Philippine Journal of Science
151 (5): 1725-1735, October 2022
ISSN 0031 - 7683
Date Received: 17 Mar 2022

Molecular Characterization and Antimicrobial Resistance Profiling of *Salmonella* spp. from Onion Leaves Collected from Wet Markets in Metro Manila, Philippines

Glaezel Angelique T. Barredo[1]*, **Gil M. Penuliar**[2], and **Maria Auxilia T. Siringan**[3]

[1]Institute of Biological Sciences, University of the Philippines Los Baños,
Laguna 4031 Philippines
[2]Institute of Biology, University of the Philippines Diliman,
Quezon City 1101 Philippines
[3]Natural Sciences Research Institute, University of the Philippines Diliman,
Quezon City 1101 Philippines

***Salmonella* spp. is the causative agent of salmonellosis outbreaks in poultry and meats, but recent studies have implicated this pathogen in foodborne outbreaks associated with vegetables. Salmonellosis is commonly managed with antibiotics, but antimicrobial resistance has complicated the treatment of the disease. The pathogenesis of *Salmonella* spp. is based on virulence factors such as plasmid-encoded genes and lipopolysaccharide moieties, which can be detected by PCR and serotyping, respectively. Thirteen (13) isolates previously identified and confirmed as *Salmonella* spp. using *invA* gene-targeting assay were isolated from onion leaf samples collected from wet markets in Metro Manila, Philippines. The isolates were characterized based on the presence of plasmid-encoded virulence gene, serogroup and serotype, and antibiotic resistance profiles using the VITEK®2 system. All 13 *Salmonella* spp. isolates were *spvC*-negative. O-serotyping revealed the presence of isolates belonging to serogroups C1 (30.8%), C2 (23.0%), and E1 (30.8%). Two isolates (15.4%) did not belong to the serogroups examined in the study. Further, sequence analysis revealed the presence of serovars Newport, Weltevreden, Tennessee, and Anatum. All the isolates were resistant to at least five antimicrobial agents. To our knowledge, this is the first study in the Philippines to establish baseline data for the molecular features and antibiogram profiles of *Salmonella* spp. isolated in onion leaf samples from selected wet markets in Metro Manila, Philippines. Furthermore, this study is the first report on the occurrence of four different *Salmonella* serovars from onion leaves. The data obtained in this study could be used as baseline information and guidance in the implementation of the GAP, monitoring programs of the Department of Agriculture, and the formulation of laws and regulations by policymakers that will address safety issues in fresh produce and quality management principles in farm operations.**

Keywords: antimicrobial resistance, onion leaves, *Salmonella*, serogroup, virulence genes

*Corresponding author: gtbarredo@up.edu.ph

INTRODUCTION

Fruits and vegetables are important parts of a healthy diet. In recent years, the increased consumption of fresh produce has been associated with an increase in the number of illnesses and outbreaks caused by foodborne pathogens (Carstens *et al.* 2019). Most of these outbreaks are caused by *Salmonella* spp., the causative agent of salmonellosis. While the common food vehicles of salmonellosis are meat products, including poultry products (eggs and poultry meat) (Antunes *et al.* 2016), recent reports have implicated various vegetables in *Salmonella* outbreaks (CDC 2022). Infections caused by non-typhoidal serovars of *Salmonella* spp. cause food poisoning and self-limiting gastrointestinal diseases. Some serovars, however, can become invasive and cause bloodstream infections. Infections caused by *Salmonella* spp. are generally treated with antibiotics like fluoroquinolones and expanded spectrum cephalosporins (Carattoli *et al.* 2002; Chiu *et al.* 2002). Recently, however, treatments have become less effective and more complicated due to antimicrobial resistance (AMR), thus creating problems in food safety and public health (Wadamori *et al.* 2016; Kilonzo-Nthenge and Mukuna 2018). While the antibiotic resistance profiles of bacteria associated with food animals are well-documented, published reports regarding those from fresh produce are very limited. This is alarming considering that fruits and vegetables – which are often consumed raw – are generally contaminated by bacteria from irrigation water, soil, animal manure, fertilizers, equipment, and farm workers (Carstens *et al.* 2019).

