Journal of Nature Studies 18(2): 60-71
Online ISSN: 2244-5226

DETECTION OF MULTI-DRUG RESISTANT *Salmonella* IN ORGANIC SOIL AMENDMENTS DISTRIBUTED IN THE PHILIPPINES

Angella Melissa A. Carlos*, Arianne Nicole N. Manuel and Rina B. Opulencia

University of the Philippines Los Baños

*Corresponding author: aacarlos@up.edu.ph

ABSTRACT – Multidrug resistance of foodborne pathogens like *Salmonella* is a global concern to human health. Organic fertilizer and compost can harbor pathogens since these amendments are subjected to minimal physical and chemical treatments. This study aimed to detect multidrug-resistant *Salmonella* in 27 samples, representing nine brands, of organic fertilizer and compost using international standard protocols, combining phenotypic and PCR-based methods. Nine isolates from four uncertified brands were confirmed as *Salmonella* by PCR amplification of the *invA* gene, and subjected to Kirby-Bauer antibiotic susceptibility test. All isolates were resistant to ampicillin and amoxicillin, but only one was also resistant to two other classes of antibiotics, namely, nalidixic acid and ofloxacin. This shows that multidrug-resistant *Salmonella* can occur in organic fertilizer, which can potentially contaminate organically grown fresh produce

Keywords: compost, organic fertilizer, multidrug resistance, Salmonella

INTRODUCTION

Salmonella continues to be one of the leading causes of foodborne diseases worldwide. In the United States alone, annual data estimates by the Centers for Disease Control and Prevention (CDC) reveal 1.2 million cases, 23,000 hospitalizations, and 450 deaths caused by this pathogen. Although global statistics show that norovirus was the leading cause of foodborne diseases in 2010 with 125 million cases, *Salmonella enterica* infections resulted in the highest burden equivalent to 4.07 million Disability Adjusted Life Years (DALY). According to the World Health Organization (WHO), DALY is a metric computed as the sum of the Years of Life Lost (YLL) due to premature mortality in the population and the Years Lost due to Disability (YLD). It measures the burden of disease expressed as the number of years lost due to illness, disability or premature death. It is interesting to note that the Southeast Asian region where the Philippines belongs, registered the second highest DALYs per 100,000 people (Kirk, 2015).

During the first six months of 2018, there have been 9,201 reported typhoid fever cases resulting in 18 deaths here in the Philippines according to the Epidemiology Bureau of the Department of Health (Epidemiology Bureau, 2018). This disease is caused by *Salmonella enterica* serovar Typhi. Unfortunately, the surveillance report does not include foodborne and waterborne cases due to non-typhoidal *Salmonella*.

Molecular detection methods of *Salmonella* employ the use of specific genes amplified through PCR-based methods. Among the widely used genes for detection of *Salmonella* are *invA, iroB, hns, hisJ, hilA,* and *fimY*. The study by Li et al. (2012) demonstrated that use of primer for *invA* gene produced specific PCR products. Moreover, a study by Mainar-Jaime et al. (2013) described the specificity and sensitivity of PCR-based method using *invA* gene primers in the detection of *Salmonella* species compared

To cite this paper: Carlos, A.M.A., Manuel, A.M.N. & Opulencia, R.B. 2019. Detection of Multi-Drug Resistant Salmonella in Organic Soil Amendments Distributed in the Philippines. *Journal of Nature Studies*. 18(2), 60-71.

to international standards. From the *Salmonella* samples tested, the international standard failed to detect 17.7% of the PCR-positive samples. The sensitivity and specificity of *invA*-based PCR method was 83.6% and 97.4%, respectively.

InvA gene is part of an operon *inv* found in *Salmonella* responsible for encoding proteins for invasion of the host's intestinal epithelial cells (Galan et al., 1992). This operon of *Salmonella* encodes at least three proteins for invasion of the host's intestinal epithelial cells. *InvA* encodes for one of these proteins, referred to as invA. InvA is a member of a set of several inner membrane proteins that form the Type III Secretion System (T3SS). *Salmonella* spp. utilize this highly specialized nanomachine to translocate virulence proteins into the cytoplasm of the target host. The ability of *Salmonella* species to invade epithelial cells is one of the common features in its pathogenesis.

Organic agriculture is taking root globally in response to consumer demands for pesticide-free produce and other agricultural products. In the Philippines, the movement is spearheaded by the Department of Agriculture's National Organic Agriculture Board (NOAB). In contrast to its Asian neighbors, the organic agriculture sector in the country is still in its infancy. As stated in the metadata provided by the Department of Agriculture, the total land allocation for organic farming in the country has increased from 14,140 hectares in 2006 to 234,642 hectares in 2015. This land area represents just 1.9% of the country's agricultural lands (DAR, 2017).

