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Identification of antimicrobial resistance in *Salmonella enterica* isolates from swine slaughterhouses in the Philippines through different antimicrobial susceptibility test protocols

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Abstract

The spread of antimicrobial resistance (AMR) is an impending crisis highlighted by the emergence of multidrug-resistant (MDR) pathogenic foodborne bacteria, such as MDR *Salmonella enterica*, due to the misuse and overuse of antibiotics in agricultural and livestock industries. Hence, quick, and accurate identification of AMR and resistance genes are of utmost importance to treat infections, monitor or safeguard food production, and trace the sources of AMR outbreaks. Conventional methods of antimicrobial susceptibility testing (AST) such as disk diffusion assays are relatively inexpensive but are labor-intensive, slow, and limited to phenotypic detection. Conversely, modern AST methods include polymerase chain reaction (PCR) and DNA sequencing, which are faster and provide more accurate genotypic detection. This study sought to detect resistance genes in *S. enterica* isolated from swine from Philippine slaughterhouses through various protocols of conventional and modern AST methods. Resistance to five antibiotic classes was examined. It was found that 50% (14/28) of the isolates were MDR, and resistance to tetracycline was found in all isolates. The most common genes detected from the isolates were *tet*(A) (39.3%), followed by *tet*(C) (28.6%), and *tet*(E) (25%). Also, 25% (7/28) and 25% (7/28) of isolates were resistant to one and two antibiotic classes, respectively. PCR methods were used only for detection of tetracycline resistance genes, as a model for molecular investigation. The results of this study demonstrated the growing prevalence of MDR in the agricultural industry and the necessity for improvement of its detection.

Keywords: Antibiotic resistance genes, Antimicrobial resistance, Disk diffusion assay, Multidrug resistance, Polymerase chain reaction, *Salmonella enterica*

1. Introduction

The rise of multidrug resistance in bacteria is an emerging issue in the field of medical microbiology and an impending public health crisis since fewer antibiotics remain effective due to their misuse and overuse [1]. One of the major causes of the proliferation of antibiotic resistance in bacteria and their subsequent spread to humans is the rampant use of antibiotics in the agricultural sector. This occurs through a sequence of events beginning with the practice of including antibiotics in the diets of farm animals and livestock to prevent disease, decrease mortality, and increase profits [2]. This overuse of antibiotics suppresses susceptible bacteria but allows resistant strains to thrive in food-producing animals such as swine, cattle, and poultry. These multidrug-resistant (MDR) bacteria are then transferred to humans along the food chain through contaminated meat or other animal products. Once MDR bacteria from contaminated food products manage to infect humans, this then causes various adverse effects that are difficult to treat. An example of this is *Salmonella enterica* which is a species of Gram-negative bacteria from the *Enterobacteriaceae* family that commonly contaminates meat and egg products. It is also one of the leading foodborne pathogens in developing countries [3]. In fact, a recent study in the Philippines by

Calayag et al. discovered the presence of MDR *S. enterica* in contaminated swine raised and slaughtered in the Philippines with multidrug resistance occurring in up to 67.8% of isolates [4].

There are numerous ways to prevent the emergence of multidrug resistance; one of which is improving diagnosis [5]. Improved microbiological diagnosis is considered one of the most effective ways to reduce inappropriate antibiotic use at the clinical level. Also, improved detection of MDR bacteria in food can aid in the management of outbreaks at the public health level. The improved diagnosis is assisted by recent advances that shifted away from slow and tedious phenotypic detection methods, such as measuring growth on defined agar media to newer and more efficient molecular or genotypic detection methods, such as real-time polymerase chain reaction (qPCR) and DNA sequencing. These methods can detect genotypic differences in the AMR genes between strains of resistant bacteria. This enhances the specificity of detecting phenotypic resistance and identifying its root genotypic causes. Moreover, today is an era of DNA sequencing techniques, therefore it is recommended to use these advanced molecular tools for accurate detection and diagnosis.

Proper identification of AMR genes offers additional advantages by allowing researchers and policymakers to detect and identify new genes responsible for resistance, trace their sources during an outbreak, and thus allow them to properly respond. Unfortunately, the Philippines, being a developing country, is at a greater risk for foodborne illness outbreaks, especially from bacteria like MDR *Salmonella*, compared to developed countries [4]. This is because of less efficient sanitation facilities and policies as well as a lack of funding for more advanced diagnostic methods. Thus, this study aimed to detect AMR in five classes of antibiotics in *Salmonella* isolated from swine in slaughterhouses in the Philippines and to confirm resistance genotypes using different antimicrobial susceptibility testing (AST) protocols.