Salmonella spp. is a gram-negative, non-spore-forming bacillus classified under Family Enterobacteriaceae (Velge *et al.* 2005). It frequently inhabits the intestines of humans, chickens, and other warm-blooded animals. Some studies also cited the presence of this pathogen in fresh produce, including vegetables. *Salmonella* spp. can be detected phenotypically using validated conventional methods (including the use of selective media) and PCR-based detection methods, which amplify species-specific genes.

Salmonella spp. virulence factors include lipopolysaccharide moieties (O antigen), flagellar protein (H antigen), and capsular polysaccharide (Vi antigen) (Grimont and Weill 2007). These factors are either chromosome-encoded or plasmid-encoded (Okamoto *et al.* 2009). As these virulence factors are encoded by genes, PCR-based methods have been developed to identify and characterize *Salmonella* spp. based on virulence genes, in addition to serogroup and serotype. For example, the *invA* and *spvC* have been used as markers for the detection and screening of chromosome-encoded and plasmid-encoded virulence, respectively, in *Salmonella* spp. from feces (Chiu and Ou 1996) and avian material (Okamoto *et al.* 2009).

This study aimed to determine the presence of a plasmid-encoded gene, serogroup, and serotype, as well as to examine the antibiotic resistance profiles of 13 *Salmonella* spp. isolated from onion leaf samples obtained from wet markets in Metro Manila, Philippines. This is the first study in the Philippines to characterize *Salmonella* spp. from onion leaf samples based on the presence of molecular markers and their antimicrobial profiles. Initial findings of this study reported the presence of *Salmonella* spp. in 13.3% of onion leaf samples collected from Metro Manila wet markets (Siringan and Torres 2019). The presence of *Salmonella* spp. in 16.7% of the fresh produce obtained from open-air markets and supermarkets in the Philippines was also reported in the study of Vital and co-authors (2014). Thus, the results of this study may help address safety concerns about the increasing incidence and outbreaks of *Salmonella* infection associated with fresh produce like onion leaf samples, which are often minimally processed prior to consumption.

MATERIALS AND METHODS

Collection of Onion Leaf Samples and Acquisition of Reference Strain

Sample collection was done through the DABIOTECH-P1201 project titled "Incidence and Determination of Foodborne Pathogens in Vegetables: Towards the Development of Microbiological Standards for Product" funded by the Department of Agriculture–Bureau of Agricultural Research (DA-BAR) and DA–Biotechnology Program Implementing Unit (DA–Biotech PIU). Briefly, a total of 98 onion leaf samples consisting of 85 samples from 15 wet markets and 13 samples from two farms in Luzon, Philippines were collected from 2012–2014. Each sample was placed in a labeled clean Ziplock bag and then stored in a cooler with an ice gel pack. The samples were transported to the laboratory for immediate processing and analysis to determine the presence of *Salmonella* spp. (Siringan and Torres 2019).

Salmonella enterica serovar Typhimurium ATCC 14028 (Lyfocult, bioMérieux, France) was acquired from the Microbiological Research and Services Laboratory (MRSL), Natural Sciences Research Institute (NSRI), University of the Philippines, Diliman (UPD), Quezon City.

Isolation of *Salmonella* spp.

Detection and isolation of *Salmonella* spp. using the standard conventional method (Andrews *et al.* 2011) and Analytical Profile Index 20E (API 20E; bioMerieux, France), as well as confirmation using PCR-based assay targeting *invA* gene (Rahn *et al.* 1992), were done as

previously reported (Siringan and Torres 2019). Briefly, onion leaf samples were homogenized using a stomacher (Bagmixer, Intescience, UK) for 2 min prior to incubation in Tryptic Soy Broth (TSB, Difco), and K_2SO_3 (potassium sulfite) at 35 ℃ for 24 h. From TSB cultures exhibiting turbidity, 0.1 mL was aseptically transferred to 10 mL Rappaport-Vassiliadis broth (RV), which was then incubated at 42 ℃ for 24 h. From RV tubes exhibiting turbidity, a loopful of culture was streak-plated onto each of these three selective media – Xylose Lysine Dextrose Broth, Bismuth Sulfite Agar, and Hektoen Agar. Colonies exhibiting typical *Salmonella* colonies were then isolated, purified, and subjected to the following verification tests: [a] sugar fermentation test using Triple Sugar Iron Agar, [b] lysine decarboxylation using Lysine Iron Agar, and [c] urease test. Identification of the 13 isolates that yielded positive results in these verification tests was confirmed using the API 20E identification system (bioMerieux, France) and *invA* gene-targeting PCR assay.