One of the benefits of organic type of farming is soil improvement through acidity reduction and air circulation, which also supports useful microorganisms. However, pathogenic bacteria such as *Salmonella*, *Listeria monocytogenes*, and *Escherichia coli* O157:H7, to name a few, may be introduced to the land through the use of organic soil amendments. These amendments may contain various types of animal manure, which in turn are known sources of pathogenic organisms (Marlina, et al.; Chen and Jiang, 2014). Moreover, these pathogens may survive the composting process and thrive in the fertilizer for several months (Lemunier et al., 2005).

Some *Salmonella* strains have acquired adaptive mechanisms that give them resilience and the ability to survive under extreme environmental conditions. Their ability to grow at elevated temperatures (e.g., 54ºC) has been reported (Matthews, et al., 2017) and this raise concerns in the composting process if it is done incorrectly. To compound the problem, reports on the increasing trend in the numbers of antibiotic-resistant *Salmonella* serotypes have reached alarming proportions. Furthermore, multiple drug-resistant types, defined as exhibiting resistance to three or more antibiotic classes (Magiorakos et al., 2012), like *Salmonella enterica* serovar Typhimurium DT104, endangers infected consumers because of limited drug options to combat infections. This may result in more expensive therapies, increased risk of complications, higher mortality and morbidity rates, which lead to economic loss (Doyle, 2014).

This study was done to evaluate the microbiological safety of organic soil amendments by detecting the presence of *Salmonella* spp. using cultural and molecular methods, and to determine if the isolates have acquired resistance to antibiotics commonly used in treating salmonellosis.

MATERIALS AND METHODS

Isolation and Detection of Salmonella

A total of 27 samples, representing nine local brands, were purchased from commercial suppliers. Among these brands, four were certified by the Organic Certification Center of the Philippines (OCCP) while the rest were uncertified.

Portions from different sites in the fertilizer bag were taken and placed in a sterile plastic bag. The portions were mixed together by shaking to obtain the 25-g analytical sample. This process was used only for samples in solid form. For the liquid type, a 25 ml sample portion was directly taken from the bottle after shaking.

Samples were analyzed for the presence of *Salmonella* using the methods published in ISO 6579-1:2017 (Microbiology of the food chain – Horizontal method for the detection, enumeration and serotyping of *Salmonella* – Part 1: Detection of *Salmonella* spp.) except that only Rappaport Vassiliadis (RV; Himedia, India) broth was used in the selective enrichment step, and serotyping was not conducted. Briefly, 25-gram aliquots from solid analytical samples were pre-enriched using Buffered Peptone Water (Conda, Spain) and pummeled at 200 rpm using a stomacher (Seward stomacher 400, UK) for 1.5 – 2 minutes. For the liquid samples, 25 ml aliquots were directly added to the pre-enrichment medium and shaken vigorously. Incubation was done at 35ºC for 24 hours (without shaking) afterwhich 0.1 ml of the pre-enriched culture was transferred to RV broth. The enrichment tubes were incubated in a circulating water bath set at 41.5ºC for 24 hours. Selective plating was performed on Xylose Lysine Desoxycholate Agar (XLDA; Conda, Spain) and Brilliant Green Agar (BGA; Conda, Spain). Pink colonies with or without black centers, and yellow colonies with or without black centers on XLDA plates, as well as colonies that were pink on BGA plates were picked. In cases where no expected colony morphologies were observed, four colonies from each plate were picked. The isolates were further purified by streaking onto Tryptic Soy Agar (TSA; Himedia, India). To confirm the purity of the isolates, Gram-staining was performed on 18 to 24-hour cultures and slides were observed under the oil immersion objective.

The following biochemical tests were performed on pure cultures of the 29 isolates: sugar utilization using Triple Sugar Iron Agar (TSIA; Conda, Spain), lysine decarboxylation test using Lysine Decarboxylase Broth (LDB; Conda, Spain), indole test, and urease test using Urea Broth (UB; Oxoid, UK). The biochemical tests were performed using a 24-hour pure culture of the isolates grown in TSA. *Salmonella enterica* subspecies *enterica* serovar Choleraesuis (JCM 1651) culture obtained from the RIKEN BRC through the National BioResource Project of the MEXT/AMED, Japan, was used as positive control. An uninoculated control was also prepared. Interpretation of the biochemical tests and the presumption of putative *Salmonella* sp. identities were based on ISO 6579-1:2017.

