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Original Article

Antibiogram and molecular insights into quinolone resistance in *Salmonella* spp. from food-producing animals in the Philippines

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Abstract

Introduction: The increasing quinolone resistance in food animals in the Philippines has been documented in previous studies. This study aims to investigate the prevalence and underlying molecular mechanisms responsible for quinolone non-susceptibility in *Salmonella* isolated from food-producing animals.

Methodology: A total of 227 *Salmonella* isolates were obtained from food-producing animals and their derived food products in the Philippines. These isolates were subjected to antimicrobial susceptibility testing against 17 different agents. Quinolone non-susceptible isolates were screened for mutations in the quinolone resistance-determining region (QRDR) and plasmid-mediated quinolone resistance (PMQR) genes. QRDR- and PMQR-positive isolates were also screened for virulence genes and genotyped using the multilocus sequence typing (MLST) method.

Results: Most isolates showed high resistance to tetracycline, ampicillin, streptomycin, sulfamethoxazole-trimethoprim, and nalidixic acid. About 68.3% of the isolates showed resistance to at least one antimicrobial agent, and 44.9% exhibited multidrug resistance. Out of the 74 quinolone non-susceptible isolates, 74.3% carried amino-acid substitutions in the QRDR of GyrA and ParC, 4.1% harbored PMQR genes, and 14.9% had both. High-level resistance was associated with double amino-acid substitutions in GyrA (Ser83Phe + Asp87Tyr) and ParC (Thr57Ser + Ser80Ile), while PMQR-positive isolates displayed variable low resistance. Moreover, 17 unique sequence types (STs) were identified, predominantly *S.* London ST155, *S.* Infantis ST32, and *S.* Anatum ST64. All *Salmonella* isolates exhibit QRDR mutations and/or PMQR genes that possess virulence factors *invA* and *agfA*.

Conclusions: The findings highlight the widespread fluoroquinolone-resistant *Salmonella* in food-producing animals and their products, posing a significant threat to the effective treatment of *Salmonella*-related food-borne diseases.

Key words: *Salmonella* spp.; antimicrobial resistance; quinolone; DNA gyrase mutations; plasmid-mediated quinolone resistance (PMQR).

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Introduction

Non-typhoidal *Salmonella* (NTS) remains a significant threat to public and animal health worldwide due to its broad host range [1], diverse transmission routes [2], zoonotic nature [3], and the ability to cause food-borne illnesses [2,4]. Globally, substantial disease burden in humans and animals is attributed mainly to the consumption of contaminated feeds or food of animal origin, particularly from livestock and poultry [4,5]. While typically responsible for mild, self-limiting gastroenteritis, the Gram-negative bacterium can occasionally progress to a more severe invasive infection where antimicrobial intervention is often warranted [6,7].

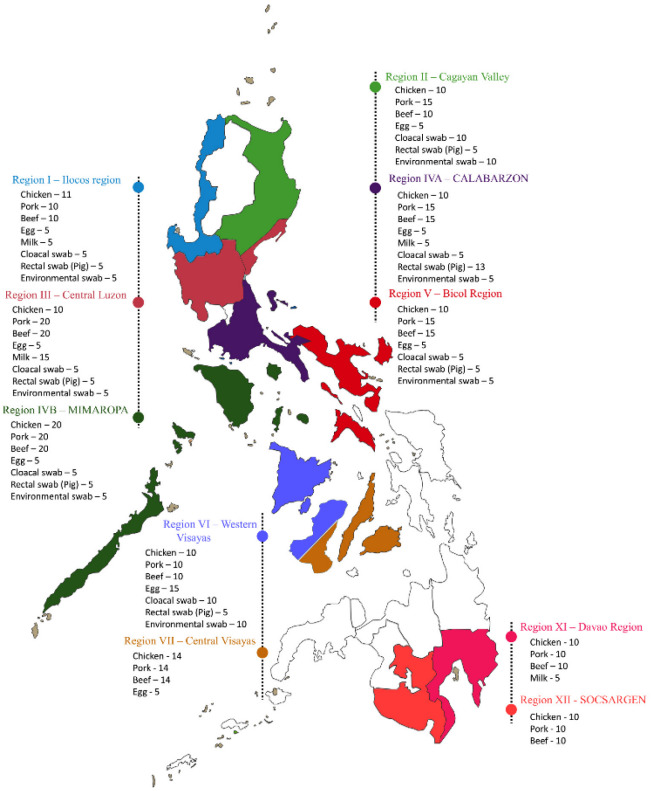
Although fluoroquinolones remain the antimicrobial of choice for salmonellosis, their indiscriminate use has led to the emergence and spread of antimicrobial resistance [8,9]. Alterations in DNA

gyrase and topoisomerase IV, the enzymes essential for bacterial DNA replication, are a primary mechanism of resistance in *Salmonella* [9,10]. These chromosomal changes are also often accompanied by the additional presence of plasmid-mediated quinolone resistance (PMQR) genes, which protect the fluoroquinolone binding sites [11,12]. The severity of the problem increases with the growing prevalence of multidrug resistance (MDR) in *Salmonella* [13], which further limits available therapeutic options and complicates the discovery of alternative treatments and new antibiotics [14].