Molecular Characterization of *Salmonella* spp.

DNA extraction. DNA was extracted using the boiling method, as previously described in Shanmugasamy and co-authors (2011) with some modifications. Briefly, 1–2 colonies from 18–24-h NA culture were suspended in 200 µL of sterile distilled water in a 1.5-mL microcentrifuge tube. The suspension was heated at 99 °C for 10 min, followed by centrifugation at 6,000 rpm for 5 min at room temperature. The clear supernatant was transferred to a new 1.5-mL microcentrifuge tube and used as a DNA template for PCR. DNA samples were stored at 0 °C until further use.

Detection of spvC. The plasmid-associated virulence gene, *spvC*, was detected by PCR amplification in the *Salmonella* spp. isolates using the primers listed in Table 1. PCR was performed in a 25-µL reaction mixture containing 12.5-µL master mix (Vivantis Technologies, USA), 1.0 µL each of the forward (spvC-1) and reverse (spvC-2) primers, 8.5-µL nuclease-free water, and 2.0-µL DNA template. PCR tubes were centrifuged for 10 s and placed in a Si96 Thermocycler (Quanta Biotech, UK). PCR conditions used in this assay consisted of the following steps: initial denaturation at 95 °C for 2 min; 40 cycles of denaturation at 95 °C for 30 s, annealing at 64 °C for 30 s, and extension at 72 °C for 30 s; and a final extension at 72 °C for 5 min (S.A. Soguilon, pers. comm., 21 Aug 2014).

O-typing of Salmonella spp. Isolates that were PCR-positive for the *invA* gene were characterized by multiplex PCR through the amplification of genes unique to the following serogroups of the O antigen: A, B, C1, C2, D, and E (Hirose *et al.* 2002; Hong *et al.* 2008) using the primers listed in Table 1. PCR was performed in a 25-µL reaction mixture containing 12.5 µL KAPA2G fast multiplex mix (2X) (Kapa Biosystems, USA), 0.5 µL (0.2 µM) each of the primers, 3.5 µL nuclease-free water, and 2 µL DNA template. The PCR tubes were centrifuged for 10 s and were placed in a Si96 Thermocycler (Quanta Biotech, UK). PCR conditions used in this assay consisted of the following steps: initial denaturation at 95 °C for 3 min; 30 cycles of denaturation at 95 °C for 15 s, annealing at 60 °C for 30 s, and extension at 72 °C for 60 s; and a final extension at 72 °C for 10 min (S.A. Soguilon, pers. comm., 21 Aug 2014).

H-typing of Salmonella spp. *Salmonella* spp. isolates were further characterized by multiplex PCR through the amplification of genes unique to four different *Salmonella enterica* serovars based on the H antigen (Greisen *et al.* 1994; Agron *et al.* 2001; Herrera-Leon *et al.* 2004; Levy *et al.* 2008; Tennant *et al.* 2010), using the primers listed in Table 1. PCR was performed in a 25-µL reaction mixture containing 12.5 µL KAPA2G fast multiplex mix (2X), 1.0 µL (0.2 µM) each of the serovar specific primers, 1.0 µL (2.5 µM) each of the 16S primers; 2.0 µL nuclease-free water; and 1.5 µL DNA template (S.A. Soguilon, pers. comm., 21 Aug 2014).

Agarose gel electrophoresis. PCR products from O- and H-typing (10 µL) with 2-µL 6x loading dye (Vivantis Technologies, USA), and 5 µL of 100 bp DNA ladder (Vivantis Technologies, USA and Kapa Biosystems, USA) were electrophoresed in 1.5% agarose gel with 1X Gel Red Nucleic Acid (Biotium, CA) at 100 V for 25–30 min using a gel electrophoresis system (Mupid One, UK). The gel was then viewed and photographed using an Alpha Imager Mini documentation system (Protein Simple, UK).

