiotic-resistant genes and can be horizontally transferred to other bacteria even without antibiotic exposure, thus becoming a source of quinolone resistance

during infection in humans [18,69]. However, in the present study, a plasmid carrying the *qnr* was not implemented, whether polymerase chain reaction assay, whole genome sequencing, or S1 nuclease pulsed field gel electrophoresis; hence, this should be further elucidated.

Molecular typing studies of *E. coli* are of limited scope in the Philippines. In the present study, ST155, ST162, ST359, and ST354 were predominantly detected in *E. coli* isolates having mutations in QRDR and/or PMQR genes. These clones were previously reported to be associated with human and animal infections [73–75]. In addition, *E. coli* ST38 and ST155 are considered high-risk clones that disperse antibiotic resistance on a global scale. These clones have acquired adaptive traits that increase pathogenicity to colonize, spread, and thrive in a variety of niches [76,77]. Clones ST10, ST48, ST162, and ST206, detected in the present work in beef, chicken, pork, and cloacal swab samples, were previously associated with carbapenemase-producing *E. coli*, which was isolated from hospital sewage and river samples in the Philippines, and they showed a reduced to high level of resistance to levofloxacin [78]. Moreover, ST10 and ST117 have been associated with emerging extraintestinal pathogenic *E. coli* (ExPEC) lineages that cause infections in humans [79]. In the present study, all ST10 isolates were MDR and mostly carried *qnrs* and a virulence gene, whereas ST117 was recovered from two different samples (chicken and pork), with both isolates being multidrug-resistant with mutations in QRDRs. ST117 is a well-recognized avian pathogenic *E. coli* with zoonotic potential [80]. In addition, in the present work, 13 STs were observed in multiple sources, seemingly indicating a possible interspecies transmission that potentially poses a risk to humans via direct contact with food-producing animals and/or consumption of contaminated animal-derived food. Therefore, to prevent possible infection outbreaks, there is a need to screen for the presence of these pathogenic STs in food-producing animal and their food products.

In the present study, the majority of the isolates belonged to phylogenetic groups A, B1, and D. These results are similar to those reported in previous work on *E. coli* not susceptible to quinolones [64]. Although *E. coli* belonging to phylogenetic groups A and B1 are classified as environmental and commensal *E. coli*, strains that belong to group D are classified as potential extraintestinal pathogenic strains [53]. In the present work, it was found that 15.7% of *E. coli* isolates harboring mutations in QRDR and/or PMQR genes belonged to phylogenetic group D. These isolates include *E. coli* clonal lineages (ST38, ST117, and ST354), which are human-associated, fluoroquinolone-resistant lineages that cause extraintestinal infection [80,81]. These findings indicate that these isolates may also carry pathogenic characteristics of ExPEC strains. Furthermore, 22.6% of the *E. coli* isolates harboring mutations in QRDR and/or PMQR genes in the present study harbored *astA*, encoding EAST1. It can be, therefore,

hypothesized that *astA* could elicit a cyclic guanosine monophosphate increase, leading to the loss of electrolytes and water from the epithelial intestinal cells, similar to the heat-stable enterotoxin mechanism [82]. Past epidemiological studies have shown that this gene was also present in other major pathogenic *E. coli* and even in a commensal strain [83]. In addition, the association of *astA* with foodborne outbreaks has been reported in many countries including Japan, Chile, Thailand, and Kenya [84–87]. The presence of EAST1 in food-producing animals and their food products is of great concern due to the notion that the spread of enteroaggregative *E. coli* strains may increase food-borne diarrheic infections. Therefore, these isolates have the potential to cause human infection given the ideal conditions, whereby it is difficult to render treatment due to predominantly MDR isolates carrying quinolone-resistance determinants.

5. Summary

Antimicrobial resistance to quinolones, which constitutes a threat to public health, has been increasing worldwide. In this study, we investigated the prevalence and quinolone-resistant determinants in *E. coli* not susceptible to quinolones and isolated from food-producing animals and food derived from them, in the Philippines. A total of 791 *E. coli* strains were isolated in 56.4% of 601 beef, chicken, pork, egg, and milk samples, as well as environmental, cloacal, and rectal swab-collected samples from supermarkets, open markets, abattoirs, and poultry, swine, and buffalo farms. Using the disc diffusion method, it was determined that 78.6% and 55.4% of the isolates were resistant to at least one antimicrobial and multiple drugs, respectively. In 141 isolates not susceptible to quinolones, 115 (81.6%) harbored quinolone-resistant determinants and had mutations predominantly in the QRDRs of *gyrA* and *parC*. PMQR and Qnr family (*qnrA1*, *qnrB4*, and *qnrS1*) genes were detected in all isolates. Forty-eight sequence types were identified in isolates harboring mutations in QRDR and/or PMQR genes by multilocus sequence typing analysis. Moreover, 26 isolates harboring mutations in QRDR and/or PMQR genes belonged mostly to phylogroup B1 and Enteroaggregative *E. coli*. In conclusion, a high prevalence of *E. coli* was found in food-producing animals and products derived from them, which could potentially spread high-risk clones harboring quinolone-resistance determinants.

Table 1. Prevalence of *Escherichia coli* in food-producing animals and their food products.

Sample source	Sample type	Number of samples		95% confidence interval
		Examined	Positive (%)	
Supermarket	Beef	54	31 (57.4)	0.432 – 0.708
	Chicken	47	41 (87.2)	0.743 – 0.952
	Pork	68	44 (64.7)	0.522 – 0.759
Open market	Beef	52	26 (50.0)	0.358 – 0.642
	Chicken	68	50 (73.5)	0.614 – 0.835
	Pork	48	34 (70.8)	0.559 – 0.831
Abattoir	Beef	28	15 (53.6)	0.339 – 0.725
	Pork	23	11 (47.8)	0.268 – 0.694
	Environmental swab	4	4 (100.0)	0.398 – 1.000
Swine farm	Rectal swab	43	29 (67.4)	0.515 – 0.809
	Environmental swab	31	23 (74.2)	0.554 – 0.881
Poultry farm	Cloacal swab	45	24 (53.3)	0.379 – 0.683
	Environmental swab	10	6 (60.0)	0.262 – 0.878
	Egg	50	1 (2.0)	0.001 – 0.107
Buffalo farm	Milk	30	0 (0.0)	0.000 – 0.116
Total tested		601	339 (56.4)	0.523 – 0.604

Table 2. Antimicrobial resistance profiles of *E. coli* isolates in samples.

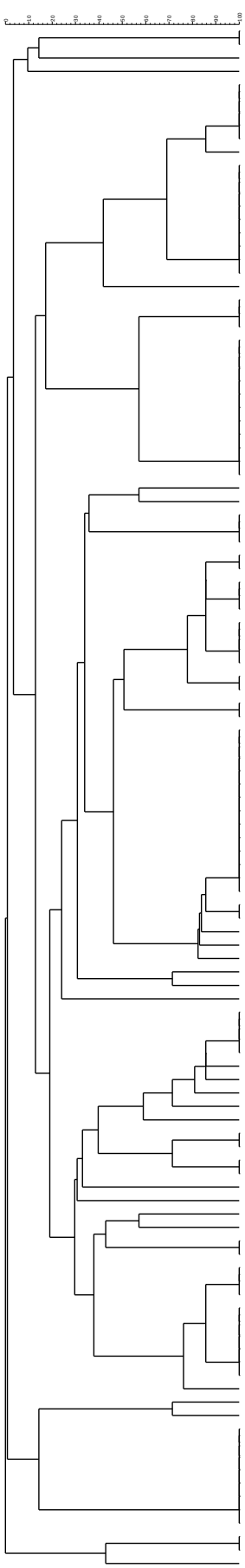
Antimicrobial agents	Sample, *n* (%)							Total (*n*=791)
	Beef (*n*=191)	Chicken (*n*=224)	Pork (*n*=188)	Egg (*n*=8)	Cloacal swabs (*n*=52)	Rectal swabs (*n*=55)	Environmental swabs (*n*=73)	
TET	66 (34.6)	148 (66.1)	93 (49.5)	3 (37.5)	40 (76.9)	53 (96.4)	52 (71.2)	455 (57.5)
AMP	63 (33.0)	143 (63.8)	104 (55.3)	7 (87.5)	34 (65.4)	46 (83.6)	51 (69.9)	448 (56.6)
SXT	32 (16.8)	104 (46.4)	87 (46.3)	2 (25.0)	39 (75.0)	37 (67.3)	45 (61.6)	346 (43.7)
CHL	37 (19.4)	86 (38.4)	65 (34.6)	1 (12.5)	16 (30.8)	39 (70.9)	40 (54.8)	284 (35.9)
STR	32 (16.8)	92 (41.1)	49 (26.1)	0	21 (40.4)	19 (34.5)	24 (32.9)	237 (30.0)
NAL	11 (5.8)	101 (45.1)	23 (12.2)	0	43 (82.7)	13 (23.6)	9 (12.3)	200 (25.3)
KAN	9 (4.7)	64 (28.6)	7 (3.7)	0	15 (28.8)	15 (27.3)	6 (8.2)	116 (14.7)
CIP	2 (1.0)	57 (25.4)	6 (3.2)	0	21 (40.4)	3 (5.5)	3 (4.1)	92 (11.6)
CST	13 (6.8)	38 (17.0)	9 (4.8)	2 (25.0)	6 (11.5)	5 (9.1)	5 (6.8)	78 (9.9)
AMC *	6 (6.8)[a]	12 (10.3)[b]	10 (11.4)[c]	0[d]	0[e]	4 (10.0)[f]	0[g]	32 (8.9)
IPM	3 (1.6)	43 (19.2)	5 (2.7)	0	15 (28.8)	2 (3.6)	2 (2.7)	70 (8.8)
GEN	5 (2.6)	42 (18.8)	11 (5.9)	0	2 (3.8)	2 (3.6)	5 (6.8)	67 (8.5)
FOX	10 (5.2)	28 (12.5)	13 (6.9)	0	9 (17.3)	3 (5.5)	3 (4.1)	66 (8.3)
CTX*	4 (4.5)[a]	6 (5.1)[b]	2 (2.3)[c]	0[d]	6 (60.0)[e]	2 (20.0)[f]	0[g]	20 (5.5)
CAZ	5 (2.6)	9 (4.0)	3 (1.6)	0	3 (5.8)	1 (1.8)	3 (4.1)	24 (3.0)
FEP	3 (1.6)	6 (2.7)	1 (0.5)	0	4 (7.7)	3 (5.5)	1 (1.4)	18 (2.3)
MEM*	0[a]	2 (1.7)[b]	1 (1.1)[c]	0[d]	0[e]	0[f]	0[g]	3 (0.8)

KAN – kanamycin; STR – streptomycin; GEN – gentamicin; FOX – cefoxitin; CTX – cefotaxime; FEP – cefepime; CAZ – ceftazidime; IPM – imipenem; MEM – meropenem; AMP – ampicillin; AmC – amoxicillin-clavulanic acid; CHL – chloramphenicol; TET – tetracycline; STX – trimethoprim-sulfamethoxazole; CST – colistin. (*): Only 361 samples were tested to AM C, CTX, MEM: [a] Beef = 88; [b] Chicken = 117; [c] Pork = 8; [d] Egg = 10; [e] Cloacal swab = 40; [f] Rectal swab = 10; [g] Environmental swab = 88 samples, respectively.

Table 3. Distribution of multidrug-resistant E. coli isolates in samples.