Genomic DNA Isolation

Pure cultures of the putative *Salmonella* isolates were grown in TSB for 24 hours at 37ºC. The overnight cultures were then transferred to sterile 1.5-ml microfuge tubes and the cells were harvested by centrifugation at 9,600 x g for 45 seconds. The CTAB (cetyl trimethylammonium bromide) method (Doyle and Doyle, 1987) as modified by Green and Sambrook (2012) was utilized for the purpose of isolating genomic DNA. In summary, the modification involves addition of sodium dodecyl sulfate (10%) and protease K (20 mg/ml) to cell suspension prior to treatment with CTAB.

The presence and quality of the genomic DNA was confirmed by running 3 µl of the DNA solution on 1% agarose gel stained with GelRed® (Biotium, USA; 2.5 ul of GelRed (initial concentration: 10,000x) were added to 25 ml of agarose gel (final concentration: 1x)), and then viewed using the Gel Doc™ XR + Gel Documentation System (Bio-Rad, USA). DNA concentration and 260nm/280nm ratio were checked using the Epoch Microplate Spectrophotometer and Take3 Software (Biotek Instruments, USA).

PCR Amplification of the invA Gene

A polymerase chain reaction (PCR) assay was conducted to confirm the identity of the putative

Salmonella isolates. To amplify the *invA* gene, primers Fw (5'– AGTGCTCGTTTACGACCTGAA -3') and Rv (5'- TGATCGATAATGCCAGACGA -3') designed by Mainar-Jaime et al. (2013) were utilized. The reaction mixture included 100 ng genomic DNA, 0.2 mM dNTPs, 1x buffer (Invitrogen, USA; contains 20 mM Tris-HCl pH 8.4, 50 mM KCl), 1.5 mM $MgCl_2$, 0.5 U *Taq* polymerase (Invitrogen, USA) and 0.4 µM of each primer. PCR was performed as follows: initial denaturation at 94ºC for five minutes, 40 cycles of denaturation at 94ºC for 30 seconds, annealing at 55ºC for one minute, extension at 72ºC for 30 seconds, and a final extension at 72ºC for ten minutes. *Salmonella enterica* JCM 1651 and *E. coli* TG 1 were used as positive and negative controls, respectively.

The PCR products were checked by running 5 µl of the PCR product on 1.5% agarose gel stained with GelRed®. Separation of fragments was performed by gel electrophoresis at 100 volts for 30 minutes then at 50 volts for another 30 minutes in 0.5x Tris-Acetate-EDTA (TAE) buffer. The gels were viewed using the Gel Doc™ XR + Gel Documentation System (Bio-Rad, USA). A fragment size of 229 bp was expected for *Salmonella* isolates.

Representative *invA* PCR products were sent for sequencing at First Base Laboratories (now Apical Scientific, Malaysia). Resulting sequences were analyzed using the Basic Local Alignment Search Tool (BLAST) program of the National Center for Biotechnology Information (NCBI) database.

Evaluation of Antibiotic Susceptibility of the Salmonella Isolates

Antibiotics were selected based on guidelines provided by CLSI document M100-S25 (CLSI, 2015) for the family *Enterobacteriaceae*. Antibiotics were purchased from Bioanalyse® (USA) and included amoxicillin (25 µg), cefoperazone (75 µg), nalidixic acid (30 µg), levofloxacin (5 µg), ofloxacin (5 µg) and ampicillin (10 µg). Amoxicillin, ampicillin and cefoperazone are β-lactam antibiotics; nalidixic acid is a quinolone while levofloxacin and ofloxacin are fluoroquinolones.

Antibiotic susceptibilities of the *Salmonella* isolates were determined according to the guidelines described in CLSI document M02-A12 (CLSI, 2017). Colonies grown on NA plates were suspended in 0.85% saline solution and turbidity was adjusted to the 0.5 McFarland standard (1 to 2 x 10^8 CFU/ml). A sterile swab was dipped into the adjusted suspension and swabbed on the surface of Mueller Hinton agar (MHA; Himedia, India) plate. Antibiotic-impregnated disks were placed 24 mm away from each other and no more than six antibiotic-impregnated disks were placed on the surface of MHA. The plates were then incubated at 35ºC for 24 hours. The tests were performed in triplicates.

Zones of inhibition were measured to the nearest millimeter and interpreted as susceptible, resistant or intermediate using the guide provided by the Clinical and Laboratory Standards Institute (2006). The intermediate category, as defined by CLSI, includes isolates with antimicrobial agent minimal inhibitory concentrations that approach usually attainable blood and tissue levels and for which response rates may be lower than for susceptible isolates. Furthermore, multidrug-resistant *Salmonella* isolates were those identified to be resistant to three or more classes of antibiotics (Magiorakos et al., 2011).