Sequencing. Amplicons from O-typing were submitted to the Philippine Genome Center, UPD, Quezon City for sequencing. DNA sequences were aligned using Molecular Evolutionary Genetics Analysis 6 and identities were determined through Basic Local Alignment Search TOOL (BLAST®) in the National Center for Biotechnology Information database. Isolates with at least 97% similarity were considered for the identification.

Antimicrobial Susceptibility Testing (AST)

AST was performed using the VITEK®2 (bioMérieux, France) system according to the manufacturer's instructions. Briefly, suspensions of overnight cultures of *Salmonella* spp. in 0.45% sterile sodium chloride solution with turbidity equivalent to 0.4–0.6 McFarland standard were prepared. The bacterial suspensions and VITEK®2 AST-N261 antimicrobial susceptibility cards were loaded in the VITEK®2 system. Generation of the antibiotic resistance and/or susceptibility profiles for the following antibiotics: amikacin (AN), amoxicillin/clavulanic acid (AMC), ampicillin (AM), cefepime (FEP), cefoxitin (FOX), ceftazidime (CAZ), ceftriaxone (CRO),

Table 1. Primers for the detection of *Salmonella* plasmid virulence gene, *Salmonella* serogroups, and *Salmonella enterica* serovars.

Primer	Sequence (5'-3')	Amplification target	Amplicon length (bp)	Reference[a]
***Salmonella* plasmid virulence gene**				
spvC-F	ACTCCTTGCACAACCAAATGCGGA	*spvC* gene	571	1
spvC-R	TGTCTTCTGCATTTCGCCACCATCA			
***Salmonella* serogroups**				
F-prt	CTTGCTATGGAAGACATAACGAACC	A and D groups	256	2
R-prt	CGTCTCCATCAAAAGCTCCATAGA			
F-abe1	GGCTTCCGGCTTTATTGG	B group	561	3
R-abe1	TCTCTTATCTGTTCGCCTGTTG			
F-wbaD-manC	ATTTGCCCAGTTCGGTTTG	C1 group	341	3
R-wbaD-manC	CCATAACCGACTTCCATTTCC			
F-abe2	CGTCCTATAACCGAGCCAAC	C2 group	397	3
R-abe2	CTGCTTTATCCCTCTCACCG			
F-tyv	GAGGAAGGGAAATGAAGCTTTT	D group	614	2
R-tyv	TAGCAAACTGTCTCCCACCATAC			
F-wzx – wzy	GATAGCAACGTTCGGAAATTC	E1	281	3
R-wzx – wzy	CCCAATAGCAATAAACCAAGC			
***Salmonella enterica* serovars**				
H-for	ACTCAGGCTTCCCGTAACGC			4
Hgp	ATTAACATCCGCCGCGCCAA	*S. enterica* serovar Dublin (H:g,p)	779	5
Hi	ATAGCCATTTACCAGTTCC	*S. enterica* serovar Typhimurium (H:i)	551	6
Hz4,z23F	TTTGTCAAAGATGTTACTGCG	*S. enterica* serovar Stanleyville (H:z4,z23)	427	5
Hz4,z23R	AGGTTAGTGATGGCAGATTC			
sdfF	TGTGTTTTATCTGATGCAAGAGG	*S. enterica* serovar Enteritidis	333	7
sdfR	CGTTCTTCTGGTACTTACGATGAC			
16SF	ATACGTTCCCGGGCCTTG	Universal bacterial 16S rRNA gene	167	8
DG74	AGGAGGTGATCCAACCGCA			

[a]1 – Chiu and Ou (1996); 2 – Hirose *et al.* (2002); 3 – Hong *et al.* (2008); 4 – Levy *et al.* (2008); 5 – Tennant *et al.* (2010); 6 – Herrera-Leon *et al.* (2004); 7 – Agron *et al.* (2001); 8 – Greisen *et al.* (1994)

cefuroxime (CXM), ciprofloxacin (CIP), colistin (CS), ertapenem (ETP), ESBL, gentamicin (GM), imipenem (IP), meropenem (MEM), piperacillin/tazobactam (TZP), and trimethoprim-sulfamethoxazole (SXT) using VITEK®2 Gram-negative Susceptibility Card (AST-N261) were performed after VITEK®2 system analysis.

