Antimicrobial Susceptibility and Prevalence of bla and qnr Genes in Salmonella Enterica Isolated from Slaughtered Pork

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Research article

Abstract

Background: *Salmonella enterica* is known as one of the most common foodborne pathogens worldwide. While salmonellosis is usually self-limiting, severe infections may require antimicrobial therapy. However, increasing resistance of *Salmonella* to antimicrobials, particularly fluoroquinolones and cephalosporins, is of utmost concern. The present study aimed to investigate the antimicrobial susceptibility of *S. enterica* isolated from pork, the major contributor in Philippine livestock production.

Results: Our results show that 61.2% of the isolates carried antimicrobial resistance genes *qnrS* and *bla*$_{TEM}$. While *qnrA* (12.9%) and *qnrB* (39.3%) were found less frequently, co-carriage of *bla*$_{TEM}$ and one to three *qnr* subtypes was observed in 45.5% of the isolates. Co-carriage of *bla*$_{TEM}$ and *bla*$_{CTX-M}$ was also observed in 3.9% of the isolates. Antimicrobial susceptibility testing revealed that majority of the isolates were non-susceptible to ampicillin and trimethoprim/sulfamethoxazole, and 13.5% of the isolates were multidrug-resistant.

Conclusions: High prevalence rates of *S. enterica* carrying antimicrobial resistance genes (ARG), specifically the presence of isolates co-carrying resistance to both ß-lactams and fluoroquinolones, raise a concern on antimicrobial use in the Philippine hog industry and on possible transmission of ARG to other bacteria.

Background

Salmonella infections, or salmonellosis, are commonly acquired through consumption of contaminated food of animal origin. In the Philippines, *Salmonella enterica* was shown to be the leading cause of foodborne disease outbreaks from 1995–2018 [1, 2]. While the disease is usually self-limiting, it may require antimicrobial therapy when the infection becomes invasive. The fluoroquinolone ciprofloxacin and the extended-spectrum cephalosporin (ESC) ceftriaxone are the current treatments of choice because the emergence of antimicrobial resistance (AMR) has rendered several drugs such as ampicillin, chloramphenicol, and trimethoprim/sulfamethoxazole obsolete in salmonellosis therapy [3, 4].

Resistance to ß-lactams, such as ESCs, is most commonly attributed to the *bla* genes of subtypes TEM, SHV, and CTX-M which encode for ß-lactamases that hydrolyze the ß-lactam ring, thereby rendering the drug inactive [5, 6]. In contrast to ß-lactam resistance, fluoroquinolone resistance is typically attributed to chromosomal mutations in the quinolone targets DNA gyrase and topoisomerase IV, and overexpression of efflux pumps that reduce drug accumulation [7]. However, plasmid-mediated quinolone resistance (PMQR), such as *qnr* genes, may also occur. These genes are broadly distributed worldwide and are commonly found in association with genes encoding for ß-lactamases [8–11]. Consequently, *bla* and *qnr* genes have been increasingly found in bacteria isolated from livestock animals [8, 9, 12–17]. If motile, resistance determinants may accelerate the spread of AMR when these are taken up by non-pathogenic or pathogenic bacteria alike.

There is evidence that substantial use of antimicrobials in food-producing animals may drive the emergence of drug-resistant strains [12, 18, 19]. While the use of certain antimicrobials such as nitrofurans and chloramphenicol has been banned in livestock production in several parts of the world, AMR in agriculture remains a global challenge [12, 19]. Monitoring AMR development in livestock and meat allows early detection of AMR emergence and prevalence [20] which can be used to design interventions to improve antimicrobial therapy and reduce resistance selection pressure [21, 22]. This is generally accomplished by antimicrobial susceptibility testing (AST) and detection of antimicrobial resistance genes (ARG).

In the Philippines, pork makes up the majority of livestock production and amounts to a 3.8M USD industry [23]. The country's rapidly growing population is expected to further increase pork consumption and production. If left unchecked, AMR may lead to challenges in food production, food security, food safety, economic losses to the hog industry, and AMR spillover to the surrounding environment [12, 18, 22]. Therefore, this study aimed to investigate the antimicrobial susceptibility and prevalence of ß-lactamase-encoding genes (bla_{CTX-M}, bla_{SHV}, and bla_{TEM}) and plasmid-mediated quinolone resistance ($qnrA$, $qnrB$, and $qnrS$) in *S. enterica* from slaughtered pork in Metro Manila, Philippines.