No. of antimicrobials classes	Number (%) of quinolone-resistant isolates							
	Beef (*n*=191)	Chicken (*n*=224)	Pork (*n*=188)	Egg (*n*=8)	Cloacal swabs (*n*=52)	Rectal swabs (*n*=55)	Environmental swabs (*n*=73)	Total (*n*=791)
0	89 (46.6)	20 (8.9)	51 (27.1)	0 (0.0)	2 (3.8)	1 (1.8)	6 (8.2)	169 (21.4)
1-2	50 (26.2)	52 (23.2)	46 (24.5)	6 (75.0)	5 (9.6)	6 (10.9)	19 (26.0)	184 (23.3)
3-4	38 (19.9)	67 (29.9)	52 (27.7)	2 (25.0)	20 (38.5)	19 (34.5)	27 (37.0)	225 (28.4)
5-6	13 (6.8)	60 (26.8)	37 (19.7)	0	16 (30.8)	25 (45.5)	18 (24.7)	169 (21.4)
7-8	1 (0.5)	24 (10.7)	2 (1.1)	0	8 (15.4)	4 (7.3)	3 (4.1)	42 (5.3)
>9	0	1 (0.4)	0	0	1 (1.9)	0	0	2 (0.3)
Resistance ≥1	102 (53.4)	204 (91.1)	137 (72.9)	8 (100.0)	50 (96.2)	54 (98.2)	48 (65.8)	622 (78.6)
MDR ≥3	52 (27.2)	152 (67.9)*	91 (48.4)	2 (25.0)	45 (86.5)	48 (87.3)	48 (65.8)	438 (55.4)

(*): The *P* value for beef, chicken and pork sample data was significant ($p < 0.05$).