RESULTS

Molecular Characterization of *Salmonella* spp.

From the 98 onion leaf samples, 13 samples (13.3%) tested positive for *Salmonella* based on the API20E-based phenotypic identification and *invA* gene-targeting PCR assay, as reported in our previous study (Siringan and Torres 2019). From these *Salmonella*-positive samples, 13 confirmed *Salmonella* spp. isolates were used in this study.

All *Salmonella* isolates (13/13) that were positive for the *invA*, as previously reported (Siringan and Torres 2019), were negative for the *spvC* (data not shown). Serogrouping (O-typing) results showed that 30.8% (4/13) of the isolates belonged to the C1 serogroup, whereas 23% (3/13) to the C2 group and 30.8% (4/13) to the E1 group. The remaining 15.4% (2/13) did not belong to any of the groups targeted in the study and belonged to other serogroups. *Salmonella* spp. isolates that tested positive for the C1, C2, and E1 groups showed the expected 341 bp, 397, and 281 bp bands, respectively (Figure 1).

Among the C1 group-positive *Salmonella* isolates, three (2OCm1, 2OCm2, and 2OCm3) were obtained from

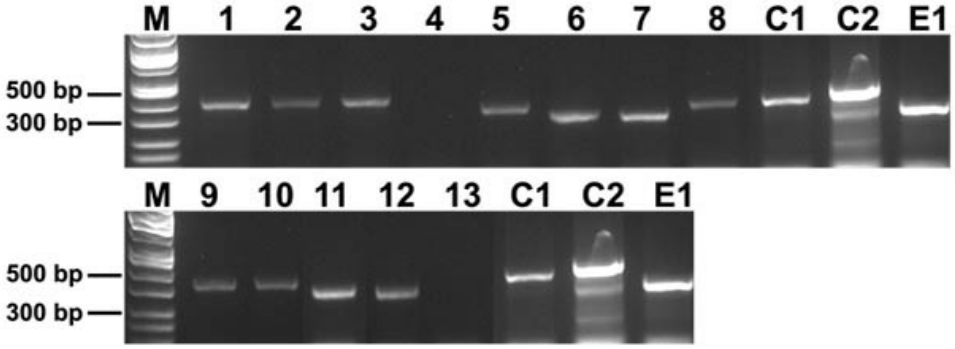

Figure 1. Serogrouping/ O-typing results of *Salmonella* isolates by multiplex PCR. Lanes M: 100 bp molecular marker; 1–13: isolates (1 – OB2, 2 – OB4, 3 – OB5, 4 – OC9, 5 – OQ5, 6 – 2ODp1,7: 2ODp2, 8 – 2OCm1, 9 – 2OCm2, 10 – 2OCm3, 11 – 2OCm4, 12 – 2OCm5, and 13 – 2OCm6); C1 (341 bp), C2 (397 bp), and E1 (281 bp) as positive controls for the serogroups.

onion leaf samples purchased from Cm Market samples, whereas one isolate (OQ5) was obtained from onion leaf samples purchased from Q Market. The three *Salmonella* isolates belonging to the serogroup C2 group (OB2, OB4, and OB5) were obtained from B Wet Market. Among the E1 group-positive *Salmonella* spp. isolates, two isolates (2ODp1 and 2ODp2) were obtained from onion leaf samples purchased from Dp Market, whereas the other two isolates (2OCm4 and 2OCm5) were obtained from samples from Cm Market (Table 2).

H-typing of the *Salmonella* spp. isolates were conducted to determine if the isolates belong to any of the following *S. enterica* serovars: Dublin, Typhimurium, Stanleyville, and Enteritidis. All 13 isolates yielded negative results to all PCR assays that targeted the H antigens of the above-mentioned serovars (data not shown).