Results

A total of 178 isolates were analyzed in this study. Vitek® 2 AST revealed that the isolates were generally resistant to ß-lactams, but susceptible to quinolones. A large number were non-susceptible to ampicillin (72.5%) and trimethoprim/sulfamethoxazole (70.8%). Non-susceptibility to key drugs, ceftazidime, ceftriaxone, and ciprofloxacin were observed in 8.4%, 7.9%, 15.7% of the isolates, respectively (Table 1). Multidrug resistance was observed in 24 (13.5%) isolates; most of which were non-susceptible to four classes of antimicrobial agents (Table 2). One ESBL-producing isolate was also detected.

Table 1

Non-susceptibility levels of 178 *S. enterica* isolates against different antimicrobial agents. Antimicrobials are classified into categories based on the recommendations of Magiorakos et al. [24]. Non-susceptibility to non-ESCs, cephamycins, and aminoglycosides are not shown as these antimicrobial agents are not clinically effective, although they may appear active in vitro.

Class	Antimicrobial	% Non-susceptibility
Penicillin	Ampicillin	71.9% (128)
Penicillin / ß-lactamase inhibitor	Amoxicillin / clavulanic acid	10.1% (18)
Antipseudomonal penicillin / ß-lactamase inhibitor	Piperacillin / tazobactam	0.6% (1)
Extended-spectrum cephalosporin	Ceftazidime	8.4% (15)
	Ceftriaxone	7.9% (14)
Carbapanem	Ertapenem	0.0% (0)
	Imipenem	1.7% (3)
	Meropenem	0.0% (0)
Fluoroquinolone	Ciprofloxacin	15.7% (28)
Folate pathway inhibitor	Trimethoprim / sulfamethoxazole	70.8% (126)

Table 2
Multidrug resistance patterns of 24 *S. enterica* isolates.

Number of *S. enterica* isolates	Multidrug resistance pattern[a]
1	Pen, Pen/BI, APen/BI, ESC, Flu
14	Pen, Pen/BI, FPI, ESC
1	Pen, Pen/BI, FPI, Car
1	Pen, Pen/BI, FPI, Flu
6	Pen, FPI, Flu
1	Pen, Pen/BI, FPI

[a]Pen, penicillin; Pen/BI, penicillin/ß-lactamase inhibitor; APen/BI; antipseudomonal penicillin / ß-lactamase inhibitor; ESC, extended-spectrum cephalosporin; Flu, fluoroquinolone; FPI, folate pathway inhibitor; Car, carbapenem.

Polymerase chain reaction (PCR) assays targeting *bla* genes revealed that 61.2% and 5.1% of the isolates were harboring the bla_{TEM} and bla_{CTX-M} genes, respectively. No isolate carried bla_{SHV}. CTX-M variant typing revealed that 6/9 bla_{CTX-M} carrying isolates carried $bla_{CTX-M-1}$, and 3/9 carried $bla_{CTX-M-2}$. Co-carriage of bla_{CTX-M} (four under the CTX-M-1 group, three under the CTX-M-2 group) and bla_{TEM} was observed in seven isolates. For *qnr* genes, 12.9%, 39.3%, and 61.2% were harboring the *qnrA*, *qnrB*, and *qnrS* genes, respectively. Co-carriage of bla_{TEM} and one to three *qnr* subtypes were found in 45.5% of the isolates (Fig. 1).

Discussion

Since it has been established that ampicillin and trimethoprim/sulfamethoxazole have become obsolete in salmonellosis therapy, high non-susceptibility rates to these antimicrobials were expected. In many countries, aminopenicillins, which include ampicillin, trimethoprim, sulfamethoxazole, and trimethoprim/sulfonamide combinations are among the most frequently used antimicrobials in livestock production [12, 26]. These antimicrobials are generally administered in all phases of hog production [26]. In this study, non-susceptibilities to ampicillin and trimethoprim/sulfamethoxazole were observed in 71.9% and 70.8% of *S. enterica*, respectively. Phongaran et al. [13] reported that 69.0% of *Salmonella* isolated from hogs in Thailand were resistant to ampicillin. However, in this study, only 35.7% were resistant to trimethoprim/sulfamethoxazole. One study conducted among hogs in Vietnam reported that 36.7% of *Salmonella* isolates were resistant to trimethoprim/sulfamethoxazole and only 41.3% to ampicillin [27]. On the other hand, low rates of resistance (< 5%) to ESC and ciprofloxacin were reported in both studies [13, 27], while this present study reported rates which were slightly higher (< 10%).