2. Materials and methods

2.1 Culture preparation

Test cultures were previously isolated by Calayag et al. from the lymph nodes, thymus, and jejunum of swine carcasses at several accredited and non-accredited slaughterhouses in Metro Manila, Philippines [4]. The uncharacterized isolates of *S. enterica* in the study of Calayag et al. were revived from frozen glycerol stock cultures via enrichment into Rappaport-Vassiliadis soya peptone broth (RVS broth) and incubated at 42°C for 18-24 h. This step was followed by subculturing the isolates into tryptic soy broth (TSB) and then incubated at 37°C for 18-24 h. These were then streaked onto xylose lysine deoxycholate (XLD) agar and then incubated at 37°C for 18-24 h. Isolated colonies of *Salmonella* were then subcultured onto nutrient agar (NA) plates. The uncharacterized isolates in this study were taken from the same batch in which multidrug resistance or resistance to three or more classes of antibiotics in most isolates were also previously detected [4]. Twenty-eight randomly selected and previously uncharacterized isolates were prepared in total.

2.2 Disk diffusion assay

The following procedure was based on Clinical Laboratory Standards Institute (CLSI) [6]. The 28 isolates were tested for resistance to five antibiotics not tested previously [4]. For each of the 28 pure cultures, three or four colonies were inoculated into tubes containing 4-mL saline (0.9%) solution using McFarland Standard No. 0.5 for comparison of turbidity.

A sterile cotton swab was dipped into the inoculated saline solution. It was pulled out of the fluid and rotated several times along the inside of the tube to remove excess fluid. The cotton swab was then used to streak the entire surface of a Mueller Hinton II (MH) agar plate. Afterward, the plate was rotated 60° and the streaking motion was repeated. It was then turned 60° again and the streaking motion was repeated a third time. The solidified medium was approximately 4-mm deep in the 100-mm-diameter disposable plates that were used. The lid of the MH agar plate was left ajar for 5 min after inoculation to allow excess fluid to be absorbed before five antibiotic disks were placed on the medium. The antibiotic disks were tetracycline (30 μg), streptomycin (10 μg), chloramphenicol (30 μg), nalidixic acid (30 μg), and amoxicillin with clavulanic acid (20/10 μg). *Escherichia coli* ATCC 25922 was used as a control. After 16-18 h of incubation at 37°C, inhibition zone diameters were measured, and the cultures were given susceptible, intermediate, resistant (SIR) scores based on published CLSI AST standards [7]. The antibiotic with the highest rates of resistance from the disk diffusion assay and had previously sequenced genes and primers available was selected for subsequent PCR procedures. A total of 28 isolates were tested in all with three trials per isolate and control, with a new control being prepared for each different day the assay was performed.

2.3 DNA extraction

Fresh or overnight subcultures of the isolates were prepared by streaking onto NA plates and incubated for 18-24 h at 37°C. From each newly prepared isolated subculture, 6-10 colonies were inoculated into 200 µl of Tris-EDTA buffer. This was then heated in a dry digital water bath at 95°C for 10 min. The samples were then centrifuged at 6000 rpm for 5 min. The liquid portion, containing the DNA, was then decanted into a new container.

2.4 PCR

We performed PCR testing on the most common antibiotic-resistant *Salmonella*, and tetracycline was selected as the representative antibiotic class. The genes targeted were *tet*(A), *tet*(B), *tet*(C), *tet*(D), and *tet*(E). The primer sequences in Table 1 were used to detect these genes, and the optimized PCR conditions were adapted from those of Adesiji et al. [8]. The PCR mixtures comprised a total volume of 15 µL, with a DNA sample volume of 1 µL, the manufacturer's recommended amount of GoTaq™ Green PCR Master Mix (Promega Corporation, Madison, WI, USA), the forward and reverse primers each at a final concentration 0.5 µM, and the remaining volume filled using nuclease-free water.

Table 1 PCR primers used in this study.