The patterns of *Salmonella* occurrence and resistance vary among geographical regions. In the Philippines, fluoroquinolone-resistant *Salmonella* strains are increasingly being detected to be affecting both human and food-producing animal populations [15,16]. A 15-year study also identified that ciprofloxacin resistance, in combination with other antibiotics such as ampicillin and cefotaxime, was common among multidrug-resistant *Salmonella* serotypes [17]. In a 2013-2014 study, over half of NTS isolates (48/65) showed resistance to at least one antimicrobial and had mutations linked to fluoroquinolone resistance from hospitals [15]. In food-producing animals, 61.2% of NTS isolates from abattoir-sourced swine tissue samples from tonsils and jejunum were found to harbor *qnrS*, either independently or along with *β*-lactamase resistance markers [18]. Among *Salmonella* strains isolated from chicken meat in public markets, 13.68% showed ciprofloxacin resistance, harboring both DNA gyrase mutations and plasmid-mediated quinolone resistance (PMQR) genes [16]. Apparently, most livestock studies on antimicrobial-resistant *Salmonella* primarily focus on detection, phenotypic resistance patterns, and virulence profiling [16,19,20].

Understanding the molecular mechanisms responsible for quinolone resistance in *Salmonella* strains isolated from food animals is crucial for developing effective strategies to mitigate the spread of resistance and safeguard public health. Despite the growing concerns over antimicrobial resistance, limited information still exists on the resistance profiles, resistance mechanisms, and molecular characteristics of *Salmonella* spp. Specifically, research on the prevalence of molecular mechanisms harbored by quinolone-resistant *Salmonella* spp. in food animals and their products remains notably scarce in the Philippines. Therefore, this study was undertaken to describe the antimicrobial susceptibility and molecular characteristics of *Salmonella* spp. isolated from food animal production in the country with a focus on determining the prevalence of non-typhoidal *Salmonella* spp., identifying quinolone resistance mechanisms to nalidixic acid and ciprofloxacin, which are both superior markers for the detection of fluoroquinolone resistance, and a global priority antibiotic for humans and animals. Lastly, the classification of serotypes and the exploration of virulence profiles were investigated.

Figure 1. Map showing the geographical distribution of samples collected from 10 regions across the Philippines.



Methodology

Sample collection, bacterial enrichment, and isolation

A total of 601 samples were collected in retail meat markets (supermarkets and public markets), abattoirs, and farms in the Philippines from November 2017 to July 2018, following a convenience sampling approach where samples from accessible locations within the target areas were obtained (Figure 1). Table 1 provides details on the number of samples collected. Each sample was pre-enriched by aseptically collecting 25 g or an equal volume into a 1 L homogenizer filter plastic bag with 225 mL of Buffered Peptone Water (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan), and homogenized using a sample blender. The homogenates were incubated for 18–24 h at 37°C. After incubation, 0.1 mL of pre-enrichment was added to 10 mL

Rappaport Vassiliadis (RV) broth (Nihon Pharmaceutical Co. Ltd., Tokyo, Japan) and incubated again for 18 – 24 h at 42°C. A loopful of the culture from the RV broth was streaked on Xylose Lysine Deoxycholate (XLD) agar (Becton Dickinson Co., Ltd., NJ, USA) plates and incubated for 18–24 h at 37 °C. Five presumptive *Salmonella* colonies (red color with a black center) were selected and identified using a standard biochemical test as previously described [21]. Confirmed *Salmonella* isolates were stored at −20 °C in Luria–Bertani broth (Merck, Darmstadt, Germany) containing glycerol 50% *v*/*v* (Difco Laboratories, Inc., Detroit, MI, USA).

Antimicrobial susceptibility testing

Following the guidelines of the Clinical and Laboratory Standards Institute [9], an antibiogram assay was performed on the isolated *Salmonella* spp. using the Kirby-Bauer disk diffusion method. The antimicrobial discs used in the study contained kanamycin (30 µg), gentamicin (10 µg), streptomycin (10 µg), cefoxitin (30 µg), cefepime (30 µg), ceftazidime (30 µg), imipenem (10 µg), nalidixic acid (30 µg), ciprofloxacin (5 µg), ampicillin (10 µg), chloramphenicol (30 µg), tetracycline (30 µg), and sulfamethoxazole/trimethoprim (23.75 µg/1.25 µg) (Becton Dickinson Co., Ltd.). Using the CLSI breakpoints, the antimicrobial susceptibility of the isolates was classified as either susceptible, intermediate, or resistant. Isolates were considered multidrug-resistant (MDR) if they were resistant to at least one agent from three or more antimicrobial categories [10,11]. In addition, the minimum inhibitory concentrations (MICs) of nalidixic acid and ciprofloxacin [12] for isolates harboring mutations in QRDR and/or PMQR genes were determined using the broth microdilution method, and the results were interpreted according to the CLSI breakpoints [13]. *Salmonella enterica* Typhimurium L2 strain was used as the control strain.

Table 1. Oligonucleotide sequences of primers used in this study.