In this study, multidrug resistance was observed in 13.5% of *S. enterica* isolates. Reports of (multidrug-resistant) MDR *Salmonella* isolated from hogs in other Southeast Asian countries are higher (30–40%) (13,27). In other countries, even higher rates (70–80%) of MDR *Salmonella* isolated from pork and the pork production chain were observed [15, 28]. Out of 24 MDR *S. enterica* isolates in the present study, 15

and 8 were non-susceptible to ESC and fluoroquinolones, respectively, the current drug options in treating salmonellosis. Multidrug resistance is a challenge as it narrows down the options for antimicrobial therapy.

Majority of studies on *bla* genes and livestock animals in Southeast Asian countries are focused on *E. coli* in which bla_{TEM} and bla_{CTX-M} are the most frequently identified *bla* genes [12]. In *Salmonella*, bla_{TEM} appears to be the most common. In India, Lalruatdiki et al. [14] observed that 30% of *Salmonella* isolated from a pig population were carrying bla_{TEM}, and 10% were carrying bla_{CTX-M}. Co-carriage of bla_{CTX-M} and bla_{TEM} has also been observed in extended-spectrum ß-lactamase (ESBL)-producing *Salmonella* from pigs [14, 29]. In the present study, co-carriage of bla_{TEM} and bla_{CTX-M} was found in seven (3.9%) isolates. However, none of these isolates were ESBL-producing which could suggest that these are only carrier of silent *bla* genes. The only ESBL-producing *Salmonella* in this study was carrying only bla_{TEM}. While most bla_{TEM} in the study possibly confer only broad-spectrum ß-lactam resistance considering the high rates of non-susceptibility to ampicillin, its presence in combination with other resistance determinants could render an isolate multidrug-resistant.

We report in this study that 71.3% of *S. enterica* isolates harbored PMQR. The genes *qnrA*, *qnrB*, and *qnrS* were observed in 12.9%, 39.3%, and 61.2% of the isolates, respectively. While Qnr proteins offer only low resistance against quinolones, the high incidence of PMQR may be a cause for concern since it has been shown to broaden the mutant selection window in bacteria [7]. Lin et al. [16] demonstrated that ciprofloxacin resistance conferred by PMQR is even comparable to that of quinolone target mutations. Prevalence rates of *qnr* genes appear to vary among samples and geographical locations. Cameron-Veas et al. [15] reported that 15% of *S. enterica* isolated from a pork production chain in Brazil were carrying *qnrB*, and none were carrying *qnrA* and *qnrS*. A separate study in China reported the prevalence of *qnrA* (0%), *qnrB* (16%), and *qnrS* (66%) [16] in foodborne *Salmonella*. In Thailand and in Laos, Sinwat et al. [17] found only 1–8% of *S. enterica* isolated from pork to be carrying the same *qnr* genes. This highlights the importance of a national surveillance of ARG since it appears individual countries seem to have different prevalence rates.

Several studies have also reported the association of *qnr* genes with *bla* genes. One MDR *Salmonella* isolated from a piglet in Spain was carrying both *qnrB* and bla_{CTX-M}. Moawad et al. [8] found that 33% of *Salmonella* from poultry and beef in Egypt were carrying *qnr* genes and either bla_{CTX-M}, bla_{TEM}, or both. Whether *qnr* and *bla* genes reside within the same plasmid was not confirmed in either of the studies. However, Penha Filho et al. [9] recently isolated *Salmonella* from poultry in Brazil which carried both $bla_{CTX-M-2}$ and *qnrB* in the same plasmid. In clinical isolates of *S. enterica*, *E. coli*, and *K. pneumoniae*, *qnr* genes have also been found within the same plasmid as that of bla_{TEM} or bla_{CTX-M} [10, 11]. In the present study, 81 bla_{TEM} carrying isolates and all 9 bla_{CTX-M} carrying isolates were harboring one to three *qnr* subtypes.

Conclusions

The increasing prevalence of MDR *Salmonella* in livestock animals has been widely reported [13, 15, 27, 28] and is mainly attributed to the inappropriate use of antimicrobial agents in veterinary medicine [18, 19]. We report that 89.4% of *S. enterica* isolated from slaughtered pork were non-susceptible to at least one antimicrobial agent and 13.5% were MDR. Majority of the isolates were also harboring bla_{TEM} which possibly encode broad-spectrum ß-lactamases, and *qnrS* which could facilitate emergence of mutations that target quinolone resistance. While worldwide AMR surveillance has allowed the determination of the evolution of resistance, national surveillance will allow countries to create policies that would fit their needs. Generation of local information on AMR and antimicrobial consumption in the veterinary and agricultural sectors will allow the development of relevant approaches to tackle AMR [21, 22]. This is highly important for low- and middle-income countries (LMICs) as strategies proven effective to work in developed countries may not be suitable for LMICs. Attention to AMR in the agricultural sector began in the Philippines only recently, and further surveillance is necessary to identify emerging resistant *S. enterica* in the pork production chain.