				MIC (ug/ml)			Gyrase and topoisomerase substitutions						
ST	CC	Origin	Sample	NAL	CIP	PMQR gene	gyrA	gyrB	parC	parE	Phylogroup	astA	Resistance profile
ST117	Singleton	Public market	Chicken	>128	8	qnrB4	S83L	S492N	S80R	WT	D	-	[KAN],STR,GEN,FOX,CTX,[CAZ],NAL,[CIP],AmC,AMP,CHL,TET,SXT
ST117	Singleton	Public market	Pork	>128	0.5	-	S83L	S492N	WT	WT	D	+	STR, GEN, NAL, AMP, TET, SXT
ST1607	Singleton	Supermarket	Chicken	>128	2	qnrS1	D87Y	WT	WT	WT	D	+	KAN, STR, GEN, NAL, AMP, CHL, TET, SXT
ST38	CC38	Public market	Chicken	8	<0.03	qnrB4	D87Y	WT	S80I	WT	D	+	KAN, STR, FOX, [CTX], [CAZ], NAL, AmC, AMP, [CHL], TET, SXT
ST101	CC101	Supermarket	Pork	>128	0.5	-	D87Y	WT	WT	WT	B1	-	NAL, TET
ST101	CC101	Swine farm	Rectal swab	64	2	qnrS1	WT	WT	WT	WT	B1	-	KAN,[NAL],AMP,CHL,TET,SXT
ST101	CC101	Swine farm	Rectal swab	16	1	qnrS1	WT	WT	WT	WT	B1	-	KAN,[NAL],AMP,CHL,TET,SXT
ST101	CC101	Supermarket	Pork	>128	32	-	S83L, D87N	WT	S80I	WT	B1	-	[STR],[IPM],NAL,[CIP],CHL,TET
ST101	CC101	Swine farm	Rectal swab	>128	32	-	S83L, D87N	WT	S80I	WT	B1	-	IPM,NAL,AMP,CHL,TET
ST5229	CC101	Supermarket	Pork	>128	32	-	S83L, D87N	WT	S80I	WT	B1	+	[KAN], STR, GEN,NAL, CIP, AMP, CHL, TET, SXT
ST359	CC101	Supermarket	Pork	>128	32	-	S83L, D87N	WT	S80I	WT	D	-	NAL, CIP, CHL, TET, SXT
ST359	CC101	Public market	Chicken	>128	>32	-	S83L, D87N	WT	S80I	WT	B1	-	[STR],IPM,NAL,[CIP],AMP,CHL,TET,SXT
ST359	CC101	Public market	Chicken	>128	32	-	S83L, D87N	WT	S80I	WT	B1	-	[STR],IPM,NAL,[CIP],AMP,CHL,TET,SXT
ST359	CC101	Public market	Chicken	>128	32	-	S83L, D87N	WT	S80I	WT	B1	-	STR,IPM,NAL,[CIP],AMP,CHL,TET,SXT
ST359	CC101	Supermarket	Chicken	>128	32	-	S83L, D87N	WT	S80I	WT	B1	-	[STR],IPM,NAL,CIP,AMP,CHL,TET,SXT
ST359	CC101	Supermarket	Chicken	>128	4	-	S83L, D87N	WT	S80I	WT	B1	-	[STR],IPM,NAL,[CIP],AMP,CHL,TET,SXT
ST359	CC101	Supermarket	Chicken	>128	>32	-	S83L, D87N	WT	S80I	WT	B1	-	[STR],IPM,NAL,[CIP],AMP,CHL,TET,SXT
ST359	CC101	Supermarket	Chicken	>128	>32	-	S83L, D87N	WT	S80I	WT	B1	-	[STR],IPM,NAL,CIP,AMP,CHL,TET,SXT
ST359	CC101	Supermarket	Chicken	>128	32	-	S83L, D87N	WT	S80I	WT	B1	-	STR,IPM,NAL,CIP,AMP,CHL,TET,SXT
ST2144	Singleton	Public market	Chicken	>128	32	qnrS1	S83L	WT	S80I	WT	D	+	[STR], NAL, CIP, AMP
ST1431	Singleton	Poultry farm	Cloacal swab	>128	>32	-	S83L, D87N	WT	S80I	S458A	B1	-	IPM,NAL,CIP,AMP
ST1431	Singleton	Poultry farm	Cloacal swab	>128	>32	-	S83L, D87N	WT	S80I	S458A	B1	-	STR,NAL,AMP,CHL,TET,SXT
ST1431	Singleton	Poultry farm	Cloacal swab	>128	>32	-	S83L, D87N	WT	S80I	S458A	B1	-	KAN,[IPM],NAL,[CIP],AMP,TET,SXT
ST162	CC469	Public market	Chicken	>128	4	-	S83L, D87N	WT	S80I	WT	B1	+	STR,IPM,NAL,[CIP],[AMP],TET,SXT
ST162	CC469	Supermarket	Chicken	>128	4	-	S83L, D87N	WT	WT	WT	B1	+	STR,IPM,NAL,[CIP],AMP,TET,SXT
ST162	CC469	Poultry farm	Cloacal swab	>128	32	-	S83L, D87N	WT	S80I	WT	B1	-	STR,IPM,NAL,[CIP],AMP,TET,SXT
ST162	CC469	Poultry farm	Cloacal swab	>128	16	-	S83L, D87N	WT	S80I	WT	B1	-	STR,NAL,[CIP],SXT
ST162	CC469	Poultry farm	Cloacal swab	>128	32	-	S83L, D87N	WT	S80I	WT	B1	-	[STR],[IPM],NAL,[CIP],AMP,TET,SXT
ST162	CC469	Poultry farm	Cloacal swab	>128	32	-	S83L, D87N	WT	S80I	WT	B1	-	[STR],[IPM],NAL,[CIP],AMP,TET,SXT
ST162	CC469	Poultry farm	Cloacal swab	>128	16	-	S83L, D87N	WT	S80I	WT	B1	-	[STR],[IPM],NAL,[CIP],AMP,TET,SXT
ST162	CC469	Poultry farm	Cloacal swab	>128	32	-	S83L, D87N	WT	S80I	WT	B1	-	[STR],[IPM],NAL,[CIP],AMP,TET,SXT
ST162	CC469	Poultry farm	Cloacal swab	>128	16	-	S83L, D87N	WT	S80I	WT	B1	-	[STR],[IPM],NAL,[CIP],AMP,TET,SXT
ST162	CC469	Poultry farm	Cloacal swab	>128	16	-	S83L, D87N	WT	S80I	WT	B1	-	[STR],[IPM],NAL,AMP,TET,SXT
ST162	CC469	Poultry farm	Cloacal swab	>128	16	-	S83L, D87N	WT	S80I	WT	B1	-	[IPM],NAL,[CIP]
ST8081	Singleton	Public market	Chicken	>128	>32	-	S83L, D87N	WT	S80I, E84G	WT	B1	-	KAN, STR, NAL, CIP, AMP, CHL, TET, SXT
ST5409	Singleton	Swine farm	Environment swab	8	0.25	qnrS1	WT	WT	WT	WT	B1	+	[NAL],AMP,CHL,TET
ST1324	Singleton	Supermarket	Beef	32	1	qnrS1	WT	WT	WT	WT	A	-	STR,[CIP]
ST1324	Singleton	Public market	Chicken	16	1	qnrS1	WT	WT	WT	WT	A	-	STR,CTX,[FEP],MEM,[IPM],[NAL],AmC
ST1324	Singleton	Public market	Chicken	32	1	qnrS1	WT	WT	WT	WT	A	-	[KAN],STR,[FOX],[NAL],[CIP],TET,CST
ST5712	Singleton	Public market	Chicken	>128	>32	-	S83L, D87N	WT	S80I	S458A	B1	+	KAN,STR,[GEN],IPM,NAL,CIP,AMP,CHL,TET,SXT
ST5712	Singleton	Public market	Chicken	>128	>32	-	S83L, D87N	WT	S80I	S458A	B1	+	KAN,STR,[GEN],[FOX],IPM,NAL,CIP,AMP,[CHL],TET,SXT
ST3753	Singleton	Public market	Chicken	>128	>32	-	S83L, D87N	WT	S80I	S458A	B1	+	KAN,STR,GEN,IPM,NAL,CIP,AMP,CHL,TET,SXT
ST3753	Singleton	Public market	Chicken	>128	>32	-	S83L, D87N	WT	S80I	S458A	B1	+	KAN,STR,IPM,NAL,CIP,AMP,CHL,TET,SXT
ST3753	Singleton	Public market	Chicken	>128	>32	-	S83L, D87N	WT	S80I	S458A	B1	+	KAN,STR,GEN,IPM,NAL,CIP,AMP,CHL,TET,SXT
ST224	Singleton	Swine farm	Environment swab	>128	>32	-	S83L, D87N	WT	S80I	S458A	B1	-	STR,[GEN],IPM,NAL,CIP,AMP,CHL,[TET],SXT
ST224	Singleton	Public market	Chicken	>128	>32	-	S83L, D87N	WT	S80I	S458A	B1	+	KAN,STR,GEN,IPM,NAL,CIP,AMP,CHL,TET,SXT
ST224	Singleton	Public market	Chicken	>128	>32	-	S83L, D87N	WT	S80I	S458A	B1	+	KAN,STR,[GEN],NAL,CIP,AMP,CHL,TET,SXT
ST224	Singleton	Supermarket	Chicken	>128	>32	-	S83L, D87N	WT	S80I	S458A	B1	+	KAN,STR,[GEN],IPM,NAL,CIP,AMP,CHL,TET,SXT
ST7081	Singleton	Public market	Chicken	>128	>32	-	S83L, D87N	WT	S80I	S458A	B1	-	KAN, STR, [GEN], [CAZ], NAL, CIP, AMP, CHL, TET, SXT
ST7081	Singleton	Poultry farm	Cloacal swab	128	0.5	-	S83L, D87N	WT	S80I	S458A	B1	+	KAN,STR,FOX,IPM,NAL,CHL,TET,SXT
ST1196	Singleton	Public market	Chicken	>128	>32	-	S83L, D87N	WT	S80I	S458A	B1	+	KAN, STR, GEN, NAL, CIP, AMP, CHL, TET, SXT
ST1196	Singleton	Supermarket	Chicken	>128	>32	-	S83L, D87N	WT	S80I	S458A	B1	-	KAN, STR, NAL,CIP, AMP, CHL, TET, SXT
ST155	CC155	Poultry farm	Cloacal swab	>128	32	-	S83L, D87N	WT	S80I	WT	B1	+	KAN,STR,IPM,NAL,[CIP],AMP,[CHL],TET,SXT
ST155	CC155	Slauhterhouse	Pork	32	1	qnrS1	WT	WT	WT	WT	B1	-	KAN,STR,NAL,AMP,TET,[CST],SXT
ST155	CC155	Supermarket	Pork	64	1	qnrS1	WT	WT	WT	WT	B1	-	STR,[NAL],SXT
ST155	CC155	Supermarket	Chicken	32	1	qnrS1	WT	WT	WT	WT	B1	-	STR,GEN,FOX,FEP,CAZ,IPM,NAL,CIP,AMP,CHL,TET,CST,[SXT]
ST155	CC155	Supermarket	Chicken	32	1	qnrS1	WT	WT	WT	WT	B1	-	[NAL],[CIP],AMP
ST155	CC155	Public market	Pork	32	1	qnrS1	WT	WT	WT	WT	B1	-	[NAL],AMP
ST155	CC155	Public market	Pork	16	1	qnrS1	WT	WT	WT	WT	B1	-	[KAN],[IPM],[NAL],AMP
ST155	CC155	Public market	Chicken	>128	2	qnrB4	S83L	WT	WT	WT	B1	-	KAN,GEN,NAL,AMP,CHL,SXT
ST155	CC155	Public market	Chicken	>128	2	qnrB4	S83L	WT	WT	WT	B1	-	KAN,GEN,NAL,AMP,CHL,SXT
ST155	CC155	Public market	Chicken	>128	1	qnrB4	S83L	WT	WT	WT	B1	-	KAN,GEN,NAL,AMP,CHL,SXT
ST155	CC155	Public market	Chicken	>128	2	qnrB4	S83L	WT	WT	WT	B1	-	KAN,GEN,IPM,NAL,AMP,CHL,TET,SXT
ST155	CC155	Public market	Chicken	>128	2	qnrB4	S83L	WT	WT	WT	B1	-	KAN,GEN,NAL,AMP,CHL,SXT
ST155	CC155	Public market	Chicken	>128	2	qnrB4	S83L	WT	WT	WT	B1	-	KAN,STR,GEN,NAL,AMP,CHL,SXT
ST58	CC155	Supermarket	Beef	>128	1	-	S83L	WT	WT	WT	B1	-	[CTX],NAL,AMP,TET
ST58	CC155	Supermarket	Pork	>128	1	-	S83L	WT	WT	WT	B1	-	STR,NAL,AMP,TET
ST949	CC155	Slauhterhouse	Beef	>128	0.5	-	S83L	WT	WT	WT	B1	-	NAL,AMP,TET
ST4482	Singleton	Public market	Chicken	>128	2	qnrB4	S83L	WT	WT	WT	B1	-	KAN,GEN,NAL,AMP,CHL,SXT
ST3140	Singleton	Supermarket	Chicken	>128	2	qnrB4	S83L	WT	WT	WT	B1	-	KAN,STR,GEN,NAL,AMP,CHL,TET,SXT
ST345	Singleton	Public market	Pork	64	1	qnrS1	WT	WT	WT	WT	B1	-	[STR],[NAL],AMP,CHL,TET,SXT
ST423	CC23	Slauhterhouse	Pork	>128	2	qnrS1	WT	WT	WT	WT	A	-	[NAL],TET,SXT
ST641	CC86	Supermarket	Chicken	>128	>32	-	S83L, D87N	WT	S80I	WT	D	-	KAN, STR, NAL, CIP, AMP, CHL, TET, SXT
ST48	CC10	Supermarket	Chicken	>128	>32	qnrA1, qnrS1	WT	WT	WT	WT	A	-	[STR], [NAL], CIP, AMP, CHL, TET, SXT
ST48	CC10	Poultry farm	Cloacal swab	64	1	qnrS1	WT	WT	WT	WT	A	-	[STR],IPM,NAL,CIP,AMP,SXT
ST48	CC10	Public market	Pork	32	0.5	qnrS1	WT	WT	WT	WT	A	-	[NAL],AMP,CHL,TET,SXT
ST48	CC10	Slauhterhouse	Beef	>128	4	qnrS1	S83L	WT	WT	WT	A	-	STR,NAL,AMP,CHL,TET,SXT
ST43	CC10	Poultry farm	Cloacal swab	64	2	qnrS1	WT	WT	WT	WT	A	+	FOX,FEP,CAZ,NAL,CIP,AMP,TET,SXR
ST10	CC10	Poultry farm	Cloacal swab	32	1	qnrS1	WT	WT	WT	WT	A	-	FEP,CAZ,[IPM],[NAL],AMP,CHL,TET,SXT
ST5909	CC10	Public market	Beef	>128	0.5	-	S83L	WT	WT	WT	A	-	NAL,CHL,TET
ST1397	CC10	Public market	Beef	32	1	qnrS1	WT	WT	WT	WT	A	-	[KAN].FOX,[IPM],[CIP],AMP,CHL,TET
ST2207	CC10	Public market	Chicken	>128	32	-	S83L, D87N	WT	S80I	WT	A	-	KAN, STR, NAL, CIP, AmC, AMP
ST710	Singleton	Poultry farm	Environment swab	>128	1	qnrS1	WT	WT	WT	WT	A	-	STR,FOX,FEP,CAZ,NAL,AMP,CHL,TET,CST,SXT
ST710	Singleton	Swine farm	Environment swab	64	1	qnrS1	WT	WT	WT	WT	A	-	[KAN],STR,[NAL],AMP,CHL,TET,SXT
ST93	CC168	Public market	Chicken	>128	>32	-	S83L, D87N	WT	S80I	WT	A	-	KAN, STR, NAL, CIP, AMP, CHL, TET, SXT
ST93	CC168	Public market	Chicken	>128	>32	-	S83L, D87N	WT	S80I	WT	A	-	KAN, STR, GEN, NAL,CIP, AMP, CHL, TET, SXT
ST542	Singleton	Swine farm	Environment swab	>128	2	qnrS1	WT	WT	WT	WT	A	-	CAZ,[NAL],AMP,CHL,TET,SXT
ST409	Singleton	Public market	Beef	16	0.25	qnrS1	WT	WT	WT	WT	A	-	[NAL]
ST1178	CC165	Public market	Chicken	>128	2	qnrS1	WT	WT	WT	WT	A	-	[FOX],[NAL],AMP,CHL,TET
ST1112	Singleton	Slauhterhouse	Beef	32	1	qnrS1	WT	WT	WT	WT	A	-	KAN,[NAL],AMP,TET,SXT
ST57	CC350	Public market	Chicken	>128	>32	-	S83L, D87N	WT	S80I	I464F	D	-	KAN, STR, NAL, CIP, AMP, TET
ST57	CC350	Public market	Chicken	>128	>32	-	S83L, D87N	WT	S80I	I464F	B1	-	KAN, [STR], NAL, CIP, AMP, TET
ST3288	CC206	Public market	Chicken	>128	2	qnrB4	S83L	WT	WT	WT	A	+	STR,FOX,NAL,AMP
ST3288	CC206	Supermarket	Chicken	>128	4	qnrB4	S83L	WT	WT	WT	A	+	KAN,STR,GEN,NAL,AMP,CHL,SXT
ST3288	CC206	Supermarket	Chicken	>128	2	qnrB4	S83L	WT	WT	WT	A	+	FOX,CAZ,NAL,SXT
ST206	CC206	Poultry farm	Cloacal swab	>128	>32	-	S83L, D87N	WT	S80I	WT	A	-	[KAN],IPM,NAL,CIP,AMP,CHL,TET,SXT
ST206	CC206	Poultry farm	Cloacal swab	>128	>32	-	S83L, D87N	WT	S80I	WT	A	-	[FOX],IPM,NAL,CIP,AMP,CHL,TET,SXT
ST206	CC206	Poultry farm	Cloacal swab	>128	32	-	S83L, D87N	WT	S80I	WT	A	-	IPM,NAL,CIP,AMP,[CHL],TET,SXT
ST206	CC206	Poultry farm	Cloacal swab	>128	8	-	S83L, D87N	WT	S80I	WT	A	-	KAN,IPM,NAL,CIP,AMP,CHL,TET,SXT
ST206	CC206	Poultry farm	Cloacal swab	8	0.25	qnrS1	WT	WT	WT	WT	A	-	KAN,IPM,NAL,CIP,AMP,CHL,TET,SXT
ST206	CC206	Supermarket	Chicken	>128	2	qnrB4	S83L	WT	WT	WT	A	+	FOX,NAL,AMP
ST540	Singleton	Supermarket	Pork	>128	1	qnrS1	WT	WT	WT	WT	A	-	[FOX],[NAL],AMP,CHL,TET,SXT
ST1485	CC648	Public market	Chicken	>128	2	-	S83L	WT	S80I	WT	B1	-	[KAN], STR, CAZ, [IPM], NAL, AMP, SXT
ST624	CC648	Public market	Chicken	16	0.25	qnrB4	WT	S492N	WT	WT	A	-	KAN, STR, FOX, CTX, CAZ, IPM, CIP, AmC, AMP, TET, SXT
ST354	CC354	Supermarket	Chicken	>128	>32	-	S83L, D87N	S492N	S80I, E84G	WT	D	-	KAN,STR,GEN,IPM,NAL,CIP,CHL,TET
ST354	CC354	Supermarket	Chicken	>128	>32	-	S83L, D87N	S492N	S80I, E84G	WT	D	-	STR,IPM,NAL,CIP,CHL,TET
ST354	CC354	Supermarket	Chicken	>128	>32	-	S83L, D87N	WT	S80I, E84G	WT	D	-	STR,FOX,[CAZ],IPM,NAL,CIP,AMP,CHL,TET,SXT
ST354	CC354	Supermarket	Chicken	>128	>32	qnrB4	S83L, D87N	S492N	S80I, E84G	WT	D	-	STR,GEN,FOX,[CAZ],IPM,NAL,CIP,AMP,CHL,TET,SXT
ST354	CC354	Supermarket	Chicken	>128	>32	qnrB4	S83L, D87N	S492N	S80I, E84G	WT	D	-	STR,FOX,[CAZ],IPM,NAL,CIP,AMP,CHL,TET,SXT
ST354	CC354	Supermarket	Chicken	>128	>32	qnrB4	S83L, D87N	S492N	S80I, E84G	WT	D	-	STR,FOX,IPM,NAL,CIP,AMP,CHL,[TET],SXT
ST354	CC354	Supermarket	Chicken	>128	>32	-	S83L, D87N	S492N	S80I, E84G	WT	D	-	[KAN],STR,GEN,IPM,NAL,CHL,[TET]
ST354	CC354	Supermarket	Pork	>128	>32	-	S83L, D87N	S492N	S80I, E84G	WT	A	-	STR,[GEN],FOX,[CAZ],[IPM],NAL,CIP,AMP,CHL,[TET],SXT
ST770	Singleton	Poultry farm	Cloacal swab	>128	1	-	S83L	WT	WT	WT	D	+	KAN,STR,NAL,CHL,[TET]
ST770	Singleton	Swine farm	Rectal swab	16	0.125	-	S83L	WT	WT	WT	D	+	KAN,[STR],[NAL],AMP,CHL,TET,SXT
ST747	Singleton	Supermarket	Chicken	>128	1	-	S83L	WT	WT	WT	D	-	STR,NAL,AMP,CHL,TET,SXT

Figure 1. Dendrogram showing the relationship between 115 QRDR/PMQR gene-harboring *E. coli* strains isolated from beef, chicken, pork, and cloacal, rectal, and environmental swab samples, based on the MLST allele profile including information about ST, CC, MIC of nalidixic acid /ciprofloxacin, gyrase/topoisomerase substitutions, phylogroup, virulence gene (*astA*), and phenotypic resistance profile according to the Kirby–Bauer disc diffusion method. KAN – kanamycin; STR – streptomycin; GEN – gentamicin; FOX – cefoxitin; CTX – cefotaxime; FEP – cefepime; CAZ – ceftazidime; IPM – imipenem; MEM – meropenem; AMP – ampicillin; AMC – amoxicillin/clavulanic acid; CHL – chloramphenicol; TET – tetracycline; STX – sulfamethoxazole/trimethoprim; CST – colistin. Antimicrobial agents inside bracket indicate intermediate resistance. (+) – positive; (−) – negative; WT – wild type.