Primer	Sequence	Amplicon size (bp)	Reference
Sal-gyrA_F	5'-GTTAGATGAGCGACCTTGCG-3'	491	[24]
Sal-gyrA_R	5'-GGAATTTTGGTCGGCATGAC-3'		
Sal-gyrB_F	5'-GCTGGAAAACCCATCTGACG-3'	465	[24]
Sal-gyrB_R	5'-CGATAGAAGAAGGTCAACAGC-3'		
Sal-parC_F	5'-CCCTGTTAATGAGCGATATG-3'	512	[24]
Sal-parC_R	5'-GCCGTTCAGCAGGATGTTCG-3'		
Sal-parE_F	5'-CGCAAAAAGCTCACCAGC-3'	485	This study
Sal-parE_R	5'-TCCAGTACGCCCGCCTTCTC-3'		
qnrA_F	5'-ATTTCTCACGCCAGGATTTG-3'	516	[25]
qnrA_R	5'-GATCGGCAAAGGTTAGGTCA-3'		
qnrB_F	5'-GATCGTGAAAGCCAGAAAGG-3'	469	[25]
qnrB_R	5'-ACGATGCCTGGTAGTTGTCC-3'		
qnrS_F	5'-ACGACATTCGTCAACTGCAA-3'	417	[25]
qnrS_R	5'-TAAATTGGCACCCTGTAGGC-3'		
aac(6)lb-cr_F	5'-ATGACTGAGCATGACCTTG-3'	477	[27]
aac(6)lb-cr_R	5'-AACCATGTACACGGCTGG-3'		
qepA_F	5'-CGTGTTGCTGGAGTTCTTC-3'	403	[26]
qepA_R	5'-CTGCAGGTACTGCGTCATG-3'		
thrA_F	5'-ATCCCGGCCGATCACATGAT-3'	*630*	[28]
thrA_R	5'-CTCCAGCAGCCCCTCTTTCAG-3'		
purE_F	5'-GACACCTCAAAAGCAGCGT -3'	*635*	[28]
purE_R	5'-AGACGGCGATACCCAGCGG -3'		
sucA_F	5'-CCGAAGAGAAACGCTGGATC-3'	639	[28]
sucA_R	5'-GGTTGTTGATAACGATACGTAC-3'		
hisD_F	5'-GTCGGTCTGTATATTCCCGG-3'	651	[28]
hisD_R	5'-GGTAATCGCATCCACCAAATC-3'		
aroC_F	5'-GGCGTGACGACCGGCAC-3'	656	[28]
aroC_R	5'-AGCGCCATATGCGCCAC-3'		
hemD_F	5'-ATTCTGATCACCCGCCCCTC-3'	634	[28]
hemD_R	5'-GACCAATAGCCGACAGCGTAG-3'		
dnaN_F	5'-CCGATTCTCGGTAACCTGCT-3'	659	[28]
dnaN_R	5'-ACGCGACGGTAATCCGGG-3'		
invA_F	5'-TGCCTACAAGCATGAAATGG-3'	500	[29]
invA_R	5'-AAACTGGACCACGGTGACAA-3'		
agfA_F	5'-GCATTCGCAGCAATCGTAGT-3'	402	[30]
agfA_R	5'-ACCAACCTGACGCACCATTA-3'		
pefA_F	5'-GCACACGCTGCCAATGAA-3'	452	[31]
pefA_R	5'-ACTGCGAAAGATGCCACAGA-3'		
spvB_F	5'-CGGTTATAGAAGAGCTCCTGT-3'	349	[32]
spvB_R	5'-CCGGTATACGACTCTGTGATC-3'		

Detection of quinolone-resistance determinants

Genomic DNA was extracted using the boiling method [22]. Isolates not susceptible to quinolones, intermediate and/or resistant to nalidixic acid and/or ciprofloxacin, were selected by screening for quinolone resistance determinants. 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 PCR and sequencing as previously described [23–26]. PCR products were purified using ExoSAP® IT (Thermo Fisher Scientific Co., Ltd., MA, USA) and subjected to 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/). Subsequently, inferred amino-acid sequences of QRDR-encoding genes were aligned with the corresponding regions of *S.* Typhi (GenBank accession no. AL513382.1) as a reference strain using the ClustalW program by MEGA v7.0.21.

Multilocus sequence typing (MLST) analysis

All *Salmonella* isolates that harbored mutations in QRDR and/or PMQR genes were further genotyped by multilocus sequence typing as previously described for *Salmonella enterica* [27]. Seven housekeeping genes, namely, *aroC*, *dnaN*, *hemD*, *hisD*, *purE*, *sucA,* and *thrA*, were amplified using the recommended primers. 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, clonal complexes, and singleton assignments, the obtained sequences were submitted to the multilocus sequence typing database (https://pubmlst.org/salmonella-spp and enterobase.warwick.ac.uk). A minimum spanning tree unweighted pair group method with arithmetic mean (UPGMA) was generated following the cluster analysis of the multilocus sequence typing allelic profiles of the isolates using BioNumerics 6.6 software (Applied Maths, Sint-Martens-Latem, Belgium).

Detection of Virulence Genes

The *invA*, *agfA*, *pefA*, and *spvB* genes were detected to determine the presence of virulence genes, as previously reported [28–31]. The PCR conditions were as follows: for *invA*, 95 °C for 2 min, followed by 30 cycles consisting of denaturation of 95 °C for 1 min, annealing at 51 °C for 1 min, an extension at 72 °C for 1 min, and a final extension at 72 °C for 5 min; for *agfA*, 95 °C for 1 min, followed by 30 cycles consisting of denaturation of 95 °C for 15 s, annealing at 51 °C for 55 s, an extension at 72 °C for 30 s, and a final extension at 72 °C for 8 min; and finally, for *pefA*/*spvB*, 95 °C for 2 min, followed by 30 cycles consisting of denaturation of 95 °C for 45 s, annealing at 51 °C for 45 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 stained with GelRed (Biotium, Inc., CA, USA). After gel electrophoresis, images of the PCR amplicons were captured using Printgraph Classic (ATTO Corp., Tokyo, Japan).

Statistical analysis

The data were descriptively analyzed using Statistical Package for Social Sciences (SPSS) software version 22.0 (IBM Corp., Armonk, NY). Differences in the 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 of less than 0.05 was considered statistically significant.

Results

Prevalence of Salmonella spp.

Of the 601 samples collected, 85 (14.1%; 95% CI = 0.115 – 0.172) were positive for *Salmonella*. A total of 25 (14.6%; 95% CI = 0.241 – 0.385) samples from supermarkets were contaminated with *Salmonella*; the highest observed was in chicken (23.4%; 95% CI = 0.123 – 0.380). In the open market, 52 (31.0%; 95% CI = 0.241 – 0.385) samples were contaminated with *Salmonella*. These mostly came from pork samples (41.7%; 95% CI = 0.276 – 0.568). Moreover, no *Salmonella* was detected in abattoir-derived meat samples (0/51). Overall, a significant difference in *Salmonella* contamination rates between open markets and supermarkets was observed ($p = 0.0004$). On the other hand, the isolation rates of *Salmonella* were 6.7% (3/45) and 7.0% (3/43) for cloacal and rectal swab samples, respectively. Additionally, 4.4% of the environmental swab samples (0/4 from abattoirs, 1/31 from swine farms, and 1/10 from poultry farms) were

Table 2. Prevalence of Salmonella contamination among collected samples.