The DNA sequences of the 11 isolates with known serogroups were found to be 97–99% similar to reference DNA sequences. Three isolates (2OCm1, 2OCm2, and 2OCm3) were 99% similar to *S. enterica* subsp. *enterica* serovar Tennessee, whereas isolates 2OCm5 and 2OCm4 were 97 and 99% similar to *S. enterica* subsp. *enterica* serovar Anatum. Isolates 2ODp1 and 2ODp2 were 98 and 99% similar to *S. enterica* subsp. *enterica* serovar Weltevreden. Further, three isolates (OB2, OB4, and OB5) were 99% similar to *S. enterica* subsp. *enterica* serovar Newport. One isolate (OQ5) had a 99% DNA sequence similarity to the sequence of serovars belonging to the C1 group (Table 2).

Antimicrobial Susceptibility Testing (AST)

Figure 2 shows the results of the AST done on the 13 isolates. Results indicate that 100% (13/13) of the *Salmonella* isolates were resistant to cefuroxime (CXM) and cefuroxime axetil (CXA), whereas 92% (12/13) were resistant to cefoxitin (FOX), amikacin (AN), and gentamicin (GM). Further, 31% (4/13) were resistant to ampicillin (AM) and trimethoprim/sulfamethoxazole (SXT), whereas only 8% (1/13) showed resistance to ceftriaxone (CRO) and ciprofloxacin (CIP).

All *Salmonella* spp. isolates showed multidrug resistance to at least five antibiotics. Results also showed that three isolates (2OCm1, 2OCm2, and 2OCm3) that were resistant to amoxicillin/clavulanic acid were also resistant to trimethoprim/sulfamethoxazole (SXT), cefuroxime (CXM), cefuroxime axetil (CXA), cefoxitin (FOX), amikacin (AN), and gentamicin (GM); whereas one isolate (2OCm4H) was resistant to amoxicillin/clavulanic acid (AMC), trimethoprim/sulfamethoxazole (SXT), cefuroxime (CXM), cefuroxime axetil (CXA), ceftriaxone (CRO), and ciprofloxacin (CIP) (Table 3).

DISCUSSION

Meat products including poultry products (eggs and poultry meat) are the common food vehicles of salmonellosis (Antunes *et al.* 2016). Recent reports, however, indicate that vegetables such as onions, raw sprouts, papayas, and those included in prepackaged salads harbor *Salmonella*, thereby resulting in outbreaks (CDC 2022). In the Philippines, *Salmonella* outbreaks linked to fresh produce have not been reported. Only three cases of food poisoning linked to vegetables were reported from 2005–2018, but the causative agent was not identified (Azanza *et al.* 2019). However, a previous study on the microbiological quality of 300 fresh produce retailed in open-air markets and supermarkets (*e.g.* bell pepper, cabbage, carrot, lettuce, and tomato) reported the presence of *Salmonella* in 74 (24.7%) of the samples tested (Vital *et al.* 2014). The presence of *Salmonella* in fresh produce may cause foodborne disease outbreaks, similar to those reported

Table 2. Serogrouping and DNA sequencing results of *Salmonella* spp. isolates from onion leaves collected from wet markets in Metro Manila, Philippines.

Isolate code	Source (market codes)	Serogroup	BLAST identification	Similarity (%)
OB2	B Market	C2	*Salmonella enterica* subsp. *enterica* Serovar Newport	99
OB42	B Market	C2	*Salmonella enterica* subsp. *enterica* Serovar Newport	99
OB5	B Market	C2	*Salmonella enterica* subsp. *enterica* Serovar Newport	99
OC9	C Market	–	–	–
OQ5	Q Market	C1	*Salmonella enterica* subsp. *enterica* Serovar Montevideo*	99
2ODp1	Dp Market	E1	*Salmonella enterica* subsp. *enterica* Serovar Weltevreden	98
2ODp2	Dp Market	E1	*Salmonella enterica* subsp. *enterica* Serovar Weltevreden	99
2OCm1	Cm Market	C1	*Salmonella enterica* subsp. *enterica* Serovar Tennessee	99
2OCm2	Cm Market	C1	*Salmonella enterica* subsp. *enterica* Serovar Tennessee	99
2OCm3	Cm Market	C1	*Salmonella enterica* subsp. *enterica* Serovar Tennessee	99
2OCm4	Cm Market	E1	*Salmonella enterica* subsp. *enterica* Serovar Anatum	99
2OCm5	Cm Market	E1	*Salmonella enterica* subsp. *enterica* Serovar Anatum	97
2OCm6	Cm Market	–	–	–

*Showed same BLAST result for the following serovars: Montevideo, Infantis, Cholerasius, Thompson, Bareilly and Paratyphi C.