RESULTS AND DISCUSSION

The present investigation is the first report on the occurrence of *Salmonella* in organic soil amendments sold in the Philippines. Results revealed that 29 isolates suspected to be *Salmonella* were obtained from the samples tested. Table 1 shows the biochemical reactions of the different putative

Salmonella isolates. Out of the 29 isolates, 13 exhibited typical *Salmonella* reactions on the four mentioned biochemical tests. However, all isolates, including those that gave atypical reactions were subjected to PCR. Standard methods require that even isolates showing atypical reactions be subjected to serological or molecular analysis. This is due to the emergence of non-typical strains of this pathogen that can ferment either lactose or sucrose resulting in growth on XLDA and BGA, which exhibit varied reactions. Hence, an isolate cannot be definitively identified as *Salmonella* sp. unless it has undergone serological or molecular analysis.

Table 1. Reactions on TSIA, LDB, UB, and indole production of suspected *Salmonella* isolates from organic fertilizer and compost after 24 hours incubation at 37°C.

mple source (Brand)	Isolate (#)	TSIA			LDB	UB	Indole
		Slant Color	Butt Color	Gas Production (+/-)			
OS1	1	Red	Yellow	(-)	(+)	(-)	(-)
	2	Red	Yellow	(-)	(+)	(-)	(-)
	3	Red	Yellow	(-)	(+)	(-)	(-)
	4	Red	Yellow	(-)	(+)	(-)	(-)
	5	Red	Yellow	(-)	(+)	(-)	(-)
	6	Red	Yellow	(+)	(+)	(-)	(-)
	7	Red	Yellow	(-)	(+)	(-)	(-)
	8	Red	Yellow	(-)	(+)	(-)	(-)
	9	Red	Yellow	(+)	(+)	(-)	(-)
	10	Red	Yellow	(+)	(-)	(-)	(-)
	11	Yellow	Yellow	(+)	(+)	(-)	(-)
	12	Red	Yellow	(+)	(-)	(-)	(-)
	13	Red	Yellow	(-)	(+)	(-)	(-)
	14	Red	Yellow	(+)	(+)	(-)	(-)
	15	Red	Yellow	(-)	(+)	(-)	(-)
	16	Red	Yellow	(+)	(-)	(-)	(-)
	17	Red	Yellow	(-)	(+)	(-)	(-)
	18	Red	Yellow	(-)	(+)	(-)	(-)
OCS2	19	Red	Yellow	(+)	(+)	(-)	(-)
	20	Red	Yellow	(+)	(+)	(-)	(-)
	21	Yellow	Yellow	(+)	(+)	(-)	(-)
	22	Red	Yellow	(+)	(+)	(-)	(-)
OS3	23	Red	Yellow	(+)	(+)	(-)	(-)
	24	Red	Yellow	(-)	(+)	(-)	(-)
	25	Red	Yellow	(-)	(+)	(-)	(-)
OS4	26	Red	Yellow	(-)	(+)	(-)	(-)
VS1	27	Red	Yellow	(-)	(+)	(-)	(-)
	28	Red	Yellow	(+)	(+)	(-)	(-)
	29	Red	Yellow	(+)	(+)	(-)	(-)
	Salmonella enterica subsp. *enterica* JCM 1651	Red	Yellow	(-)	(+)	(-)	(-)

All putative isolates were subjected to PCR but only nine showed amplicons around 229 bp, similar to that of the positive control, as shown in Figure 1. Isolates 8, 9, 15, 16, 18, 23, 24, 26 and 29 were identified as *Salmonella* isolates based on PCR amplification of *invA*-specific primers. Five confirmed *Salmonella* isolates came from sample OS1 (isolates 8, 9, 15, 16 and 18), two from OS3 (isolates 23 and 24), one from OS4 (isolate 26) and another one from sample VS1 (isolate 29). These results reveal the importance of subjecting the isolates to PCR analysis as opposed to complete reliance on cultural and biochemical analyses.

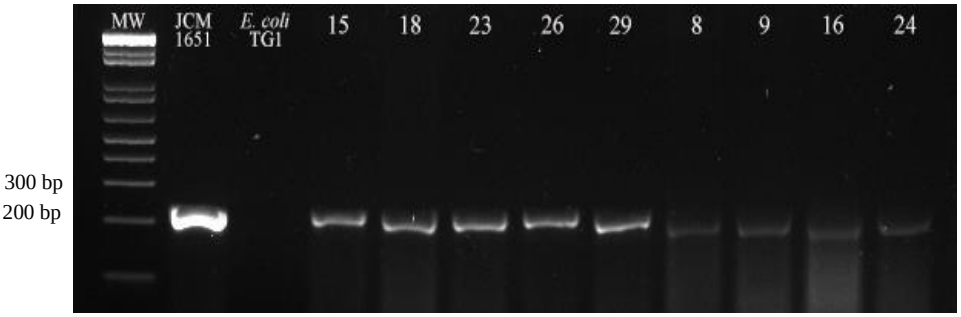

Figure 1. Confirmation of *invA* amplicons from *Salmonella* isolates as evidenced by a 229 bp band on 1.5% TAE agarose gel. MW: 1 Kb Plus DNA Ladder (ThermoFisher Scientific, Massachusetts, USA). Numbers on lanes represent the *Salmonella* isolates.