Source	Sample	Number of samples		95% CI
		Examined	Positive (%)	
Supermarket		169	25 (14.8)[a]	0.098 – 0.211
	Beef	54	9 (16.7)	0.079 – 0.293
	Chicken	47	11 (23.4)[a]	0.123 – 0.380
	Pork	68	5 (7.4)	0.024 – 0.163
Open market		168	52 (31.0)[a]	0.241 – 0.385
	Beef	52	19 (36.5)	0.236 – 0.510
	Chicken	68	13 (19.1)[a]	0.106 – 0.305
	Pork	48	20 (41.7)	0.276 – 0.568
Abattoir		55	0	0.000 – 0.065
	Beef	28	0	0.000 – 0.123
	Pork	23	0	0.000 – 0.148
	Environmental swab	4	0	0.000 – 0.602
Swine farm		74	4 (5.4)	0.015 – 0.133
	Rectal swab	43	3 (7.1)	0.015 – 0.195
	Environmental swab	31	1 (3.2)	0.032 – 0.167
Poultry farm		105	4 (3.8)	0.010 – 0.095
	Cloacal swab	45	3 (6.7)	0.014 – 0.183
	Environmental swab	10	1 (1.0)	0.003 – 0.445
	Egg	50	0	0.000 – 0.071
Cattle/Buffalo	Milk	30	0	0.000 – 0.116
Total tested		601	85 (14.1)	0.115 – 0.172

[a]$p < 0.05$.

Table 3. Prevalence of antimicrobial resistance in Salmonella isolates recovered from the abattoir, farm, and market samples.

Antimicrobial agents	No. (%) of antimicrobial-resistant isolates						Total (n = 227)
	Beef (n = 93)	Chicken (n = 65)	Pork (n = 53)	Cloacal swab (n = 4)	Rectal swab (n = 4)	Environmental swab (n = 8)	
TET	42 (45.2)	31 (47.7)	27 (50.9)	3 (75.0)	3 (75.0)	1 (12.5)	107 (47.1)
AMP	34 (36.6)	33 (50.8)	28 (52.8)	3 (75.0)	3 (75.0)	1 (12.5)	102 (44.9)
STR	41 (44.1)	14 (21.5)	16 (30.2)	0	3 (75.0)	1 (12.5)	75 (33.0)
SXT	27 (29.0)	13 (20.0)	24 (45.3)	1 (25.0)	1 (25.0)	0	66 (29.1)
NAL	20 (21.5)	15 (23.1)	15 (28.3)	3 (75.0)	3 (75.0)	1 (12.5)	57 (25.1)
CAZ	18 (19.4)	9 (13.8)	7 (13.2)	0	0	0	34 (15.0)
CHL	11 (11.8)	7 (10.8)	6 (11.3)	1 (25.0)	0	0	25 (11.0)
FOX	12 (12.9)	8 (12.3)	4 (7.5)	0	0	0	24 (10.6)
FEP	15 (16.1)	3 (4.6)	3 (5.7)	0	0	0	21 (9.3)
GEN	10 (10.8)	3 (4.6)	1 (1.9)	0	3 (75.0)	1 (12.5)	18 (7.9)
KAN	5 (5.4)	1 (1.5)	5 (9.4)	0	0	0	11 (4.8)
CIP	6 (6.5)	0	0	0	3	1 (12.5)	10 (4.4)
IPM	3 (3.2)	1 (1.5)	0	0	0	0	4 (1.8)

Antimicrobial agents: KAN – Kanamycin; GEN – Gentamicin; STR – Streptomycin; FOX – Cefoxitin; FEP – Cefepime; CAZ – Ceftazidime; IPM – Imipenem; NAL – Nalidixic acid; CIP – Ciprofloxacin; AMP – Ampicillin; CHL – Chloramphenicol; TET – Tetracycline; and SXT – Sulfamethoxazole/trimethoprim.

positive for *Salmonella*. In contrast, no *Salmonella* was detected in milk and egg samples. Fisher's exact test revealed no statistical difference among the observed *Salmonella* isolation rates among samples collected from farms ($p = 0.7194$) (Table 1).

Antimicrobial susceptibility

A total of 227 *Salmonella* isolates were obtained from the collected samples. Table 2 shows the resistance profile of all isolated *Salmonella* in this study. Of the tested antimicrobials, most of the isolates showed resistance to tetracycline (47.1%, 95% CI = 40.5 – 53.9), ampicillin (44.9%, 95%CI = 38.4 – 51.7), streptomycin (33.0%, 95%CI = 27.0 – 39.6), sulfamethoxazole-trimethoprim (29.1%, 95% CI = 23.3 – 35.5), and nalidixic acid (25.1%, 95% CI = 19.6 – 31.3). The resistance observed to kanamycin, gentamicin, ciprofloxacin, imipenem, and ceftazidime was less than 10% of the isolates. Among the samples collected from supermarkets, pork isolates exhibited a significantly higher MDR rate ($p < 0.05$) than those from chicken and beef. However, there was no significant difference in MDR rates among isolates between retail meat sources ($p = 0.7572$) and farms ($p = 0.1058$). Overall, 31.7% (95% CI = 25.7 – 38.2) of the isolates were pan-susceptible, 68.3% (95% CI = 61.8 – 74.3) were resistant to at least one antimicrobial agent, and 44.9% (95% CI = 38.4 – 51.7) were of MDR (Table 3).