Resistance gene	Primer	Nucleotide sequence 5'-3'	Product size (bp)	Annealing temp (°C)	Reference
tet(A)	F	TTGGCATTCTGCATTCACTC	494	55	
	R	GTATAGCTTGCCGGAAGTCG			
tet(B)	F	CAGTGCTGTTGTGTCATTAA	571	55	
	R	GCTTGGAATACTGAGTGTAA			
tet(C)	F	CTTGAGAGCCTTCAACCCAG	418	55	[8]
	R	ATGGTCGTCATCTACCTGCC			
tet(D)	F	GCTCGGTGGTATCTCTGCTC	546	55	
	R	AGCAACAGAATCGGGAACAC			
tet(E)	F	TATTAACGGGCTGGCATTTC	544	55	
	R	AGCTGTCAGGTGGGTCAAAC			
16S rRNA	27F	AGAGTTTGATCCTGGCTCAG	1464	50	[9]
	1492R	GGTTACCTTGTTACGACTT			

The optimized PCR conditions were an initial denaturation at 95°C for 5 min, followed by 35 cycles of denaturation at 95°C for 60 s, annealing at 55°C for 60 s, extension at 72°C for 60 s, and a final extension at 72°C for 10 min. These conditions were run using a T100™ Thermal Cycler (Bio-Rad, Hercules, CA, USA). The 16S rRNA 27F and 1492R universal primers were used as internal controls to indicate the successful extraction and presence of DNA. The internal control PCR conditions comprised of an initial denaturation at 95°C for 2 min, followed by 35 cycles of denaturation at 95°C for 40 s, annealing at 55°C for 40 s, extension at 72°C for 90 s, and a final extension at 72°C for 10 min. The amplified PCR products were resolved by gel electrophoresis with 1.5–2% agarose, stained with GelRed™ (Biotium, Hayward, CA, USA), and visualized using a UV transilluminator.

The tetracycline resistance genes examined in this study were originally detected and sequenced from other bacteria (Table 2). Moreover, the *tet* genes are usually located on mobile genetic elements such as plasmids and transposons. This indicates the possibility of the occurrence of horizontal gene transfer, which would have conferred these resistance genes onto *Salmonella*.

Table 2 GenBank data on tetracycline resistance genes.

Gene	GenBank accession number	Originally detected in
tet(A)	X75761	*Pseudomonas aeruginosa*
tet(B)	V00611	*Escherichia coli*
tet(C)	Y19114	*Escherichia coli*
tet(D)	X65876	*Salmonella enterica*
tet(E)	L06940	*Escherichia coli*

2.5 Real-time polymerase chain reaction *(qPCR)*

The presence of the most common tetracycline resistance gene in the isolates was confirmed using real-time PCR or qPCR. The following procedure was adapted from the protocols issued by Applied Biosystems, the manufacturer of StepOnePlus™ Real-Time PCR (Applied Biosystems, Foster City, CA, USA) used in this study.

For the qPCR assay, a final reaction volume of 20 µL was prepared with DNA, primer, nuclease-free water, and PowerUp™ SYBR™ Green Master Mix (Thermo Fisher Scientific, Waltham, MA, USA) concentrations following the protocols specified by the manufacturer with a final primer concentration of 0.5 µM. All "no template controls" (NTCs) consisted of all PCR reagents without the target DNA.

The primers used were the same as those listed in Table 1. The qPCR parameters were a 2-min 50°C Uracil-DNA glycosylases (UDG) activation, followed by a 2-min 95°C Dual-Lock™ DNA Polymerase activation, 50 cycles of denaturation at 95°C for 15 sec, annealing at 62°C for 15 sec, and extension at 72°C for 60 s, and then a final extension at 72°C for 10 min. The fluorescence data were analyzed using the built-in software of the Applied Biosystems StepOnePlus™ Real-Time PCR to determine the presence or absence of the tested genes by computing threshold fluorescence intensity and determining whether this was reached.

3. Results

3.1 Disk diffusion assay

The disk diffusion assay results are summarized in Table 3. The isolated names were randomly and arbitrarily assigned with numbers and letters indicating no particular differences or patterns. The disk diffusion assay directly measured the effect of antimicrobials on the isolates by measuring the zones of inhibition of their growth or lack of growth around the antibiotic disks in millimeter. These quantitative measurements were converted into their SIR classification based on the CLSI standards [7]. The results revealed a remarkably high degree of AMR in the *Salmonella* isolates. All 28 isolates showed resistance to at least one class of antibiotics. Fourteen (14) out of 28 (50%) isolates were found to be resistant to three or more classes of antibiotics, with three of them (isolates 10, 13, and 26) resistant to all five classes (Table 3). Bacteria resistant to three or more classes of antibiotics are classified as MDR [10].