Methods

Sample collection

The study population consisted of freshly slaughtered hogs from six abattoirs across the different districts of Metro Manila, Philippines. Informed consent was obtained from the Philippine National Meat Inspection Service, hence, ethics approval was waived for this particular study. Animal slaughter and evisceration were performed according to national regulations. Informed consent was also obtained from veterinarians in charge of the abattoirs, and farm owners for sample collection. Tissue samples from hog tonsils and jejunum were collected post-slaughter and under the supervision of a veterinarian. Sample collection was performed as previously described [30]. Briefly, tissues were collected from each hog upon evisceration using sterile forceps and scissors, and then immediately transferred into sterile bags. All samples were kept chilled upon collection and during transport, and were immediately processed in the laboratory.

Bacterial Isolation And Identification

Bacteria were first enriched prior to isolation as previously described [30]. Briefly, 25 g of each sample was transferred to 225 mL buffered peptone water (BPW), and incubated overnight at 35°C. Afterwards, 100 μL of pre-enriched bacterial culture in BPW was inoculated into 10 mL Rappaport-Vassiliadis broth (RVB), and then incubated overnight at 42°C for selective enrichment of *S. enterica*. RVB cultures were then inoculated onto brilliant green agar (BGA) and xylose lysine deoxycholate agar (XLD), and then incubated overnight at 35°C for isolation. Presumptive *S. enterica* were then inoculated onto nutrient agar and incubated overnight at 35°C for subsequent total DNA extraction.

Total DNA was extracted by harvesting colonies using a sterile 1 µL loop and suspending these in 100 µL TE buffer (10 mM Tris, 1 mM EDTA at pH 8.0). The suspension was boiled for 10 min, and pelleted at 6000 rpm for 5 min. The supernatant was collected and then stored at -20 °C until use. These DNA extracts were used in both PCR-based identification of *S. enterica* and detection of ARG.

Each PCR reaction for *S. enterica* identification contained 2 µL DNA, 10 pmol each of forward and reverse primers, and HiPi PCR Premix (Elpis Biotech, Daejeon, South Korea) in a final volume of 20 µL. Amplification of a 244-bp region in the species-specific *invA* gene was performed as previously described [31]. PCR products were subsequently analyzed via capillary electrophoresis. *Salmonella enterica* KCTC 2421 was used as a positive control.

Antimicrobial susceptibility testing

Vitek® 2 AST was used to generate the antimicrobial susceptibility profiles of the isolates. It automatically classifies isolates into susceptible, intermediate, or resistant to a particular antimicrobial agent based on the latest breakpoints provided by the Clinical and Laboratory Standards Institute (CLSI). Multidrug resistance was defined as non-susceptibility to at least one antimicrobial agent in three or more antimicrobial categories as recommended by Magiorakos et al. [24].

Inoculum preparation for the automated AST was followed as previously described [30]. Vitek® 2 AST-N261 cards were used which contain 15 antimicrobials including amikacin, amoxicillin/clavulanate, ampicillin, cefepime, cefoxitin, ceftazidime, ceftriaxone, ciprofloxacin, colistin, ertapenem, gentamicin, imipenem, meropenem, piperacillin/tazobactam, and trimethoprim/sulfamethoxazole. Colistin was not tested because there are currently no CLSI breakpoints available for *Salmonella* spp. Each test run was accompanied with antimicrobial susceptibility tests for *Escherichia coli* ATCC 25922 (negative control for ESBL test) and *Klebsiella pneumoniae* ATCC 600703 (positive control for ESBL test).