Table 4. Distribution of amino acid substitutions in QRDR genes, PMQR genes and MIC of tested quinolones in QRDR-PMQR harboring *E. coli* isolates

QRDR Amino-Acid Substitutions [A]				PMQR	No. of Isolates	MIC (µg/mL) [d]	
gyrA	***gyrB***	***parC***	***parE***			**Nalidixic Acid**	**Ciprofloxacin**
Ser83 Leu	-[b]	-[b]	-[b]	-[c]	7	16 – >128	0.125–1
Ser83 Leu	Ser492 Asn	-[b]	-[b]	-[c]	1	>128	0.5
Ser83 Leu	-[b]	Ser80 Ile	-[b]	-[c]	1	>128	2
Asp87 Tyr	-[b]	-[b]	-[b]	-[c]	1	>128	0.5
Ser83 Leu; Asp87 Asn	-	-[b]	-[b]	-[c]	1	>128	4
Ser83 Leu; Asp87 Asn	Ser492 Asn	Ser80 Ile; Glu84 Gly	-[b]	-[c]	4	>128	>32
Ser83 Leu; Asp87 Asn	-[b]	Ser80 Ile	-[b]	-[c]	31	>128	4 – >32
Ser83 Leu; Asp87 Asn	-[b]	Ser80 Ile	Ile464 Phe	-[c]	2	>128	>32
Ser83 Leu; Asp87 Asn	-[b]	Ser80 Ile	Ser458 Ala	-[c]	16	128 – >128	0.5 – >32
Ser83 Leu; Asp87 Asn	-[b]	Ser80 Ile; Glu84 Gly	-[b]	-[c]	2	>128	>32
Ser83 Leu	Ser492 Asn	Ser80 Arg	-[b]	*qnrB4*	1	>128	8
Ser83 Leu	-[b]	Ser80 Ile	-[b]	*qnrS1*	1	>128	32
Ser83 Leu	-[b]	-[b]	-[b]	*qnrS1*	1	>128	4
Ser83 Leu	-[b]	-[b]	-[b]	*qnrB4*	12	>128	1–4
Asp87 Try	-[b]	-[b]	-[b]	*qnrS1*	1	>128	2
Asp87 Try	-[b]	Ser80 Ile	-[b]	*qnrB4*	1	8	<0.03
Ser83 Leu; Asp87 Asn	S492 *n*	S80 I; E84 G	-[b]	*qnrB4*	3	>128	>32
-[b]	S492 *n*	-[b]	-[b]	*qnrB4*	1	16	0.25
-[b]	-[b]	-[b]	-[b]	*qnrS1*	27	8 – >128	0.25–2
-[b]	-[b]	-[b]	-[b]	*qnrA1; qnrS1*	1	>128	>32

[a] QRDR substitutions: GyrA – Ser83Leu: serine to leucine at codon 83; Asp87Asn: aspartic acid to asparagine/ tyrosine at codon 87; GyrB – Ser492Asn: serine to aspartic acid at codon 492; ParC – Ser80Ile/Arg: serine to isoleucine/arginine at codon 80; Glu84Gly: glutamic acid to glycine at codon 84; ParE – Ser458Ala: serine to alanine at codon 458; Ile464Phe: isoleucine to phenylalanine at codon 464.
[b] No substitution detected in the QRDR.
[c] No PMQR determinant detected.
[d] MIC – Minimum inhibitory concentration

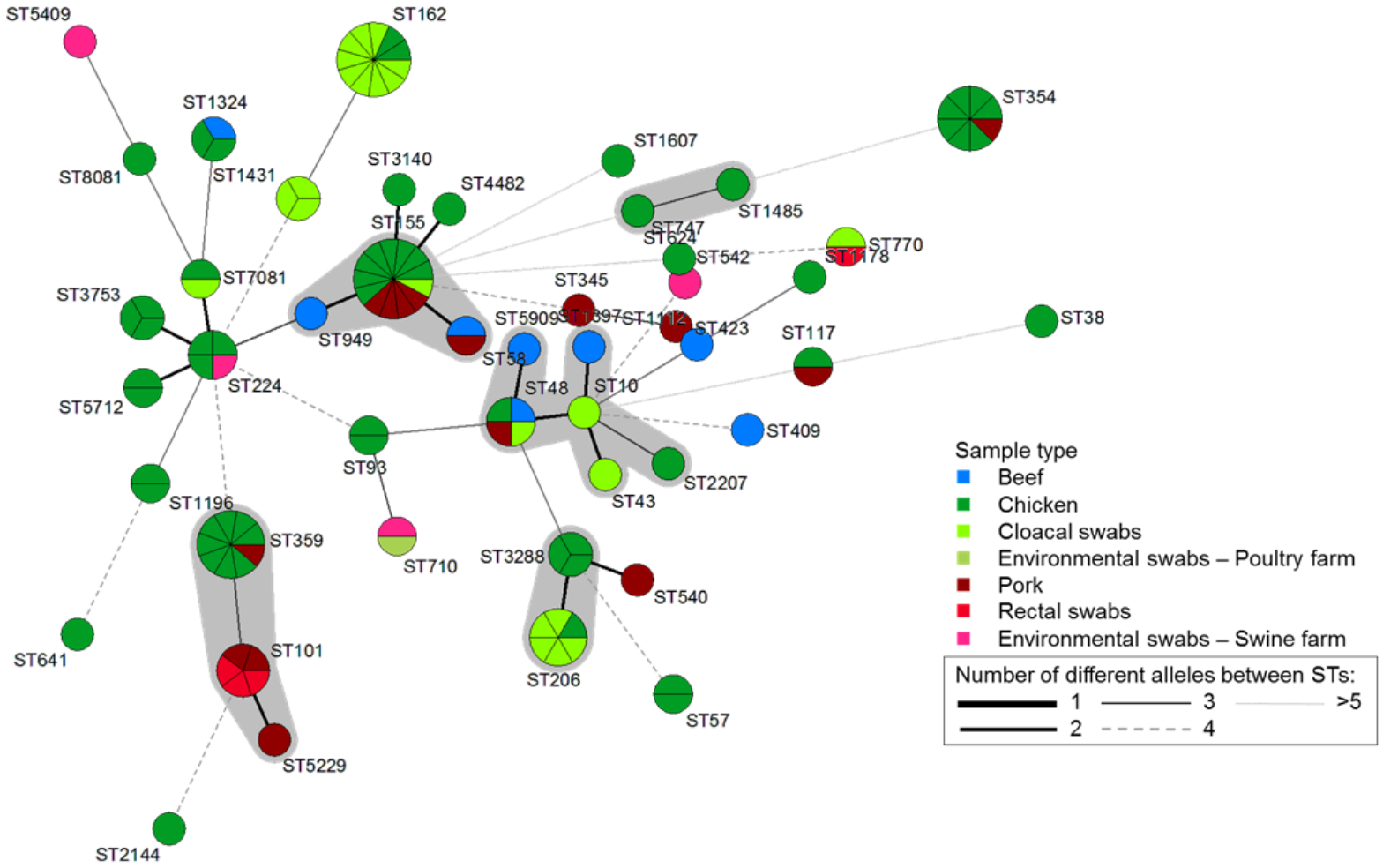


Figure 2. Minimum spanning tree based on Multilocus Sequence Typing alleles of 115 QRDR-PMQR harboring *E. coli* isolates from beef, chicken, pork, cloacal swabs, rectal swabs and environmental swabs. Each circle corresponds to an individual sequence type (ST), and the circle size indicates the number of isolates assigned to the same ST. The color of the circle denotes the sample type. The connecting lines between circles denote allelic variations between STs, and the grey shadowing indicates ST belonging to the same clonal complex (CC).

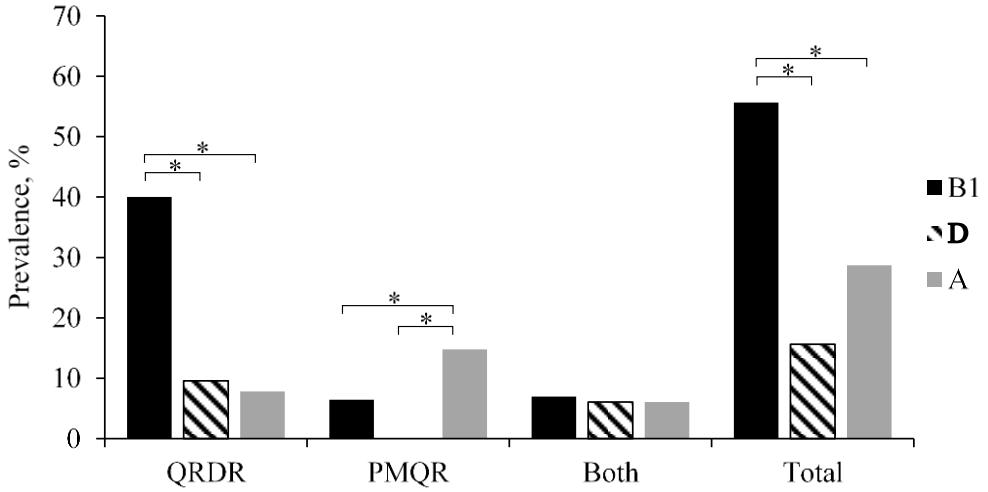


Figure 3. Prevalence of phylogenetic groups in 115 isolates not susceptible to quinolones harboring mutations in QRDR and/or PMQR genes, from food-producing animals and their food products, collected in the Philippines. * The *p*-value was significant ($p < 0.05$).