To further confirm if the PCR products obtained were specific to *Salmonella* species, representative PCR products of isolates 15, 18 and 23 were sequenced at First Base Laboratories (Malaysia). BLAST search and Clustal Omega multiple sequence alignment (Figure 2) results show that the PCR products from all of the isolates were homologous to *invA* of *Salmonella enterica* subspecies *enterica*, confirming their identity. Although there were several serovars that appeared as the possible identity, the particular serovar of the isolates were not identified.

It is noteworthy that all the samples found to carry the bacterium were uncertified brands of organic fertilizer and compost. Two of these samples contain chicken manure (OS 1 and OS 4); one includes carabao manure (OS 3) while one has vermicompost (VS 1). These data are similar with those gathered by Smith et al. in 1982. In their study, 33.3% (40/120) of garden fertilizer samples from the East Anglia region in England contained *Salmonella.* In contrast, the results are inconsistent with the data obtained by Miller (2011), which showed that *Salmonella* was absent in 103 organic fertilizers collected in the United States. However, the same study confirmed that the bacterium has the potential to grow in different types of organic fertilizers.

It can then be surmised from the present study that the pathogen, which is likely present in the manure, was able to survive the composting process. According to the Cornell University Waste Management Institute (1996), there are three phases in the composting process, namely, the mesophilic, thermophilic and maturation phases. During the thermophilic stage, the heat produced by thermophilic organisms dominated by *Bacillus* spp. may cause the temperature to reach 55-60ºC. At this temperature range, vegetative bacterial cells start dying off. However, some pathogens may survive due to non-uniform

distribution of the heat. The cells on top of the heap may not receive the same exposure to the heat. Furthermore, some may survive if they develop thermal resistance during the early stage of the composting process that may lead to survival during the heat treatment itself (Chen and Jiang, 2014).

According to the Philippine National Standards (PNS, 2016), the presence of Salmonella in organic soil amendments is unacceptable, i.e., it should be absent per 25g sample. Thus, it can be concluded that the samples certified by the Organic Certification Center of the Philippines (OCCP) were able to comply with the aforementioned microbiological criterion. The rest, however, failed to meet the microbiological standard.

```
15    ATGGTGATGATCATTTCTATGTTCGTCATTCCATTACACTACACTATCTGGTTGATTTCCTGATC
18    ATGGTGATGATCATTTCTATGTTCGTCATTCCATTACCT--ACCTATCTGGTTGATTTCCTGATC
23    ATGGTGATGATCATTTCTATGTTCGTCATTCCATTACACTACACTATCTGGTTGATTTCCTGATC
Sa    ATGGTGATGATCATTTCTATGTTCGTCATTCCATTACCT--ACCTATCTGGTTGATTTCCTGATC
      ************************************..  ..**********************

15    GCACTGAATATCGTACTGGCGATATTGGTGTTTATGGGGTCGTTCTATATTGACAGAATCCTCAG
18    GCACTGAATATCGTACTGGCGATATTGGTGTTTATGGGGTCGTTCTATATTGACAGAATCCTCAG
23    GCACTGAATATCGTACTGGCGATATTGGTGTTTATGGGGTCGTTCTATATTGACAGAATCCTCAG
Sa    GCACTGAATATCGTACTGGCGATATTGGTGTTTATGGGGTCGTTCTATATTGACAGAATCCTCAG
      ************************************************************

15    TTTTTCAA
18    TTTTTCAA
23    TTTTTCAA
Sa    TTTTTCAA
      ********
```

Figure 2. Clustal Omega sequence alignment of invA PCR products from isolates 15, 18 and 23 with *invA* gene fragment of *S. enterica* subsp. *enterica* published by Boyd et al. (1996; 1997).

The antibiotic resistance patterns of the confirmed *Salmonella* isolates were also studied. Results summarized in Table 2 show that all of the isolates were resistant to both ampicillin and amoxicillin but susceptible to cefoperazone. Only one isolate, i.e., isolate 18, was multidrug resistant (MDR), exhibiting resistance to three classes of antibiotics, namely, the penicillin-like antibiotics (ampicillin and amoxicillin), quinolone (nalidixic acid) and fluoroquinolone (ofloxacin).