QRDR and PMR determinant analysis

Out of the 74 quinolone non-susceptible isolates, 74.3% (95% CI: 62.8 – 83.8) had an amino-acid substitution in the QRDR of GyrA and ParC, 4.1% (95% CI: 0.8 – 11.4) harbored PMQR genes, and 14.9% (95% CI: 7.7 – 25.0) had both. The predominant amino acid substitution in the QRDR of GyrA were serine to phenylalanine (Ser83Phe, 7.5%) or leucine (Ser83Leu, 3.0%) or tyrosine (Ser83Tyr, 1.5%) at codon 83, and aspartic acid to tyrosine (Asp87Tyr, 6.0%) or glycine (Asp87Gly, 11.9%) or asparagine (Asp87Asn, 9.0%) at codon 87. Substitutions in ParC were mainly threonine to serine at codon 57 (Thr57Ser, 94.0%) and serine to isoleucine at codon 80 (Ser80Ile, 6.0%). In contrast, no amino acid substitution was observed in GyrB and ParE. Isolates carrying double amino acid substitutions in GyrA (Ser83Phe + Asp87Tyr) and ParC (Thr57Ser + Ser80Ile) demonstrated high-level quinolone resistance (NAL MIC: > 128 µg/mL and CIP MIC: 16 µg/mL) (Table 4 and Figure 2).

A total of 14 isolates (18.9%, 95% CI: 10.8 – 29.7) carried *qnr*-type PMQR genes, with *qnrS1* being the most frequent gene, present in 11 isolates (78.6%, 95% CI: 49.2 – 95.3). Notably, nine (9) of these *qnrS1*-

Table 4. Distribution of multidrug-resistant Salmonella isolates among samples.

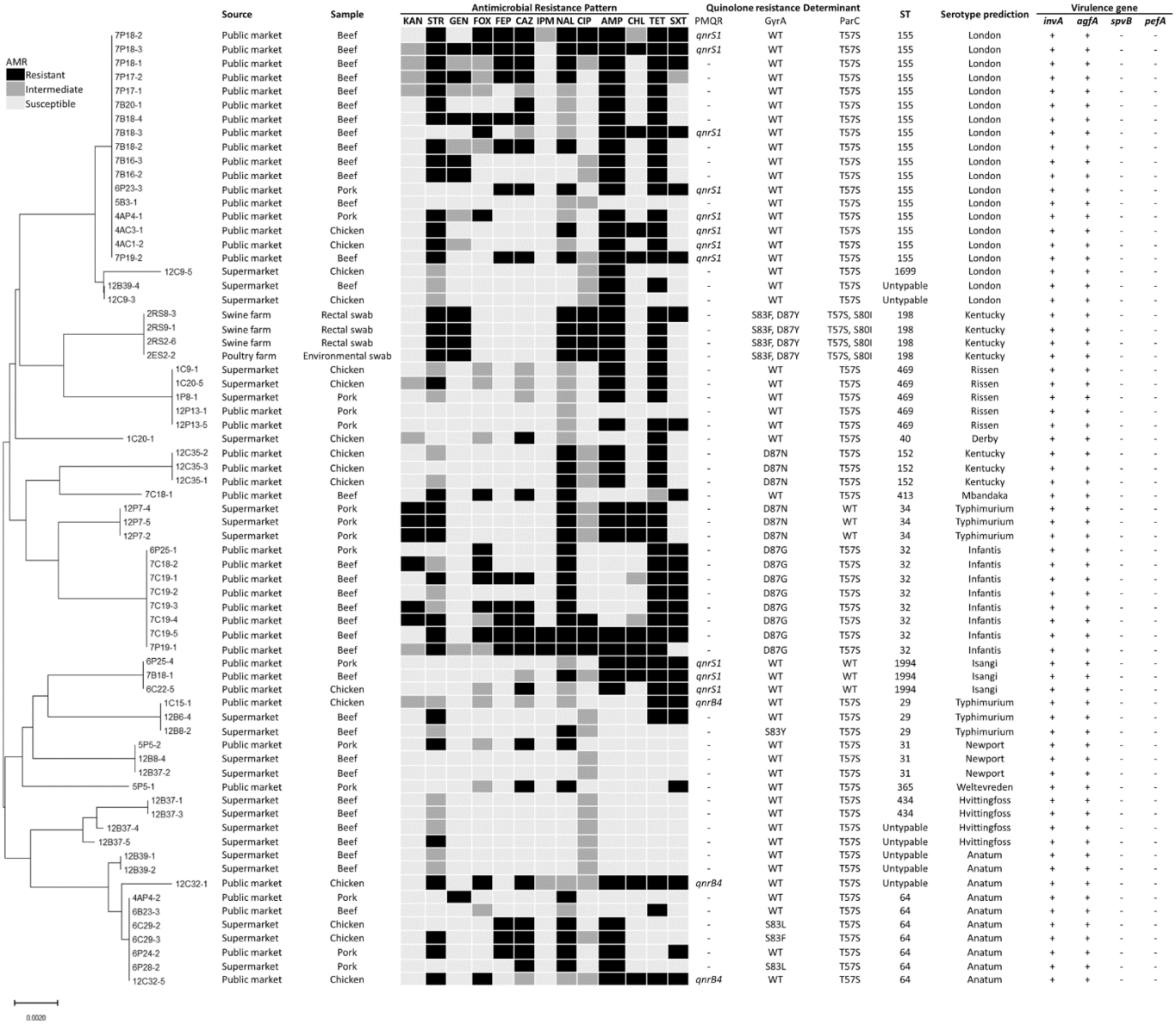
No. of antimicrobial classes	No. (%) of multidrug-resistant isolates						Total (n = 227)
	Beef (n = 93)	Chicken (n = 65)	Pork (n = 53)	Cloacal swab (n = 4)	Rectal swab (n = 4)	Environmental swab (n = 8)	
0	35 (37.6)	15 (23.1)	13 (24.5)	1 (25.0)	1 (25.0)	7 (87.5)	72 (31.7)
1-2	15 (16.1)	26 (40.0)	12 (22.6)	0	0	0	53 (23.2)
3-4	26 (28.0)	15 (23.1)	20 (37.7)	3 (75.0)	2 (50.0)	1 (12.5)	67 (29.5)
5-6	12 (12.9)	9 (13.8)	8 (15.1)	0	1 (25.0)	0	30 (13.2)
7-8	3 (3.2)	0	0	0	0	0	3 (1.3)
> 9	2 (2.2)	0	0	0	0	0	2 (0.9)
Resistance ≥ 1	58 (62.4)	50 (76.9)	40 (75.5)	3 (75.0)	3 (75.0)	1 (12.5)	155 (68.3)
MDR ≥ 3	43 (46.2)	24 (36.9)	28 (52.8)	3 (75.5)	3 (75.0)	1 (12.5)	102 (44.9)