CHAPTER II

Characterization of plasmids harboring *qnrA1*, *qnrS1*, and *qnrB4* in Quinolone Non-susceptible *E. coli* isolated from Food-producing Animals and Food Products in the Philippines

1. Introduction

Multidrug resistant bacteria are increasing globally due to the abuse and overuse of antibiotics exerting selective pressure on the development of resistance [88–90]. Naturally, a bacterium is able to establish antibiotic resistance through spontaneous mutation [91,92]. Horizontal gene transfer (HGT) is a process that allows bacteria to exchange genetic materials including antimicrobial resistance genes (ARGs) among diverse species [92,93]. This process is facilitated by the mobile genetic elements (MGEs) such as plasmid, integrons and transposons, which promote movement of genetic materials within or between bacteria [94]. Plasmids are small circular double-stranded DNA molecules, and naturally occurs in bacteria. Genes found on plasmids often provide bacteria with genetic advantages, such as resistance to antibiotics [89].

PMQR determinants had been found in plasmids with variable sizes globally [16]. In addition, PMQR determinants were often associated with different incompatibility groups (Inc) such IncL/M, A/C, HI2, FII, I1, N, ColE1, FIA, F, R, X2, U, Q, FIB, X1 and other ARGs [16,95,96]. Frequently found PMQR determinants belong to the Qnr family. To date, there were seven different Qnr families (QnrA, QnrB, QnrC, QnrD, QnrE, QnrS and QnrVC). The Qnr protein belongs to the family of pentapeptide repeat proteins, which confer resistance to quinolones by physically protecting DNA gyrase and topoisomerase IV from inhibition of quinolones [97].

In addition to conferring a low degree of resistance to quinolones, *qnr* may also facilitate the selection of additional chromosomal resistance mechanisms, leading to the emergence of bacterial strains with high resistance [18,49]. The reduced susceptibility to fluoroquinolones given by *qnr* was important cause of clinical treatment failure in humans, creating serious therapeutic problems worldwide [18,59,63]. Recently, *qnr* determinants have been found in association with β-lactamase producing Enterobacteriaceae from human clinical isolates in the Philippines [71,72,98–100]. In those clinical isolates, *qnr* determinants were localized in IncL/M, IncA/C2, IncX3 and IncFII(K) plasmids [99,100]. However, data on plasmids containing PMQR determinants in bacteria isolated from livestock animals and their

derived foods have not yet been elucidated. Therefore, this chapter aims to determine the prevalence and characteristics of plasmids carrying the PMQR gene in *E. coli* strains isolated from food-producing animals and food derived from them in the Philippines.

2. Materials and Methods

2.1. Strains used in this study

A total of 44 *qnr*-positive *E. coli* strains were analyzed in this study. Most of the isolates were from chicken meat (n = 21) followed by pork (n = 8), beef (n = 5), cloacal swab from chicken (n = 4), rectal swab from pig (n = 2). In addition, four isolates from environmental swab from pig farm (n = 1) and chicken farm (n = 3) were included. The presence of *qnr* in these isolates was previously described [101]. *qnr-S1*-positve *E. coli* belong to 19 STs mostly ST155 (n = 6), ST48 (n = 3), ST1324 (n = 3), ST101 (n = 2), ST710 (n = 2). Isolates harboring *qnrB4* belong to 7 STs mostly ST155 (n = 5), ST354 (n = 3) and ST3288 (n = 3). One *E. coli* isolate was positive for *qnrA1* and *qnrS1* belong to ST48. The DNA sequence of the QRDR of the *gyrA*, *gyrB*, *parC* and *parE* was described in Chapter I.

2.2. Antimicrobial Susceptibility Testing

The antimicrobial susceptibility of all *qnr*-harboring isolates was described in Chapter I. In addition, the MICs of nalidixic acid and ciprofloxacin were determined using broth microdilution for donor and transconjugant isolates harboring *qnr* and the results were interpreted according to the CLSI criteria [44]. *E. coli* ATCC 25922 was used as the control strain.

2.3. Detection of β-lactamase genes

All *qnr*-harboring *E. coli* were screened for the presence of bla_{TEM}, bla_{SHV}, $bla_{\text{CTX-M}}$, and bla_{OXA} by PCR amplification as described previously using primers listed in Table 5. The PCR mixture (20 μL) contained 1.25 U of Taq DNA polymerase (Promega, Madison, WI, USA), 1× PCR buffer (Promega), 0.2 mM of each Deoxynucleoside triphosphate (dNTP) (Takara Bio Inc.), 2.5 mM $MgCl_2$ (Promega), 1 μM of each primer, and 1 μL of DNA template. The reaction condition was as follows: denaturation at 94°C for 5 min followed by 35 cycles of denaturation at 94°C for 30 s, an annealing at 60°C (for bla_{TEM}, bla_{SHV}, and $bla_{\text{CTX-M}}$), 55°C (for bla_{OXA}) for 30 s, an extension at 72°C for 1.5 min, and a final extension at 72°C for 10 min. PCR amplicons were visualized on 1.5% agarose gels with GelRed (Biotium, Inc.). After the gel electrophoresis, images of the PCR amplicons were captured using Printgraph Classic (ATTO Corp.).

2.4. Plasmid analysis

Plasmid incompatibility (Inc) groups were determined by PCR as described previously

using primers listed in Table 5. The PCR mixture was the same as that mentioned in Section 2.3. Using the DNA prepared by the boiling method as a template, PCR was performed under the following conditions: denaturation at 94°C for 5 min followed by 30 cycles of denaturation at 94°C for 30 s, an annealing at 60°C (for all replicon type except $IncF_{repB}$ at 55°C) for 30 s, an extension at 72°C for 1 min, and a final extension at 72°C for 5 min. PCR amplicons were visualized on 1.5% agarose gels with GelRed (Biotium, Inc.). After the gel electrophoresis, images of the PCR amplicons were captured using Printgraph Classic (ATTO Corp.).

The localization of the *qnr* in plasmid was investigated by S1-nuclease pulsed-field gel electrophoresis (S1-PFGE) and Southern blot hybridization according to previous studies [46,102]. Briefly, S1-digested bacterial plugs were loaded into wells in 13 × 14 cm 1.5% agarose gel and run in a CHEF-Mapper (Bio-Rad Laboratories, Inc., Hercules, CA) under the following conditions: run duration, 18 h; temperature, 14°C; Angle, 120°; Initial switch time, 2.16 s; Final switch time, 63.8 s; Gradient, 6.0 v/cm. MidRange PFG Marker (New England BioLabs, Ipswich, MA) was used as a size marker.

Following PFGE electrophoresis, the DNA fragments were transferred to Zeta-probe nylon membrane (Bio-Rad Laboratories) following Southern blot hybridization standard protocol. PCR-amplified *qnrA1/B4/S1* or $IncF_{repB}$ fragments from the *qnrA1/B4/S1*- and $IncF_{repB}$-positive isolates were labeled with digoxigenin using PCR DIG Labeling Mix (Roche Diagnostic, Basel, Switzerland) according to the manufacturer's instructions and used as a specific probe for the *qnrA1/B4/S1* or $IncF_{repB}$ detection. The membranes were pre-hybridized and hybridized using DIG Easy Hyb buffer (Roche Diagnostic) following the manufacturer's recommendations. After hybridization, the membranes were stringency washed using DIG Wash and Blocking Buffer Set (Roche Diagnostic) following the manufacturer's recommendations. Following blot washing, immunological detection was conducted utilizing an anti-DIG antibody Fab fragment (antigen-binding fragment) that was coupled to alkaline phosphatase (AP), anti-DIG-AP (Roche Diagnostic). CSPD® (Disodium 3-(4-methoxyspiro {1,2-dioxetane-3,2'-(5′-chloro) tricyclo [3.3.1.13,7] decan}-4-yl)phenyl phosphate) (Roche Diagnostic) was used for chemiluminescent substrate for detection of DIG-labeled probes (Roche Diagnostic). Amersham Imager 600 (GE Healthcare, Chicago, IL) was used to analyze the digital image of chemiluminescence after 5-10 min exposure.

2.5. Plasmid-mediated quinolone resistance transferability

Broth mating assays were performed with *E. coli* J53 (Sodium azide-resistant) as recipient strain. Donors and recipient were grown in Luria-Bertani (LB) broth to logarithmic

phase, and then 0.5 mL of both cultures was added to 4 mL of fresh LB. The mating cultures were incubated overnight at 37°C without shaking. Transconjugants were selected in LB agar plate supplemented with Sodium azide (100 μg/ml), and ciprofloxacin (0.06 μg/ml). DNA of transconjugants was extracted by boiling method. PCR was performed to determine the presence of *qnrA1*, *qnrB4* and *qnrS1*, as described in section 2.4 of Chapter I, and plasmid replicons as described above.

2.6. Data Analysis

The data were descriptively analyzed using Statistical Package for Social Sciences (SPSS) software version 22.0 (IBM Corp.). Differences in proportions were compared using a χ^2 test or Fisher's exact test. All the tests were analyzed with a 95% confidence interval. A *p*-value less than 0.05 was considered statistically significant.

3. Results

3.1. Antimicrobial Susceptibility

Table 6 shows the antimicrobial susceptibility of *qnr*-positive *E. coli* isolates to 14 antimicrobial agents. Resistance was most commonly observed to ampicillin (86.4%), followed by sulfamethoxazole-trimethoprim (70.4%), chloramphenicol (63.6%), tetracycline (56.8%) and nalidixic acid (50.0%). However, resistance to imipenem (11.4%), ceftazidime (11.4%), cefepime (9.1%), and colistin (6.8%) was observed less frequently. Furthermore, 79.5% of the isolates were found to be multidrug resistant.

3.2. Prevalence of β-lactamase and plasmid replicon

Twenty-five (56.8%) of these 44 isolates carry a β-lactamase gene, bla_{TEM}. The other ESBL genes, namely bla_{SHV}, $bla_{CTX\text{-}M}$ and bla_{OXA} were not detected (Table 7).

The plasmid replicon analysis of *qnr*-harboring isolates was shown in Table 8. Thirty-four (77.3%) of the isolates were positive for variety of plasmid replicon types. Briefly, $IncF_{repB}$, IncI1 and IncFIA were detected in 29, 18 and 8 isolates, respectively. Replicon belonging to the $IncF_{repB}$ and IncI1 were frequently identified among *qnrS1*-harboring isolates. Among *qnrB4*-harboring isolates, $IncF_{repB}$, IncI1 and IncFIA were most frequently identified. Ten isolates were found un-typable replicon, one and nine isolates that harbored *qnrB4* and *qnrS1*, respectively.

3.3. S1-PFGE and Southern blot hybridization

Based on the results of plasmid replicon analysis, 18 isolates, nine from *qnrS1-,* eight from *qnrB4*-harboring isolates and an isolate harboring both *qnrS1* and *qnrA1*, were further subjected for localization of *qnr* alleles in $IncF_{repB}$ plasmid. The result of plasmid analysis by S1 nuclease-PFGE and southern blot hybridization was shown in Figure 4. Among tested isolates, *qnrS1*-harboring isolates contained one to three plasmids, whereas two plasmids were found among *qnrB4*-harboring isolates. Hybridization results showed *qnrS1* was located in $IncF_{repB}$ plasmid with varying size ranging from around 45 kbp – 242.5 kbp except for one isolate. By using *qnrB4*-specific probe, *qnrB4* were also located on $IncF_{repB}$ plasmid with size ranging from around 80 kbp – 97 kbp. S1-PFGE and Southern blot hybridization of an isolate harboring *qnrA1* and *qnrS1* was unsuccessful.