Results obtained were consistent with studies on the antibiotic susceptibility patterns of *Salmonella* species. In a study by Yoon et al. (2017), 247 *Salmonella* isolates were tested with different classes of antibiotics. Resistance to a quinolone, nalidixic acid, was the highest with 43.3%, followed by resistance against ampicillin with 40.5%, then to tetracycline (31.6%). Another study showed high prevalence of resistant *S. enterica* serovar Typhi and *S. enterica* serovar Paratyphi A isolated from patients in Japan (Hirose et al. 2001). Resistance of 18 *S. enterica* serovar Typhi and five *S. enterica* serovar Paratyphi A out of a total of 99 isolates to one or more antibiotic drug was demonstrated. It was found out that among the antibiotic resistant *S. enterica* serovar Typhi, 55.6% were resistant to nalidixic acid and had decreased susceptibility to ciprofloxacin. In the Philippines, the prevalence of antibiotic-resistant *Salmonella* and other clinically significant pathogens is being monitored by the Antimicrobial Resistance

Table 2. Average and standard deviation of sizes of zones of inhibition (mm) of Salmonella isolates tested against amoxicillin (AX), ampicillin (AMP), cefaperazone (CEF), levofloxin (LEV), nalidixic acid (NA) and ofloxacin (OFX) on MHA plates incubated at 37°C for 18 hours.

Isolate #	Antibiotic											
	AMP	AS	AX	AS	CEF	AS	LEV	AS	NA	AS	OFX	AS
8	1.3 ± 2.3	R	3.0 ± 5.2	R	21.3 ± 0.6	S	28.7 ± 0.6	S	15.3 ± 1.2	I	26.7 ± 0.6	S
9	0.0 ± 0.0	R	3.7 ± 6.4	R	30.7 ± 1.5	S	32.0 ± 1.0	S	25.3 ± 0.6	S	30.3 ± 0.6	S
15	0.0 ± 0.0	R	0.0 ± 0.0	R	21.3 ± 0.6	S	28.7 ± 0.6	S	14.0 ± 4.4	I	26.0 ± 0.0	S
16	0.0 ± 0.0	R	0.0 ± 0.0	R	30.7 ± 1.2	S	29.3 ± 0.6	S	22.7 ± 1.5	S	26.3 ± 0.6	S
18	0.0 ± 0.0	R	0.3 ± 5.7	R	22.0 ± 1.0	S	17.0 ± 1.7	I	00.0 ± 0.0	R	10.3 ± 0.6	R
23	0.0 ± 0.0	R	4.0 ± 6.9	R	32.3 ± 0.6	S	32.7 ± 0.6	S	24.3 ± 0.6	S	29.0 ± 1.0	S
24	0.0 ± 0.0	R	0.0 ± 0.0	R	32.7 ± 0.6	S	32.7 ± 1.0	S	25.3 ± 1.0	S	31.3 ± 0.6	S
26	0.0 ± 0.0	R	0.0 ± 0.0	R	30.3 ± 2.1	S	30.0 ± 1.7	S	23.7 ± 1.5	S	26.7 ± 1.2	S
29	0.0 ± 0.0	R	0.0 ± 0.0	R	32.7 ± 1.5	S	29.0 ± 0.6	S	23.0 ± 1.2	S	27.3 ± 0.6	S

*AS – antibiotic sensitivity (CLSI, 2006)
R = resistant
I = intermediate
S = susceptible

Surveillance Program of the Department of Health (DOH). In their 2016 summary report, among 150 isolates of *S. enterica* serovar Typhi, resistance to nalidixic acid was the highest (7.8%), followed by cotrimoxazole (6.8%), ampicillin (1.4%) and chloramphenicol (0.8%). There were no resistant strains to ciprofloxacin and ceftriaxone.

Increasing resistance to antibiotics has been attributed to the manure composition of the organic fertilizers used. A study by Marti et al. (2013) reported that antibiotic resistant bacteria like *E. coli, Enterococcus, Clostrididum perfringens, Yersinia, Salmonella enterica,* and *Aeromonas* were detected in manures applied to soils utilized in vegetable production. Resistance to ampicillin, amoxicillin-clavulanic acid and cefoxitin was detected in most samples such as swine and dairy manure. There were no resistant bacteria against ciprofloxacin, sulfamethoxazole, cotrimoxazole, tetracycline and norfloxacin. Moreover, the effect of manuring on the frequency of antibiotic resistance was also studied. It was established that application of manure in soils used to grow vegetables caused a significant increase in the frequency of antibiotic resistance against ampicillin, gentamicin and cefoxitin. This indicates that the use of manure as fertilizer can increase the frequency of occurrence of antibiotic resistant bacteria found in soil.