Table 3 Disk diffusion assay results with zone of inhibition measurements converted into standardized SIR classifications (CLSI).

Isolate	Susceptible, intermediate, and resistant (SIR) classification of antibiotics				
	Chloramphenicol	Streptomycin	Nalidixic Acid	Tetracycline	Amoxicillin and Clavulanic Acid
1	R	R	I	R	R
1a	R	R	I	R	R
3	R	R	I	R	R
3a	R	R	I	R	R
5	R	R	I	R	R
6	R	R	I	R	R
7	R	R	I	R	R
8	S	I	S	R	S
9	S	R	R	R	R
10	R	R	R	R	R
11	R	I	I	R	S
12	S	I	S	R	S
13	R	R	R	R	R
14	S	I	S	R	S
16	I	R	R	R	R
17	R	R	I	R	R
18	S	S	R	R	S
18a	S	S	R	R	S
18b	S	S	R	R	S
19	R	R	R	R	S
20	S	S	R	R	S
22	S	S	S	R	S
23	R	I	S	R	S
24	R	I	I	R	S
26	R	R	R	R	R
29	S	I	S	R	S
30	S	I	S	R	S
30a	S	I	S	R	S

The number of isolates resistant to each antibiotic class is shown in Figure 1. It shows that the most common antibiotic class that the *Salmonella* isolates displayed resistance to was tetracyclines with all 28 isolates displaying resistance. This was followed by the antibiotic class phenicols (chloramphenicol) with 15 resistant isolates, 14 for aminoglycosides (streptomycin), 13 for β-lactam/β-lactamase inhibitor (amoxicillin and clavulanic acid), and lastly 10 for quinolones (nalidixic acid) which showed the least number of resistant isolates.

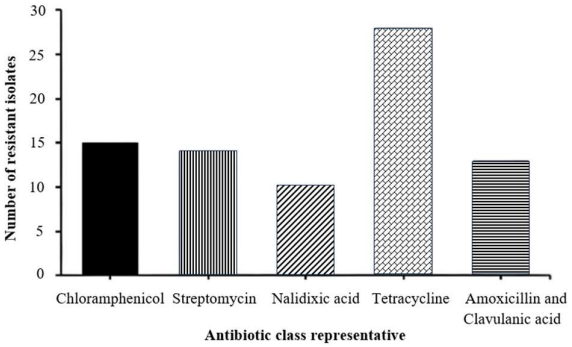

Figure 1 Disk diffusion assay results show the presence of AMR for all five antibiotic classes in Salmonella isolates, with many isolates displaying multidrug resistance, and resistance to tetracycline being the most common.

3.2 PCR

DNA extraction was successful for all isolates since all PCR tests using the universal 16S primers produced bands indicating the presence of bacterial DNA. Tetracycline became the focus of the conventional PCR assay because resistance to this antibiotic was the most prevalent of those tested in the disk diffusion assay. The results of the PCR amplification using the primers in Table 1 were visualized using gel electrophoresis, and Figure 2 shows an example of a stained gel. The appearance of bands of equal molecular weight to the expected product size in Table 1 indicates that the gene has been amplified and is thus present. The negative control (N) contains all PCR reagents except for the extracted DNA and the molecular weight (MW) marker used was the Kappa™ Universal Ladder (KAPA Biosystems, Inc., MA, USA).

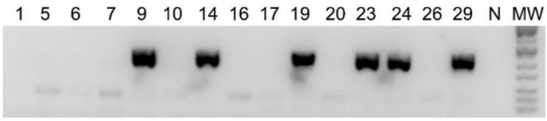

Figure 2 A sample gel showing the detection of the *tet*(A) gene in *Salmonella* isolates.

Table 4 summarizes the presence or absence of the five tetracycline resistance genes tested. *tet*(A) was the most common tetracycline resistance gene found with 11 of 28 (39.3%) isolates possessing it. This was followed closely by *tet*(C) with eight of 28 (28.6%) isolates possessing it and *tet*(E) with seven of 28 (25%) isolates. Only three (10.7%) isolates and one of 28 isolates possessed *tet*(B) and *tet*(D) genes, respectively.

Table 4 PCR results indicating the presence or absence of tetracycline resistance genes in *Salmonella* isolates.