3.4. Transferability of *qnr* and plasmid

The results of the conjugation experiment on seven representative donor strains carrying *qnr* was presented in Table 9 and Figure 5. The plasmid carrying all *qnr* alleles were transferable to the recipient strain. All transconjugant stains but one was positive for $IncF_{repB}$. In addition, two transconjugants positive for *qnrB4* were positive for IncI1. Also, five transconjugants were positives for bla_{TEM}. The MICs of ciprofloxacin and nalidixic acid against recipient were 0.0625 μg/mL and eight μg/mL, respectively, whereas those against transconjugants were 0.5 – 16 μg/mL and 32 – 128 μg/mL, respectively. *qnrA1-qnrS1*-harboring isolate yielded two transconjugants: one positive for both *qnr* and one positive for *qnrS1* only. In addition, *qnrA1-qnrS1*-harboring transconjugant was positive for both bla_{TEM} and $IncF_{repB}$, while *qnrS1*-harboring transconjugant was negative for both bla_{TEM} and $IncF_{repB}$ (Table 9 and Figure 5).

4. Discussion

The characteristics of 44 *qnr*-positive *E. coli* isolates from livestock and related samples were elucidated in this study.

In this study, 79.5% of *qnr*-harboring isolates exhibit multidrug-resistant phenotype. Most of *qnr*-harboring isolates were concurrently resistance to ampicillin, chloramphenicol, sulfonamide-trimethoprim and tetracycline. The resistance of *qnr*-harboring isolates to multiple antibiotics were consistent with those previous works in *E. coli* from clinical and retail food samples [38,103]. This finding also supports the idea of the coexistence of *qnr* and other ARGs in the same plasmid. This will be elucidated by further study.

In the current study, *qnr*-harboring isolates were positive for at least one to three plasmid replicons. Plasmid replicon type $IncF_{repB}$ and IncI1 was frequently observed among *qnrS1*-harboring isolates. In addition, $IncF_{repB}$, IncI1 and IncFIA plasmid replicon were predominantly detected in the *E. coli* isolates carrying *qnrB4*. The multiple plasmid replicons profiles detected in this study was similar to the results in India [104] and Mexico [105]. However, ten of *qnr*-harboring isolates were negative for those plasmid replicon types. Ten *qnr*-harboring isolates might carry replicons belonging to new or rare plasmid. Overall, $IncF_{repB}$ plasmid was predominantly identified among *qnr*-harboring isolates. This finding was consistent with the results in previous studies in Thailand and Lao border provinces [106] and India [104]. IncF plasmids has characteristics of low-copy number, conjugative nature with varying size (~45 to 200 kbp). And it is the most abundant plasmid type found in Enterobacteriaceae [94,96]. In addition, IncF plasmid was found sporadically in MDR Enterobacteriaceae in Asia [95,96,106]. Thus, it may play a vital role in the dissemination of *qnr* together with other ARG determinants. Therefore, monitoring resistant plasmids are indispensable in tracking the emergence and spread of *qnr*-associated plasmids in food-producing animals and their food products.

Among *qnr*-harboring isolates, 56.8% were concurrently harbor bla_{TEM}. PMRQ and β-lactamase determinant had been documented involving horizontal transmission events by several plasmids with different incompatibility groups [104]. In this study, both *qnr* and bla_{TEM} from donor strain were successfully transferred into recipient strain upon ciprofloxacin selection, similar to the previous report [38,106]. These results demonstrated that the prolonged usage of ciprofloxacin in food-producing animals can contribute to the selection not only *qnr* but also bla_{TEM}. However, co-localization of *qnr* and bla_{TEM} in same plasmid was not determined in the current study.

The localization of *qnr* alleles in $IncF_{repB}$ plasmid with varying size was further confirmed by SI-PFGE and Southern blot hybridization analysis. These results established that *qnr* determinants were primarily transmitted horizontally by $IncF_{repB}$ plasmid. In addition, $IncF_{repB}$ plasmid carrying *qnr* determinants could be transferred to *E. coli* J53 as a recipient strain by conjugation. The ability to transfer the *qnr* determinants via $IncF_{repB}$ plasmid indicated the potential spread of these resistance determinants to other organisms.

The MICs of nalidixic acid and ciprofloxacin against transconjugants harboring *qnrA1*, *qnrB4* and *qnrS1* were 8-fold to 64-fold higher than the recipient strain (Table 9). However, comparing with parent strains, transconjugants MIC were decreased at least 2-fold to 64-fold. These results supports the previous findings that Qnr exert low activity and can supplement the level of resistance caused by target mutations at QRDR against quinolone [107,108]. Interestingly, two transconjugants harboring *qnrS1* were still expressed clinical level of resistance to nalidixic acid (MIC 128 μg/mL) and ciprofloxacin (MIC 4 – 16 μg/mL) same as its parent strain. Elevation in *qnr* expression previously demonstrated for *qnrB* in response to ciprofloxacin to be regulated by the cellular SOS-response [82,95]. Nonetheless, the expression found in *qnrS* appeared to be SOS-independent [95,109]. Another study on the QnrS1 structure demonstrated that individual residues within the QnrS1 loop B are a key for interaction with gyrase necessary for quinolone resistance [110]. Other earlier studies indicate that the nature and degree of quinolone resistance conferred by *qnrS1* appear to be present on the plasmid [109,111]. Besides, other mechanism such as an alteration in cell wall permeability [20,46,61] and efflux pump encoded by *aqxAB* [61,95] perhaps on the transferred plasmid might be playing roles in developing the resistance to quinolone.

In the present study, two $IncF_{repB}$ were found in two isolates by hybridization (Figure 4C). The result was comparable with the previous work on *E. coli* isolates from Thai sewage carrying two $IncF_{repB}$ plasmids [102]. Generally, plasmids that are closely related and sharing the same replication control system cannot reproduce stably in the same cell, as they compete each other [96,112]. However, according to Van der Hoeven [113], two incompatible plasmids can coexist, but only if they follow different survival strategies; one has a high conjugative transfer rate with low adaptability and the other has a low transfer rate with higher host adaptability. In addition, IncF plasmids was usually multi-replicon and reported to co-integrated with other replicons such IncI1 and IncN [96]. Hence, the carriage of two plasmids with same replicon type was possible, later one plasmid will be selected, and finally maintained by the host cell.

Overall, 59.1% of the *qnr* positive isolates were from chicken-associated samples. The

high rate of *qnr* might due to selective pressure of fluoroquinolones usage in the poultry production. According to the previous studies, poultry production consumes huge number of fluoroquinolones compared to other livestock production in the Philippines [31,32,56]. Furthermore, *qnr* were generally localized in IncF plasmid similarly to those found in human clinical isolates in the Philippines [100]. However, further study is needed to establish the transmission of *qnr* via IncF plasmid to human. For now, these results indicate that food-producing animals and their food derived products can be reservoirs and sources of dissemination of plasmid-mediated antimicrobial resistance genes in the Philippines. Therefore, to prevent the emergence and spread of *qnr*, it is essential to practice good animal husbandry management and good hygiene at the farm and food-processing establishment

5. Summary

PMQR determinants refer to the family of related genes that confer reduced susceptibility to quinolones. The *qnr* determinants are located in a plasmid and generally carried additional ARGs against a range of antimicrobials. A high prevalence of *E. coli* harboring *qnr* in food-producing animals and food-derived found in the previous study. However, the plasmid carrying *qnr* determinants on these isolates was not determined. Hence, this study aims to determine the prevalence and characteristics of plasmid harboring *qnr* in *E. coli* isolated from food-producing animals and food-derived.

Plasmids were characterized by PCR-based replicon typing. The localization of the qnr determinants in the plasmid was determined by S1-PFGE and Southern blot hybridization. The transferability plasmid carrying qnr determinants was determined by conjugation. Furthermore, β-lactamase-encoding genes, bla_{TEM}, bla_{SHV}, $bla_{\text{CTX-M}}$ and bla_{OXA} were investigated in PMQR-positive isolates by PCR.

Overall, 34 out of 44 isolates harboring *qnr* determinants were positive for seven replicon types, and 25 were concurrently harbor bla_{TEM}. The frequent replicons among *qnrB4* and *qnrS*1 harboring isolates were $\text{IncF}_{\text{repB}}$, IncI1, or IncFIA. S1-PFGE and Southern blot hybridization analysis confirmed the localization of *qnr* determinants in $\text{IncF}_{\text{repB}}$ plasmid. In addition, the $\text{IncF}_{\text{repB}}$ plasmids carrying *qnr* determinants can be transferred by bacterial conjugation. The current study proves that *qnr*-harboring *E. coli* in food-producing animals and its food products can spread via $\text{IncF}_{\text{repB}}$ plasmid upon selective pressure by quinolone or other antimicrobials. Therefore, prudent use of antimicrobials in animal production is recommended. Implementation of good food handling practices are also necessary to prevent the emergence and spread of *qnr*-harboring bacteria.

Table 5. Primers used in this study.

Name	Primer sequence (5'–3')	Amplicon size (bp)	Reference
β-lactamase			[114]
TEM-F	TCGGGGAAATGTGCG	1,074	
TEM-R	TGCTTAATCAGTGAGGCACC		
SHV-F	GCCGGGTTATTCTTATTTGTCGC	1,016	
SHV-R	ATGCCGCCGCCAGTCA		
CTX-M-uni-F	CGATGTGCAGTACCAGTAA	585	
CTX-M-uni-R	TAAGTGACCAGAATCAGCGG		
OXA-uni-F	TCAACTTTCAAGATCGCA	610	
OXA-uni-R	GTGTGTTTAGAATGGTGA		
Replicon type			[112]
HI1-F	GGAGCGATGGATTACTTCAGTAC	471	
HI1-R	TGCCGTTTCACCTCGTGAGTA		
HI2-F	TTTCTCCTGAGTCACCTGTTAACAC	644	
HI2-R	GGCTCACTACCGTTGTCATCCT		
I1-F	CGAAAGCCGGACGGCAGAA	139	
I1-R	TCGTCGTTCCGCCAAGTTCGT		
X-F	AACCTTAGAGGCTATTTAAGTTGCTGAT	376	
X-R	TGAGAGTCAATTTTTATCTCATGTTTTAGC		
L/M-F	GGATGAAAACTATCAGCATCTGAAG	785	
L/M-R	CTGCAGGGGCGATTCTTTAGG		
N-F	GTCTAACGAGCTTACCGAAG	559	
N-R	GTTTCAACTCTGCCAAGTTC		
FIA-F	CCATGCTGGTTCTAGAGAAGGTG	462	
FIA-R	GTATATCCTTACTGGCTTCCGCAG		
FIB-F	GGAGTTCTGACACACGATTTTCTG	702	
FIB-R	CTCCCGTCGCTTCAGGGCATT		
W-F	CCTAAGAACAACAAAGCCCCCG	242	
W-R	GGTGCGCGGCATAGAACCGT		
Y-F	AATTCAAACAACACTGTGCAGCCTG	765	
Y-R	GCGAGAATGGACGATTACAAAACTTT		
P-F	CTATGGCCCTGCAAACGCGCCAGAAA	534	
P-R	TCACGCGCCAGGGCGCAGCC		
FIC-F	GTGAACTGGCAGATGAGGAAGG	262	
FIC-R	TTCTCCTCGTCGCCAAACTAGAT		
A/C-F	GAGAACCAAAGACAAAGACCTGGA	465	
A/C-R	ACGACAAACCTGAATTGCCTCCTT		
T-F	TTGGCCTGTTTGTGCCTAAACCAT	750	
T-R	CGTTGATTACACTTAGCTTTGGAC		
FIIA-F	CTGTCGTAAGCTGATGGC	270	
FIIA-R	CTCTGCCACAAACTTCAGC		
F_{repB}-F	TGATCGTTTAAGGAATTTTG	270	
F_{repB}-R	GAAGATCAGTCACACCATCC		
K/B-F	GCGGTCCGGAAAGCCAGAAAAC	160	
K-R	TCTTTCACGAGCCCGCCAAA		
B/O-R	TCTGCGTTCCGCCAAGTTCGA	159	

Table 6. Antimicrobial susceptibility of *qnr*-harboring *E. coli* isolates.