harboring isolates had additional amino acid substitutions in ParC. The second most frequent gene was qnrB4 detected in three isolates (21.4%, 95%CI: 4.7 – 50.8), which all had additional amino acid substitutions in QRDR of ParC (Figure 2). Other PMQR genes, *qepA* and *acc(6')-Ib-cr,* were not detected in any of the tested isolates. All these PMQR-positive isolates displayed variable low resistance to quinolones (CIP MIC < 0.015 – 0.5 µg/mL and NAL MIC 4 – 16 µg/mL). Overall, 69 (93.2%, 95%CI: 84.9 – 97.8) isolates were positive for quinolone resistance determinants, while five (6.8%, 95% CI: 2.2 – 15.1) lacked any of these determinants under investigation (Table 4 and Figure 2).

Multilocus sequence type analysis and serotype prediction

Seventeen unique sequence types (STs) were identified among the 69 isolates with QRDR mutations and PMQR determinants, except for seven isolates that

Figure 2. Dendrogram showing the relationship between 69 PMQR-QRDR gene-harboring Salmonella strains isolated from beef, chicken, pork, and cloacal, rectal, and environmental swab samples, based on the MLST allele profile including information about ST, quinolone resistance determinant, virulence genes (invA, agfA, spvB, pefA), and phenotypic resistance profile according to Kirby Bauer disk diffusion method.



The antimicrobial agents used in this study include KAN: Kanamycin; STR: Streptomycin; GEN: Gentamicin; FOX: Cefoxitin; FEP: Cefepime; CAZ: Ceftazidime; IPM: Imipenem; NAL: Nalidixic acid; CIP: Ciprofloxacin; AMP: Ampicillin; CHL: Chloramphenicol; TET: Tetracycline; and SXT: Trimethoprim/Sulfamethoxazole. (+) – positive; (:) – Negative; WT – wild type.

had unknown STs. The three predominant STs were ST155 (n = 17), ST32 (n = 8), and ST64 (n = 7). Serotypes were also inferred from the MLST profiles. The predominant predicted serotype was *S.* London (n = 20), followed by *S.* Anatum (n = 10), *S.* Infantis (n = 8), *S.* Kentucky (n =7), *S.* Typhimurium (n = 6), *S.* Rissen (n = 5), *S*. Hvittingfoss (n = 4), *S*. Newport (n = 3), *S*. Isangi (n = 3), *S*. Mbandaka (n = 1), and *S*. Weltevreden (n = 1). *Salmonella enterica* ser. Kentucky and *S*. Typhimurium were assigned to two different STs, ST198 and ST152, and ST29 and ST32, respectively. Among the samples, *S*. London ST155 and *S*. Infantis ST32 were significantly observed in beef samples ($P > 0.05$). Meanwhile, S. Kentucky ST198 was detected solely in swab samples, while the other serotypes were present in the meat (Figure 2).

Prevalence of virulence genes

All *Salmonella* isolates harboring QRDR mutations and/or PMQR genes carried *invA* and *agfA*, though none were positive for *spvB* and *pefA* (Figure 2).

Discussion

With an estimated 90 million cases per year, *Salmonella* spp. constitutes a major cause of food-borne disease, posing substantial risks to global human and animal health as well as significant economic challenges [7,8,32–38]. In Southeast Asia, livestock products (food derived from farm animals), particularly meats, serve as the primary source of salmonellosis transmission [39]. In this study, food products sourced from chicken exhibited the highest contamination rate (23.4%; 95% CI = 0.123 – 0.380) in the supermarket. These findings suggest that *Salmonella* was more likely to be recovered in supermarkets than in open markets. Surprisingly, these meat retailers employ superior sanitary and quality standards compared to their open market counterparts. In contrast, we also observed that pork and beef samples from the open market demonstrated a high prevalence of *Salmonella* contamination. This may be attributed to the lack of adherence to slaughter and sanitary standard protocols even at authorized abattoirs [4]. This result further confirms that samples obtained from a registered abattoir in this study were negative for *Salmonella*, but is in contrast to an earlier report in the country [40]. Overall, the *Salmonella* contamination level in the open markets was higher compared to that in supermarkets ($p = 0.0004$). Factors that could account for the high rates of *Salmonella* prevalence in open market samples include the use of unclean utensils (chopping boards and knives) when handling products, improper storage methods, cross-contamination from contaminated ice or water, and inadequate temperature display conditions [41,42]. Various reports from across the globe have consistently pointed to higher or comparable levels of *Salmonella* contamination in meat obtained from open markets than those from supermarkets [39,41,43–46]; however, a few studies have also reported opposite trends [47–49]. Despite these discrepancies in numbers, it is evident that meat sold in both types of markets presents a key risk factor for salmonellosis in humans, underscoring the need for stringent sanitary protocols and food safety standards in both market settings.