Isolate	Tetracycline resistance genes ("+"-present, "-"-absent)				
	tet(A)	*tet*(B)	*tet*(C)	*tet*(D)	*tet*(E)
1	-	-	-	-	-
1a	-	-	+	-	-
3	-	-	-	-	-
3a	-	-	-	-	-
5	-	-	-	-	-
6	-	-	+	-	-
7	-	-	-	-	-
8	+	-	-	-	+
9	+	-	-	-	-
10	-	-	-	-	-
11	+	-	-	-	+
12	+	-	-	-	+
13	-	-	-	-	-
14	+	-	-	-	+
15	-	-	-	-	-
16	-	-	-	-	-
17	-	+	-	-	-
18a	-	+	+	-	-
18b	-	+	-	-	-
19	+	-	-	+	-
20	-	-	+	-	-
22	-	-	-	-	-
23	+	-	+	-	-
24	+	-	-	-	+

Table 4 (continued) PCR results indicating the presence or absence of tetracycline resistance genes in *Salmonella* isolates.

Isolate	Tetracycline resistance genes ("+"-present, "-"-absent)				
	tet(A)	*tet*(B)	*tet*(C)	*tet*(D)	*tet*(E)
26	-	-	-	-	-
29	+	-	+	-	+
30	+	-	+	-	-

Eleven of 28 (39.3%) isolates possessed multiple tetracycline resistance genes with nine (32.1%) and two (7.1%) isolates possessing two and three different resistance genes, respectively, for the same antibiotic (Table 4). On the other hand, the five tetracycline resistance genes were absent in 11 of 28 (39.3%) isolates despite their phenotypic resistance to tetracycline.

3.3 qPCR plus/minus assay

The *tet*(A) gene became the focus of the plus/minus assay due to it being the most common gene detected in the isolates using conventional PCR. The results of the qPCR assay are compared with those of conventional PCR and summarized in Table 5.

Table 5 Real-time qPCR assay results and conventional PCR results for the presence or absence of the *tet*(A) gene.

Isolate	Conventional PCR result (+/-)	Ct value	Tm (°C)
NTC	n/a	Undetermined	n/a
NTC	n/a	Undetermined	n/a
NTC	n/a	Undetermined	n/a
NTC	n/a	Undetermined	n/a
1	-	Undetermined	n/a
1a	-	Undetermined	n/a
3	-	Undetermined	n/a
3a	-	Undetermined	n/a
5	-	Undetermined	n/a
6	-	36.6755 (LA)	74.2337 (DMC)
7	-	Undetermined	n/a
8	+	21.207	90.3272
9	+	17.8065	90.1769
10	-	Undetermined	n/a
11	+	19.18	90.1769
12	+	18.5197	90.1769
13	-	35.6472 (LA)	77.0663 (DMC)
14	+	19.1345	90.1769
16	-	Undetermined	n/a
17	-	Undetermined	n/a
18	-	45.2317 (LA)	75.2789 (DMC)
18a	-	Undetermined	n/a
18b	-	Undetermined	n/a
19	+	18.1852	90.3281
20	-	34.3091 (LA)	90.1791
22	-	Undetermined	n/a
23	+	18.8939	90.1791
24	+	18.1907	90.3281
26	-	Undetermined	n/a
29	+	19.4614	90.1801
30	+	18.7792	90.1801
30a	+	18.8665	90.1801

The NTC acts as a baseline and contains all the reagents for the plus/minus assay except for the isolate DNA. The PCR result classified as either a plus or a minus (+/-) indicates whether *tet*(A) was detected in the original PCR assay. The Ct value indicates the cycle at which the sample reached the threshold fluorescence intensity, determined in the pre-read run, for the amplicon to be considered detectable. A late amplify (LA) indicates that the DNA amplified very late and is likely due to other unwanted reactions. An undetermined Ct value indicates the absence of amplicons. Melting curve analysis determines the temperature (Tm) that the amplified DNA begins to dissociate. Similar amplicons should have similar melting curves and Tm. Samples with detected Ct values but have different melt curves (DMC) have amplified DNA other than the target.

The qPCR plus/minus assay was able to detect the presence of the *tet*(A) gene in the same 11 isolates (Ct value = ~17.35, Tm = ~90.22°C for non-LA) that were found to possess the gene after the conventional PCR tests and

was able to confirm the absence of the *tet*(A) gene in the other isolates (Ct value = undetermined, Tm = n/a). The Ct value indicated the cycle number at which point the number of amplicons produced was able to cross the required fluorescence threshold. The amplification targets were expected to have relatively low Ct values close to other wells that have amplified the correct target. This allowed wells that amplified late or at vastly different times to be excluded as they are likely to be the result of unwanted reactions between reagents. The Tm values indicate the Tm at which the amplified DNA dissociates. Similar amplicons were therefore expected to have similar Tm values and allowed wells that amplified non-target DNA to be excluded. The wells that successfully amplified the target DNA were also more easily identified as they had similar Tm values.