Antimicrobials	Number of isolates (%)		
	Resistance	Intermediate	Susceptible
AMP	38 (86.4)	0	6 (13.6)
SXT	31 (70.5)	0	13 (29.5)
CHL	28 (63.6)	0	16 (36.4)
TET	25 (56.8)	1 (2.3)	18 (40.9)
NAL	22 (50.0)	20 (45.5)	2 (4.5)
STR	17 (38.6)	4 (9.1)	23 (52.3)
KAN	14 (31.8)	5 (11.4)	25 (56.8)
GEN	12 (27.3)	0	32 (72.7)
FOX	9 (20.5)	3 (6.8)	32 (72.7)
CIP	8 (18.2)	5 (11.4)	31 (70.5)
IPM	5 (11.4)	4 (9.1)	35 (79.5)
CAZ	5 (11.4)	2 (4.5)	37 (84.1)
FEP	4 (9.1)	0	40 (90.9)
CST	3 (6.8)	1 (2.3)	40 (90.9)

KAN – kanamycin; STR – streptomycin; GEN – gentamicin; FOX – cefoxitin; CTX – cefotaxime; FEP – cefepime; CAZ – ceftazidime; IPM – imipenem; AMP – ampicillin; CHL – chloramphenicol; TET – tetracycline; SXT – sulfamethoxazole/trimethoprim; CST – colistin; NAL – nalidixic acid; CIP – ciprofloxacin.

Table 7. Characteristic of 44 *E. coli* isolates harboring *qnr*

Isolates	Sample type	PMQR gene	QRDR amino acid substitution[a]			MIC (μg/mL)		β-lactamase	Sequence Type	Phylogroup
			GyrA	GyrB	ParC	NAL	CIP			
1CS1-3	Cloacal swab	*qnrS1*	WT	WT	WT	64	2	TEM	ST43	A
1CS4-5	Cloacal swab	*qnrS1*	WT	WT	WT	32	1	TEM	ST10	A
1ES1-3	Environment swab	*qnrS1*	WT	WT	WT	>128	2	TEM	ST542	A
1ES4-1	Environment swab	*qnrS1*	WT	WT	WT	8	0.25	TEM	ST5409	B1
1RS1-1	Rectal swab	*qnrS1*	WT	WT	WT	64	2	-	ST101	B1
1RS3-2	Rectal swab	*qnrS1*	WT	WT	WT	16	1	TEM	ST101	B1
2B1-4	Beef	*qnrS1*	WT	WT	WT	32	1	TEM	ST1397	A
2B5-4	Beef	*qnrS1*	WT	WT	WT	16	0.25	-	ST409	A
2CS6-4	Cloacal swab	*qnrS1*	WT	WT	WT	8	0.25	TEM	ST206	A
2CS9-2	Cloacal swab	*qnrS1*	WT	WT	WT	64	1	TEM	ST48	A
2ES2-5	Environment swab	*qnrS1*	WT	WT	WT	>128	1	-	ST710	A
2ES8-5	Environment swab	*qnrS1*	WT	WT	WT	64	1	-	ST710	A
2P2-1	Pork	*qnrS1*	WT	WT	WT	64	1	TEM	ST345	B1
2P5-1	Pork	*qnrS1*	WT	WT	WT	32	0.5	TEM	ST48	A
4AP7-2	Pork	*qnrS1*	WT	WT	WT	64	1	-	ST155	B1
4AP33-5	Pork	*qnrS1*	WT	WT	WT	32	1	TEM	ST155	B1
4BC1-1	Chicken	*qnrS1*	WT	WT	WT	>128	2	TEM	ST1178	A
4BC19-4	Chicken	*qnrB4*	S83L, D87N	S492N	S80I, E84G	>128	>32	-	ST354	D
4BC20-2	Chicken	*qnrB4*	S83L, D87N	S492N	S80I, E84G	>128	>32	-	ST354	D
5B33-2	Beef	*qnrS1*	S83L	WT	WT	>128	4	TEM	ST48	A
5B33-3	Beef	*qnrS1*	WT	WT	WT	32	1	-	ST1112	A
5P19-2	Pork	*qnrS1*	WT	WT	WT	>128	1	TEM	ST540	A
5P34-3	Pork	*qnrS1*	WT	WT	WT	>128	2	-	ST423	A
6B29.2	Beef	*qnrS1*	WT	WT	WT	32	1	-	ST1324	A
6C21.2	Chicken	*qnrS1*	WT	WT	WT	16	1	-	ST1324	A

Table 7. (Continue)

6C22.4	Chicken	*qnrS1*	WT	WT	WT	32	1	-	ST1324	A
6C27.4	Chicken	*qnrS1*	WT	WT	WT	32	1	TEM	ST155	B1
6C30.4	Chicken	*qnrS1*	WT	WT	WT	32	1	TEM	ST155	B1
6P23.1	Pork	*qnrS1*	WT	WT	WT	32	1	TEM	ST155	B1
6P24.4	Pork	*qnrS1*	WT	WT	WT	16	1	TEM	ST155	B1
7C1-1	Chicken	*qnrB4*	S83L	WT	WT	>128	2	-	ST155	B1
7C2-2	Chicken	*qnrB4*	S83L	WT	WT	>128	2	TEM	ST155	B1
7C3-3	Chicken	*qnrB4*	S83L	WT	WT	>128	1	TEM	ST155	B1
7C6-1	Chicken	*qnrB4*	S83L	WT	WT	>128	2	TEM	ST4482	B1
7C6-2	Chicken	*qnrB4*	S83L	WT	WT	>128	2	TEM	ST155	B1
7C6-3	Chicken	*qnrB4*	S83L	WT	WT	>128	2	-	ST155	B1
7C6-5	Chicken	*qnrB4*	S83L	WT	WT	>128	2	-	ST3288	A
7C7-1	Chicken	*qnrB4*	S83L	WT	WT	>128	4	-	ST3288	A
7C7-3	Chicken	*qnrB4*	S83L	WT	WT	>128	2	-	ST206	A
7C7-5	Chicken	*qnrB4*	S83L	WT	WT	>128	2	TEM	ST3140	B1
12C1-2	Chicken	*qnrB4*	S83L	S492N	S80R	>128	8	-	ST117	D
12C9-1	Chicken	*qnrS1*	D87Y	WT	WT	>128	2	TEM	ST1607	D
12C23-5	Chicken	*qnrA1, qnrS1*	WT	WT	WT	>128	>32	TEM	ST48	A
12C32-4	Chicken	*qnrS1*	S83L	WT	S80I	>128	32	-	ST2144	D

[a] No amino acid substitution in ParE

- Negative

Table 8. Prevalence of plasmid replicon among *qnr*-harboring isolates

PMQR gene	Number of isolates	Replicon profile
qnrA1, *qnrS1*	1	F_{repB} + I1 + L/M
qnrB4	1	Not detected
	2	F_{repB}
	2	F_{repB} + FIA
	3	F_{repB} + I1
	5	F_{repB} + I1 + FIA
qnrS1	9	Not detected
	1	FIC
	5	F_{repB}
	1	I1/I-ɣ
	3	Y
	1	F_{repB} + FIA
	1	F_{repB} + HI2
	8	F_{repB} + I1
	1	F_{repB} + Y

Table 9. Characteristic of transconjugant and the donor and recipient strain.

Sample ID	PMQR gene	QRDR amino acid substitution				MIC (μg/mL)		β-lactamase	Plasmid replicon
		GyrA	GyrB	ParC	ParE	NAL	CIP		
Donor/Transconjugant (T)[a]									
5B33-2	*qnrS1*	S83L	WT	WT	WT	>128	4	TEM	F_{repB} + HI2
T5B33-2	*qnrS1*	WT	WT	WT	WT	128	4	TEM	F_{repB}
12C9-1	*qnrS1*	S83L	WT	WT	WT	>128	2	TEM	F_{repB}
T12C9-1	*qnrS1*	WT	WT	WT	WT	64	1	TEM	F_{repB}
12C32-4	*qnrS1*	S83L	WT	WT	WT	>128	2	-	F_{repB}
T12C32-4	*qnrS1*	WT	WT	WT	WT	128	1	-	F_{repB}
7C2-2	*qnrB4*	S83L	S492N	S80R	WT	>128	8	TEM	F_{repB} + I1 + FIA
T7C2-2	*qnrB4*	WT	WT	WT	WT	128	0.5	TEM	F_{repB}+I1
7C3-3	*qnrB4*	S83L	WT	WT	WT	>128	1	TEM	F_{repB} + I1 + FIA
T7C3-3	*qnrB4*	WT	WT	WT	WT	32	0.5	TEM	F_{repB}+I1
12C1-2	*qnrB4*	S83L	WT	S80I	WT	>128	32	-	F_{repB} + FIA
T12C1-2	*qnrB4*	WT	WT	WT	WT	128	0.5	-	F_{repB}
12C23-5	*qnrA1, qnrS1*	WT	WT	WT	WT	>128	>32	TEM	F_{repB} + I1 + L/M
T12C23-5a	*qnrA1, qnrS1*	WT	WT	WT	WT	128	8	TEM	F_{repB}
T12C23-5b	*qnrS1*	WT	WT	WT	WT	128	16	-	Not detected
Recipient									
E. coli J53Az^{R}	-	-	-	-	-	8	0.062	-	-