Clinical resistance to ampicillin, tetracycline, nalidixic acid, sulfamethoxazole-trimethoprim, and streptomycin were observed in this study. Though consistent with previous reports from the Philippines, other Southeast Asian nations, and even the USA [7,13,50,51], resistance to the aforementioned antimicrobials occurred at much lower rates than those recorded in earlier works [13,52,53]. Differences in the

Table 5. Distribution of amino-acid substitutions in QRDR genes, PMQR genes, and MIC of tested quinolone-resistant isolates.

QRDR amino acid substitution[a]		PMQR	MIC (µg/mL)[d]		Serotype (No. of isolate)
GyrA	ParC		Nalidixic acid	Ciprofloxacin	
Asp87→Asn	-[b]	-[c]	>128	0.25	Typhimurium (3)
Asp87→Gly	Thr57→Ser	-[c]	2 -128	<0.015- 0.25	Infantis (8)
Asp87→Asn	Thr57→Ser	-[c]	128	0.25	Kentucky (3)
Ser83→Leu	Thr57→Ser	-[c]	>128	0.25	Anatum (2)
Ser83→Phe	Thr57→Ser	-[c]	>128	0.25	Anatum (1)
Ser83→Tyr	Thr57→Ser	-[c]	>128	0.25	Typhimurium (1)
Ser83→Phe, Asp87→Tyr	Thr57→Ser, Ser80→Ile	-[c]	>128	16	Kentucky (4)
-[b]	Thr57→Ser	-[c]	2 - 4	< 0.015 - 0.03	Anatum (5); Derby (1); Hvittingfoss (4); London (14); Mbandaka (1); Newport (3); Rissen (5); Typhimurium (1); Weltevreden (1)
-[b]	Thr57→Ser	*qnrB4*	8 - 16	0.5	Anatum (2)
-[b]	Thr57→Ser	*qnrS1*	4	< 0.015 - 0.25	London (6)
-[b]	Thr57→Ser	*qnrA1*, *qnrB4*	16	0.25	Typhimurium (1)
-[b]	-[b]	*qnrS1*	4 - 8	0.25 - 0.5	Isangi (3)

[a] QRDR substitution: GyrA – Ser83→Leu/Phe/Try: Serine to Leucine/ Phenylalanine/Tyrosine at codon 83; Asp87→Asn/Gly/Tyr: Aspartic acid to Asparagine/Glycine /Tyrosine at codon 87; ParC – Ser80→Ile: Serine to Isoleucine at codon 80; Thr57→Ser: Threonine to Serine at codon 57. [b] No substitution detected in the QRDR. [c] No PMQR determinant detected. [d] MIC – Minimum inhibitory concentration.

antimicrobial resistance patterns and trends among countries can likely be traced to varying farm practices, particularly the frequency and indication of the use of antimicrobials, which are shaped by local conditions and existing agricultural policies [54]. Hence, the ongoing emergence and spread of quinolone-resistant *Salmonella*, particularly in food products, continues to be a significant global threat to public health [8,50,55]. The present work also found that pork isolates had the highest MDR rate at 44.9% among all the samples. This is comparatively much lower than the 73-73.9% MDR rates recorded in neighboring Southeast Asian nations such as Thailand and Laos [18,36]. Nevertheless, these findings can be attributed to the dependence of intensive and extensive swine production systems on antimicrobials, along with the improper use and easy access to antibiotics [50,54,56], practices which are employed to enhance farm performance, prevent disease in animals subjected to stress (e.g., changes in weather conditions, pig movement, post-vaccination, among others), and control infection spread among pigs in close contact [57]. Projections indicate that the Philippines, which ranks among the highest users of antimicrobials in livestock and poultry production, will further increase its usage by 2030 [58]. Hence, the growing prevalence and potential alarming impacts of multidrug-resistant *Salmonella* strains on the meat supply chain highlight the need for the stringent implementation of an antimicrobial use policy that will help combat concerns regarding MDR [37].

Indeed, the improper use of veterinary antimicrobials, such as antibiotics, could lead to the development of antibiotic-resistant bacteria that might be transmitted to humans [59]. In the Philippines, livestock farmers can easily buy veterinary antimicrobial drugs at local agricultural veterinary supply and retail outlets, even without a prescription [60,61]. However, despite existing policies, the issuance of veterinary prescriptions has not been effectively enforced. Furthermore, there is a scarcity of animal health practitioners in the Philippines, which forces livestock farmers to “self-medicate” and use antimicrobials without prescription [61]. This could result in drug misuse when treating sick animals without a proper diagnosis and administering improper doses. Both can reduce the drugs' clinical effectiveness and may also promote the development of drug resistance [59]. Many authors have also noted that livestock farmers often misuse antibiotics due to a lack of knowledge on the responsible use, potential negative effects, and failure to follow manufacturers' instructions [60,62]. Therefore, as observed in this study, indiscriminate, improper, and irrational use of veterinary antimicrobials in the Philippine livestock sector can potentially cause the emergence and spread of antimicrobial-resistant pathogens, such as *Salmonella*.