The MDR isolate from this study was obtained from a sample containing chicken manure. Chicken manure is an established source of *Salmonella,* hence, this magnifies the current concern about increased health risks posed by organically grown produce to consumers due to the possible transfer of the organism from the soil to the plant.

Commercial poultry production utilizes antibiotics for the purpose of increasing meat production. This is realized through higher feed conversion, growth rate improvement and disease prevention (Mehdi and Godbout, 2018). The widespread use of sub-therapeutic doses of antibiotics in the poultry industry as a growth enhancer can cause selective pressure that may eventually result in the development of MDR pathogenic bacteria (Chen and Jiang, 2014).

The study indicated the occurrence of *Salmonella* in commercially available organic fertilizers. This is a grave concern since the pathogen can transfer from the land amended by the fertilizer to the produce. If the produce is a ready-to-eat product, which does not receive a kill step, then salmonellosis infections may ensue. The isolates also exhibited resistance to at least two antibiotics and one was found to be multidrug-resistant. This MDR strain was isolated from organic fertilizer containing chicken manure. Its acquisition of the MDR trait may have been the result of injudicious use of antibiotics in the poultry industry as a growth promoter.

CONCLUSION AND RECOMMENDATIONS

Four uncertified organic soil amendment brands tested positive for *Salmonella*. No *Salmonella* isolates were detected in certified brands, indicating that the standards set by the Philippine National Standards were met by these samples.

Multidrug resistance of the confirmed *Salmonella* isolates was evaluated using Kirby-Bauer antibiotic susceptibility test. Six antibiotics commonly used to treat salmonellosis were used namely, ampicillin, amoxicillin, cefoperazone, levofloxacin, nalidixic acid and ofloxacin. Only one out of nine isolates (11.1%) showed multidrug resistance to ampicillin, amoxicillin, nalidixic acid and ofloxacin. The rest of the isolates were resistant to only ampicillin and amoxicillin. The multidrug-resistant *Salmonella*

came from sample OS1 showing that it is indeed possible to have multidrug resistant strains in organic fertilizer. This is of great concern if the fertilizer is applied to soil that is used for growing organic produce that are eaten raw. There is a risk of infection by these multidrug-resistant strains of *Salmonella* found in organic fertilizer, which cannot be treated by the usual line of drugs commonly used for antibiotic therapy.

It is, therefore, recommended that more studies of this nature be conducted to help establish good agricultural practices (GAP) particularly in the composting process as well as baseline data for risk analysis studies. All these will help protect not only consumers but also all stakeholders in the organic farming value chain.

STATEMENT OF AUTHORSHIP

All three authors participated in writing and reviewing the manuscript. The first author conceptualized and designed the study as well as prepared the initial write up. The second author performed the experiments and gathered data. The third author provided the expertise in the conduct of the molecular biology experiments and analyses.

REFERENCES

Boyd, E.F., Li, J., Ochman, H., Selander, R.K. (1997). Comparative genetics of the inv-spa invasion gene complex of Salmonella enterica. Journal of Bacteriology. 179: 1985-1991.

Boyd, E.F., Wang, F.S., Whittam, T.S. and Selander, R.K. (1996). Molecular genetic relationships of the salmonellae. Applied and. Environmental Microbiology. 62:804-808.

[CDC] Centers for Disease and Control. (n.d.). National Outbreak Reporting System. Retrieved 9 May 2017, from https://www.cdc.gov.

Chen, Z, and Jiang, X. (2014). Microbiological Safety of Chicken Litter or Chicken Litter-Based Organic Fertilizers; A Review. Agriculture. 4:1-29.

[CLSI] Clinical and Laboratory Standards Institute. (2006). M100-S16 Performance Standards for Antimicrobial Susceptibility Testing; 16th Informational Supplement. Retrieved April 23, 2018 from https://clsi.org.

Cornell Waste Management Institute. (1996). The Science of Composting. cwsi.css.cornell.edu/chapter1.pdf.

Dar, W. (2017). Organic Agriculture guided by Inclusive Agribusiness. www.pressreader.com.

Doyle, J.J. and Doyle, J.L. (1987). A rapid DNA isolation procedure for small quantities of fresh leaf tissue. Phytochemical Bulletin. 19:11-15.

Doyle, M.E. (2014). White paper: Public health impact of multi-drug resistant pathogens. www.fri.wisc.edu.

Epidemiology Bureau, Department of Health. (2018). Typhoid Morbidity Report. www.doh.gov.ph/statistics.

Galan, J.E., Ginochio, C., Costeas, P. (1992). Molecular and functional characterization of the *Salmonella* invasion gene *invA*: Homology of *InvA* to members of a new protein family. Journal of Bacteriology 174: 4338–4349.