4. Discussion

The results of the disk diffusion assay, as summarized in Table 3, confirmed the fears of widespread AMR and even multidrug resistance in pathogens found in Philippine slaughterhouses [1,2]. The disk diffusion assay also reinforced the conclusions of Calayag et al. that multidrug resistance was highly prevalent in swine found in Philippine slaughterhouses [4]. This makes the matter even more pressing since these MDR *Salmonella* were isolated from agricultural livestock that were meant for human consumption, making the chances of food contamination, infection, or any contact between pathogenic MDR bacteria and humans more likely.

As previously predicted, a major source of the development of antimicrobial-resistant bacteria is the agricultural industry [11,2]. The large presence of MDR bacteria isolated from agricultural livestock is likely a product of persistent misuse and overuse of antibiotics in the agricultural industry. Owners of livestock are enticed to supplement even healthy animals with copious amounts of antibiotics since these allow the animals to avoid common illnesses and thus grow large and more rapidly, in turn increasing profits for the agricultural industry [2]. However, the consequence of this is the development of antibiotic-resistant bacteria, which in turn leads to livestock owners turning to more or different antibiotics, which the bacteria eventually also develop resistance to. This perpetuates a cycle of misuse and overuse of antibiotics that results in the MDR *Salmonella* we observed in this study. This contributes to the impending antibiotic crisis not only in the losses surely to be accrued in the agricultural industry but also in the possible difficulty in treating infections when people eat food contaminated by the MDR bacteria.

However, as previously suggested, there are numerous ways to combat multidrug resistance and one of these is through improved detection of AMR [5]. Most advances recently in AST have been in the field of molecular or genotypic methods [12]. These AST methods have numerous advantages over their traditional counterparts. Molecular methods are fast, accurate, and elucidate information about the genetic causes of AMR. Molecular or genotypic methods provide more than just phenotypic detection, they also allow for the identification and characterization of AMR genes themselves.

The ability to elucidate resistance genotypes, rather than just phenotypes, allows in-depth diagnosis and research, detection and identification, and effective responses and treatment. Examples of these are the use of molecular AST methods in the discovery of novel AMR using next-generation sequencing (NGS) and metagenomics, the development of rapid AMR gene assays for patients with sepsis based on qPCR, and microbial source tracking (MST) through the targeting of resistance genes for better diagnosis, just to name a few [13, 14]. More modern AST methods such as microarrays, NGS, PCR, and qPCR technologies are becoming more advanced and accessible [12]. It is for these reasons that this study did not stop at phenotypic detection and continued to genotypic detection using PCR and qPCR.

The tetracycline class of antibiotics was chosen for further identification of resistance genes since it was the class for which resistance was most prevalent among the isolates. The five most common tetracycline resistance genes were chosen as reported by Adesiji et al. [8]. The discovery of these five genes in the *Salmonella* isolates provides more information about the nature of the AMR in the isolates than mere phenotypic detection. One such observation that can be made is that all genes conferring resistance to tetracycline detected in this study were all originally sequenced in other bacteria. Based on previous studies, most tetracycline resistance genes were usually located on mobile genetic elements such as plasmids or transposons. As predicted by previous researchers, this gene pattern is highly indicative of the occurrence of horizontal gene transfer between different species of bacteria leading to the conferment and propagation of tetracycline resistance in different microorganisms including the *Salmonella* isolates in this study [1,2]. Moreover, the genes located on mobile genetic elements, or any vector that facilitates horizontal gene transfer are more prevalent than those found on the bacterial chromosome [1,2]. To add, all the tetracycline resistance genes detected in this study are tetracycline efflux genes (*tet*(A), *tet*(B), *tet*(C), *tet*(D), and *tet*(E) while other tetracycline resistance genes which are not included in this study are targeting ribosomal protection genes [15].