Amino acid substitutions in the QRDR of GyrA and ParC, along with the presence of PMQR genes in *Salmonella* isolates, were also noted in this study. Mutations that occurred on codons 83 and 87 were linked to the non-susceptibility of *Salmonella* to quinolones [63]. Specifically, a mutation in the Ser 83 codon led to a higher ciprofloxacin MIC than a mutation at the Asp87 codon [64]. The double mutations affecting Ser83 and Asp87 found in the isolates of this study were also consistent with previous findings [53], which were associated with an increased MIC of *Salmonella* to fluoroquinolones [65]. Moreover, ciprofloxacin-resistant *Salmonella* strains were found to harbor a single mutation in the gyrA gene, while strains carrying double mutations within the same gene did not demonstrate such resistance [64].

The amino acid substitution Thr57-Ser was predominant in *Salmonella* quinolone non-susceptible isolates. This particular mutation, located outside the QRDR of *ParC*, resulted in *Salmonella* strains that remained susceptible or only slightly less susceptible to ciprofloxacin (MIC 0.015 – 0.03 µg/mL) and nalidixic acid (MIC 2 – 4 µg/mL). Alone, it conferred low susceptibility, providing only marginal protection against quinolones. Previous studies found that Thr57-Ser alone had a low MIC to ciprofloxacin but not to nalidixic acid [65–67]. Interestingly, observations in the present study contradicted earlier findings, as the presence of the Thr57-Ser mutation alone did not lead to an increase in ciprofloxacin MIC when compared to non-mutant strains [68,69]. Furthermore, additional mutations in the *GyrA* and *Qnr* genes also increase ciprofloxacin and nalidixic acid resistance. Therefore, it was noted that the mutation outside the ParC QRDR likely functions as a naturally occurring compensatory mutation with little bacterial fitness cost, explaining its prevalence in the *Salmonella* populations examined [9,63].

A total of 14 quinolone-resistant isolates in this study have PMQR genes from the Qnr family. The PMQR determinant Qnr gene was a plasmid-encoded and chromosomally-determined protein that protects DNA gyrase and topoisomerase IV against the inhibitory effect of quinolones [64,70–73]. In the present study, *Salmonella* strains that carry this gene were found to have reduced susceptibility to ciprofloxacin while at the same time promoting

mutation development in the QRDR of the *GyrA* gene [18,64,73]. Also, a variable low resistance to quinolones was exhibited by PMQR-positive isolates (CIP MIC < 0.015 - 0.5 µg/mL and NAL MIC 4-16 µg/mL). Calayag *et al.* [18] previously reported that the most common *qnr* genes of *Salmonella* isolates in the country were *qnrS*, *qnrB,* and *qnrA* genes, in that order. This result corroborates the observed occurrence rates of the same genes being studied in the present work. While the *aac(6')-lb-cr4* gene was reported to be present in *Salmonella* isolates in China [34,63], this was not detected from the present study's tested isolates. Hence, these observations demonstrate that the ubiquity of *qnr* genes varies depending on samples and regions [18].

Multiple salmonellosis outbreaks in humans and animal farms have been documented throughout the Philippines [51]. Humans usually become infected through the ingestion of food contaminated with feces from an infected animal [18]. The majority of the non-typhoidal *Salmonella* serotypes found in these patients were Enteritidis, Typhimurium, Weltevreden, Stanley, Anatum, Heidelberg, and Choleraesuis [15,17]. *S.* Anatum, *S.* Saint Paul, and *S.* Kentucky were also frequently detected in food of animal origin [40]. Similarly, *S.* London, *S.* Anatum, *S.* Infantis, *S.* Kentucky, *S.* Typhimurium, and *S.* Rissen have been frequently identified in this study. Consistent with previous research, these findings validate the identified serotypes, which were widely distributed in the country and caused salmonellosis through multiple transmission routes. These include consumption of contaminated water, food animal products, or fresh produce, and contact with animals or their environment.

Many *Salmonella* strains are detrimental due to the presence of various virulence genes [74]. Numerous investigations on Salmonella virulence have consistently reported a high prevalence of *invA*, aligned with the findings of this study. This virulence gene facilitates the invasion of the host's epithelial tissues [75]. Concurrently, *agfA,* an aggregative fimbriae gene detected in this work, was responsible for the sliding motility, adherence to epithelial cells, and accumulation of *Salmonella* [76]. It is also an important gene for biofilm formation and bacterial survival in the environment [77]. However, the absence of plasmid-related *spvB* and *pefA* genes among the tested *Salmonella* isolates points to a lack of plasmids related to these virulence markers. These results are parallel with the prior investigations from the Philippines and other countries, which similarly reported that certain virulence genes were lacking or only present in a few *Salmonella* strains [78–81]. Given their potential significance in disease pathogenesis, further investigation of these genes remains critical for elucidating the mechanisms behind *Salmonella* virulence.

Conclusions

In conclusion, this study demonstrated the high prevalence of *Salmonella* spp. in food-producing animals and their varying degrees of resistance to different antibiotics. In addition, certain quinolone-resistant isolates showed amino acid substitutions in the QRDR and harbored PMQR genes, both contributing to their reduced susceptibility and resistance to quinolones. Furthermore, the QRDR and PMQR-positive isolates were identified as distinct serovars, *S.* Anatum ST64, *S.* London ST155, and *S.* Infantis ST32, which were frequently present in pork and chicken meat from wet markets and supermarkets. Taken altogether, this present work underscores the significant role of food-producing animals as reservoirs for antimicrobial-resistant *Salmonella* in the Philippines. The emergence and potential spread of quinolone resistance in *Salmonella* emphasize the urgent need for stricter control measures on antibiotic use in animal production to curb the further development and dissemination of antimicrobial resistance. Moreover, adopting hygiene and biosecurity practices on animal farms and retail meat markets is crucial to limiting *Salmonella* transmission risks. Finally, increased surveillance and further research are needed to improve the global understanding of quinolone-resistant *Salmonella*'s presence, transmission, and evolution.

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Conflict of interest

No conflict of interest is declared.

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