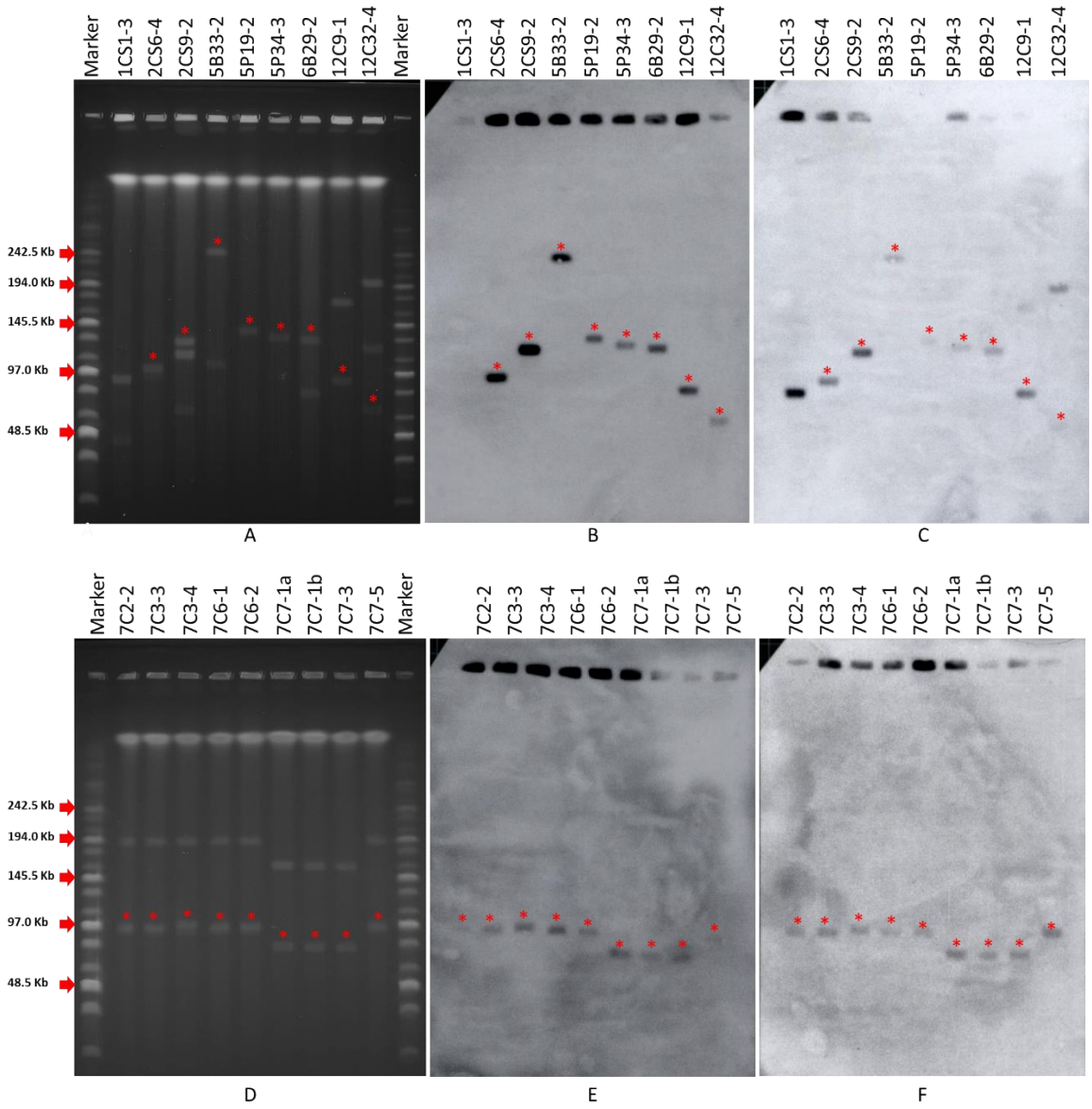
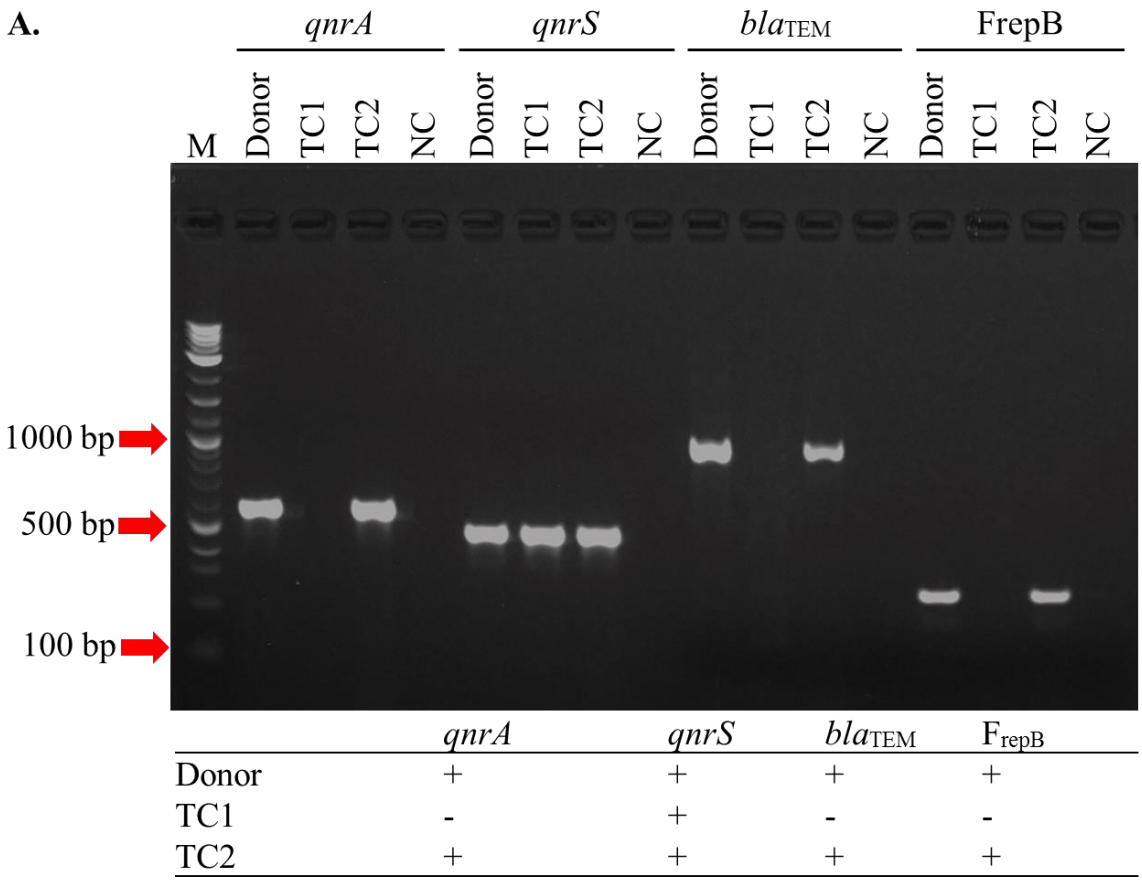


Figure 4. Plasmid profiles of *qnr*-harboring *E. coli*. S1-PFGE profiles of isolates with (A) *qnrS1* and (D) *qnrB4* stained by Ethidium bromide. Lanes 1 and 11: Midrange marker; Southern blot hybridization with a (B) *qnrS1*-specific, (E) *qnrB4*-specific and (C and F) $IncF_{repB}$-specific probes. Lanes 2 – 10: nine and eight selected E. coli harboring *qnrS1* and *qnrB4*, respectively. (∗)- indicate location of *qnr* determinants in plasmid.



	qnrA	*qnrS*	bla_{TEM}	F_{repB}
Donor	+	+	+	+
TC1	-	+	-	-
TC2	+	+	+	+

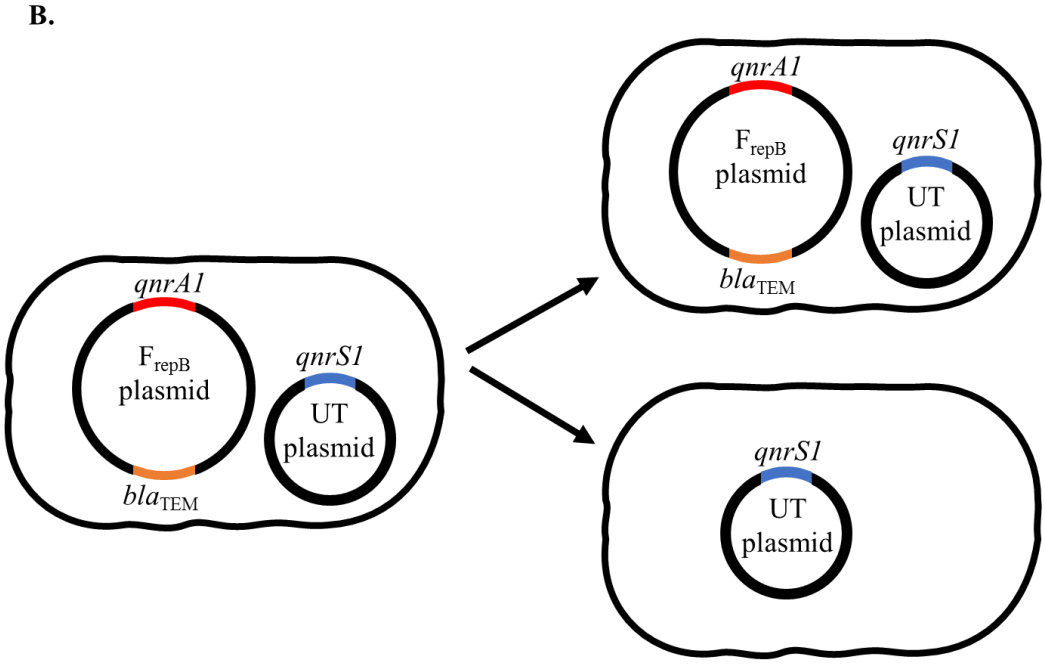


Figure 5. (A) PCR analysis of *E. coli* harboring both *qnrA1* and *qnrS1* and its transconjugant. (B) Schematic representation of plasmid segregation of resistant *E. coli* harboring *qnrA1* and *qnrS1*. UT – un-typable

CONCLUSION

In the Philippines, the prevalence of AMR pathogen is currently increasing, posing a significant threat to public health. Antimicrobial resistance increases mortality, morbidity, and health costs in humans and animals. The inappropriate use of antibacterial agents leads to the emergence of resistant microorganisms in agriculture and humans. Previous studies found an increasing trend of AMR in food animals in the Philippines. Thus, requires effective antimicrobial resistance surveillance programs. However, the national antimicrobial surveillance in the animal health sector has not yet been established. Therefore, this study aims to investigate the prevalence of AMR in *E. coli*, which is the sentinel organism of AMR in animals intended for human consumption and their food-derived products, to delineate the situation of AMR in the livestock sector, and to elucidate the resistance acquisition mechanism to quinolones in the Philippines.

In chapter I, high rate of contamination by multidrug-resistant *E. coli* was observed not only in abattoirs, but also in animal-derived food, from both supermarkets and open markets. The quinolone-resistance determinants of *E. coli* in the Philippines were found to be mediated predominantly by amino-acid substitutions in QRDRs of GyrA and ParC. In addition, a high prevalence of PMQR genes was detected, which raises concerns about the broad dissemination of drug-resistant strains. Finally, the high diversity of ST within *E. coli* carrying mutations in QRDR and/or PMQR genes indicate that the spread of quinolone resistance strains in the Philippines is not dependent on a specific clone. This is the first study to focus on the molecular characteristics of *E. coli* with reduced susceptibility to quinolone found in food-producing animals and their food products in the Philippines.

In chapter II, I showed that *qnr*-harboring isolates carried multiple plasmid replicons and concurrently positive for bla_{TEM}. The results highlighted that *qnr* determinants were transmitted horizontally by $\mathrm{IncF}_{\mathrm{repB}}$ plasmid with difference size and contribute to spreading with other ARGs in the Philippines. Significantly, the spread of *qnr* among commensal *E. coli* seems to be high in the food-producing animals and their food products, acting as a reservoir and promoting to MDR development. Colonization of MDR commensal *E. coli* harboring *qnr* in the human gut from food animals presenting antimicrobial burden, possibly limiting therapeutic option.

The findings in this study will serve as preliminary information and guide in strategizing a more effective control of antimicrobial usage in animals and human. According

to the findings in this study, it is recommended that the hygiene laws for animal slaughter and food handling be enforced in the Philippines, as a high multidrug-resistant *E. coli* contamination rate was observed not only in abattoirs, but also in animal-derived food, from both supermarkets and open markets. Furthermore, to minimize the emergence and spread of quinolone-resistant *E. coli*, the implementation of a strict monitoring of antimicrobial use and the restriction of quinolone usage for therapeutic and farming purposes is recommended.

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