This information is potentially more helpful than merely relying on data from the disk diffusion assays since the genotypes of the tetracycline resistance in *Salmonella* inform us that the resistance to tetracycline originated from other bacteria that were selected for in environments where antibiotics are abused, such as agricultural livestock. These bacteria were able to transfer the genes onto *Salmonella,* creating the MDR strains we have

detected in this study. As noted hitherto, the same crisis faced by other countries is likewise faced by the Philippines since these MDR *Salmonella* are actively selected for in our swine farms, where antibiotics are also overused, and are thus able to propagate and even transfer those same tetracycline resistance genes onto other potentially pathogenic bacteria [1,2].

This information provided by molecular or genotypic AST methods is useful not only in understanding and studying the mechanisms of resistance, causes of its emergence, and pathway of its propagation but also in creating policies and applying technologies to help deal with multidrug resistance. For example, these data help to reinforce and pinpoint the spread of MDR *Salmonella* to the misuse and overuse of antibiotics in the Philippine agricultural industry. This information can help guide the formation of future policies that can ensure the proper use of antibiotics for livestock and thus alleviate the problem of rising antibiotic resistance. The knowledge that most MDR *Salmonella* acquire their tetracycline resistance through horizontal gene transfer, in addition to the identification of these resistance genes, would allow researchers to perform MST. Scott et al. observed that MST can target these resistance genes [13]; thus, researchers in the Philippines may be able to trace the sources of these MDR *Salmonella* back to the farms where antibiotics were overused. Scott et al. also observed that horizontal gene transfer occurred based on molecular data from food or clinical isolates [13]. This would be even more helpful during outbreaks where identifying and stopping the foodborne source of *Salmonella* as soon as possible are the recommended response [16]. Last, pioneer projects where PCR and qPCR were used in large hospital settings to identify antibiotic resistances before treatment with antibiotics were found to lead to better patient outcomes as antibiotic treatments were more specific and targeted [17]. If this could be applied to the Philippines, this could prove to greatly improve the treatment of MDR *Salmonella* outbreaks and infections in the clinical setting.

The qPCR assay was then performed to confirm the presence of the genes detected. The qPCR assay results were as expected with the assay identifying the presence of the *tet*(A) gene in all isolates that were detected using the conventional PCR and gel electrophoresis assays, indicating the accuracy of the plus/minus assay results. The use of qPCR is similar to the conventional PCR method but is far more accurate, because of the use of fluorescent dyes that provides real-time results and removes the need to run gel electrophoresis. qPCR also allows additional information such as the Ct value and melting curve analysis that allows even more accurate detection as shown in Table 5. This is an example of an emerging technology noted by Jorgensen & Ferraro that improves upon all characteristics of older technology with the exception that it is more expensive, and materials are difficult to acquire [12]. Nonetheless, like all emerging technologies, the cost and scarcity continue to decrease as reliability and accuracy increase over time. For the Philippines to remain at the forefront of advances, this study demonstrated the use of qPCR in detecting AMR genes. The immediate advantages of this technology are the higher accuracy it offers, and more rapid detection given its ability to output results in real-time.

This study recommends that immediate action be taken in response to the observed proliferation of MDR *Salmonella* in swine industry due to the questionable use of antibiotics of the agricultural sector in the Philippines. This study also recommends the continued use of and further investment in molecular or genotypic AST methods. The genotypic data gathered from this study as well as future studies can be used to direct initiatives and formulate policies to combat the impending antibiotic resistance crisis in the country. These include the application of the protocols performed in this study to analyze the horizontal gene transfers creating the MDR *Salmonella* strains and performing MST to determine the sources of MDR *Salmonella* strains and identify their resistance profiles to better treat clinical cases.

5. Conclusion

This study sought to understand AMR in *S. enterica* isolated from swine in Philippine slaughterhouses through different AST methods. About 50% of the isolates tested were resistant to three or more classes of antibiotics through disk diffusion assay, making them classifiable as MDR. All isolates were found to be resistant to tetracycline, thus were further analyzed using conventional PCR and the plus/minus qPCR assay. Among the *tet* genes detected, *tet*(A) was the most common found in 39.3% of the isolates identified with an average Ct value of 17.35 at a Tm of around 90.22°C. This study was able to demonstrate that genotypic methods, along with the conventional phenotypic methods of resistance profiling should be applied in these types of work. The results of this study demonstrated the growing prevalence of MDR in the agricultural industry and the necessity for improvement of its detection. It is recommended that the serotype identification of *Salmonella* isolates be done to assign AMR phenotype to *Salmonella* serotypes.

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7. References

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