Detection of *bla* and *qnr* genes

S. enterica isolates were screened for ß-lactamase-encoding genes (bla_{CTX-M}, bla_{SHV}, and bla_{TEM}) and quinolone resistance genes (*qnrA*, *qnrB*, and *qnrS*) using monoplex PCR assays. The primers used are listed in Table 3. For *bla* genes, each reaction contained 2 µL DNA, 10 pmol each of forward and reverse primers, and AccuPower® PCR Premix or Maxime PCR Premix (*i*-StarTaq™ GH) in a final volume of 20 µL. PCR was carried out under the following conditions: initial denaturation step at 95 °C for 3 min; 30 cycles of denaturation at 95 °C for 30 s, annealing at 58 °C for bla_{CTX-M}, 56 °C for bla_{SHV}, and 50 °C for bla_{TEM} for 30 s, extension at 72 °C for 1 min; and a final extension step at 72 °C for 10 min. For *qnr* genes, each reaction contained 2 µL DNA, 10 pmol each of forward and reverse primers, and 6.25 µL GoTaq® Green Master Mix in a final volume of 12.5 µL. PCR was carried out under the following conditions: initial denaturation step at 95 °C for 5 min; 33 cycles of denaturation at 95 °C for 1 min, annealing at 60 °C for 1 min, extension at 72 °C for 1 min; and a final extension step at 72 °C for 10 min.

Table 3
Primers used in screening for antimicrobial resistance genes.

Target gene	Nucleotide sequence (5'-3')	Amplicon length (bp)	Reference
bla_{SHV}	F: ATGCGTTATATTCGCCTGTG	747	[32]
	R: TGCTTTGTTATTCGGGCCAA		
bla_{TEM}	F: TCGCCGCATACACTATTCTCAGAATGA	445	[5]
	R: ACGCTCACCGGCTCCAGATTTAT		
bla_{CTX-M}	F: ATGTGCAGYACCAGTAARGTKATGGC	593	[33]
	R: TGGGTRAARTARGTSACCAGAAYCAGCGG		
$bla_{CTX-M-1}$	F: AAAAATCACTGCGCCAGTTC	415	[34]
	R: AGCTTATTCATCGCCACGTT		
$bla_{CTX-M-2}$	F: CGATATCGTTGGTGGTRCCAT	404	[6]
	R: CGTTAACGGCACGATGAC		
$bla_{CTX-M-9}$	F: CAAAGAGAGTGCAACGGATG	205	[34]
	R: ATTGGAAAGCGTTCATCACC		
$bla_{CTX-M-8/25}$	F: AACCCACGATGTGGGTAGC		
$bla_{CTX-M-8}$	R: TCGCGTTAAGCGGATGATGC	666	[34]
$bla_{CTX-M-25}$	R: GCACGATGACATTCGGG	327	[34]
qnrA	F: AGAGGATTTCTCACGCCAGG	580	[35]
	R: TGCCAGGCACAGATCTTGAC		
qnrB	F: GGAATAGAAATTCGCCACTG	264	[36]
	R: TTTGCTGTTCGCCAGTCGAA		
qnrS	F: GCAAGTTCATTGAACAGGGT	428	[35]
	R: TCTAAACCGTCGAGTTCGGCG		

S. enterica isolates carrying bla_{CTX-M} were subjected to further PCR assays to identify CTX-M variants. The primers used in CTX-M variant typing are listed in Table 3. Each reaction contained 2 μL DNA, 10 pmol each of forward and reverse primers, and 6.25 μL GoTaq® Green Master Mix (Promega) in a final volume of 12.5 μL. Amplification was performed as previously described. [6].

Amplicons were analyzed in 1.5% agarose gels stained either with GelRed™ Nucleic Acid Gel Stain or SYBR® Safe DNA Gel Stain (1:10,000). Amplicons were allowed to separate at 100 V for 20–30 min, and then viewed in a gel documentation system. KAPA™ Universal Ladder was used to estimate the molecular weights of the products.

Abbreviations

AMR – antimicrobial resistance

ARG – antimicrobial resistance gene

AST – antimicrobial susceptibility testing

BGA – brilliant green agar

CLSI - Clinical and Laboratory Standards Institute

ESBL - extended-spectrum β-lactamase

ESC – extended spectrum cephalosporin

LMICs – low- and middle-income countries

MDR *Salmonella* – multidrug-resistant *Salmonella*

PCR – polymerase chain reaction

PMQR – plasmid-mediated quinolone resistance

RVB - Rappaport-Vassiliadis broth

TE buffer – Tris-EDTA buffer

XLD – xylose lysine deoxycholate

Declarations

Ethics approval and consent to participate

Informed consent was obtained from the Philippine National Meat Inspection Service, hence, ethics approval was waived for this particular study. Animal slaughter and evisceration were performed according to national regulations. Informed consent was also obtained from veterinarians in charge of the abattoirs, and farm owners for sample collection.

Consent for publication

Not applicable.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

ABC and WLR conceptualized the study. ABC performed experimental assays, data analysis, and made the first draft of the manuscript. KWW carried out data interpretation and troubleshooting of the assays. WLR and KWW made the necessary revisions before manuscript submission. All authors read and approved the final manuscript.

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