Green, M.R. and Sambrook, J. (2012). Molecular cloning: a laboratory method. 4th ed. New York: Cold Spring Harbor Laboratory Press. 2,028 pp.

[ISO] International Organization for Standardization. (2017). ISO 6579-1(E): Microbiology of the food chain-Horizontal method for the detection, enumeration and serotyping of *Salmonella*. 1-48.

Kirk, M.D, Pires, S.M., Black, R.E., Caipo, M., Crump, J.A., Devleesschauwer, B. Döpfer, D., Fazil, A., Fischer-Walker, C.L., Hald, T., Hall, A.J., Keddy, K.H., Lake, R.J., Lanata, C.F., Torgerson P.R., Havelaar A.H., and Angulo, F.J. (2015). World Health Organization Estimates of the Global and Regional Disease Burden of 22 Foodborne Bacterial, Protozoal, and Viral Diseases, 2010: A Data Synthesis. Public Library of Science Medicine. 12:e1001940.

Lemunier, M., Francou, C., Rousseaux, S., Houot, S., Dantigny, P., Piveteau, P., Guzzo, J. (2005). Long-term survival of pathogenic and sanitation indicator bacteria in experimental biowaste composts. Applied and Environmental Microbiology. 71: 5779-5786.

Li, X., Yang, F., Gao, W., Song, H., Tian, H., Xu, B. (2012). Application of pyrosequencing for *Salmonella enterica* rapid identification. Journal of Microbiological Methods. 89: 49-52.

Magiorakos, A.P., Srinivasan, A. Carey,, R.B., Carmeli Y., Falagas, M.E., Giske, C.G., Harbarth, S., Hindler, J.F., Kahlmeter, G., Olsson-Liljequist, B., Paterson, D.L., Rice, L.B., Stelling, J., Struelens, M.J., Vatopaulos, A., Weber, J.T., Monnet, D.L. (2012). Multidrug-resistant, extensively drug-resistant and pandrug-resistant bacteria: an international expert proposal for interim standard definitions for acquired resistance. Clinical Microbiology and Infection 18: 268-281.

Mainar-Jaime, R.C., Andrés S., Vico J.P., San Román B., Garrido V., Grilló, M.J. (2013). Sensitivity of the ISO 6579:2002/And 1:2007 standard method for detection of *Salmonella* spp. on mesenteric lymph nodes from slaughter pigs. Journal of Clinical Microbiology, 51:89–94.

Marlina, E.T., Kurnani, T.B.A., Hidayati, Y.A., Badruzzaman, D.Z., Firman, A. (2016). Detection of Pathogenic bacteria and Heavy metal on Liquid fertilizer from Dairy Cattle Waste. Proc. Intsem. 520-525.

Matthews, K.R., Kniel, K.E. Montville, T.J. (2017). Food Microbiology An Introduction. ASM Press: Washington DC. 597 pp.

Marti R., Scott A., Tien Y.C., Murray R., Sabourin L., Zhang Y. and, Topp E. (2013). Impact of manure fertilization on the abundance of antibiotic-resistant bacteria and frequency of detection of antibiotic resistance genes in soil and on vegetables at harvest. Applied and Environmental Microbiology 79:5701–5709.

Mehdi Y., Letourneau-Montiminy M.P., Gaucher M.L., Chorfi Y., Suresh G., Rouissi T., Brar S.K., Cote C., Ramirez, A.A., Godbout, S. (2018). Use of antibiotics in broiler production: Global impacts and alternatives. Anim. Nutr. DOI: 10.1016/j.aninu.2018.03.002.

Miller C. (2011). Microbiological safety of organic fertilizers used for production. [master's thesis]. Clemson, South Carolina, USA:Clemson University. Retrieved May 9, 2017 from http://tigerprints.clemson.edu.

[PNS] Philippine National Standards. (2014). PNS BAFS 40:2014 ICS 65.080: Organic Fertilizer. Makati City: Bureau of Agriculture and Fisheries. Retrieved May 7, 2017 from http://www.bafps.da.gov.ph.

Smith H.W., Tucker J.F., Hall M.L., Rowe B. (1982). *Salmonella* organisms in garden fertilizers of animal origin. Journal of Hygiene 89: 125-128.

[WHO] World Health Organization. Metrics: Disability-Adjusted Life Year (DALY).http://www.who.int/healthinfo/global_burden_disease/metrics_daly/en/

Yoon, K.B., Song, B.J., Shin, M.Y., Lim, H.C., Yoon, Y.H., Jeon, D.Y., Ha, H., Yang, S.I., and Kim, J.B. (2017). Antibiotic Resistance Patterns and Serotypes of *Salmonella* spp. Isolated at Jeollanam-do in Korea. Osong Public Health Res Perspect 8: 211-219.