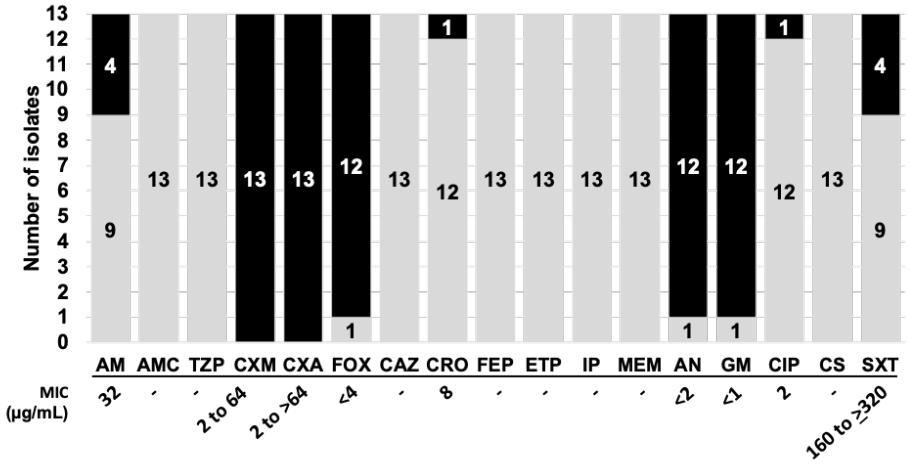

Figure 2. Antimicrobial susceptibility of *Salmonella* isolates: AM – ampicillin, AMC – amoxicillin/clavulanic acid, TZP – piperacillin/tazobactam, CXM – cefuroxime, CXA – cefuroxime axetil, FOX – cefoxitin, CAZ – ceftazidime, CRO – ceftriaxone, FEP – cefepime, ETP – ertanepem, IP – imipenem, MEM – meropenem, AN – amikacin, GM – gentamicin, CIP – ciprofloxacin, CS – colistin, SXT – trimetophrim/sulfamethoxazole. Black bars indicate the number of resistant isolates, whereas gray bars indicate the number of susceptible isolates.

Table 3. Antimicrobial resistance profile of *Salmonella* spp. isolates from onion leaves collected from selected wet markets in Metro Manila, Philippines.

Isolate code	Source (market code)	*Salmonella* serovar	Profile
OB2	B Market	Newport	CXM CXA FOX AN GM
OB42	B Market	Newport	CXM CXA FOX AN GM
OB5	B Market	Newport	CXM CXA FOX AN GM
OC9	C Market	Not determined	CXM CXA FOX AN GM
OQ5	Q Market	Montevideo*	CXM CXA FOX AN GM
2ODp1	Dp Market	Weltevreden	CXM CXA FOX AN GM
2ODp2	Dp Market	Weltevreden	CXM CXA FOX AN GM
2OCm1	Cm Market	Tennessee	CXM CXA FOX AN GM AM SXT
2OCm2	Cm Market	Tennessee	CXM CXA FOX AN GM AM SXT
2OCm3	Cm Market	Tennessee	CXM CXA FOX AN GM AM SXT
2OCm4	Cm Market	Anatum	CXM CXA AM SXT CRO CIP
2OCm5	Cm Market	Anatum	CXM CXA FOX AN GM
2OCm6	Cm Market	Not determined	CXM CXA FOX AN GM

AM – ampicillin; CXM – cefuroxime; CXA – cefuroxime Axetil; FOX – cefoxitin; CRO – ceftriaxone; AN – amikacin; GM – gentamicin; CIP – ciprofloxacin; SXT – trimetophrim/sulfamethoxazole. *Showed same BLAST result for the following serovars: Montevideo, Infantis, Cholerasius, Thompson, Bareilly, and Paratyphi C.

by the United States (US) Centers for Disease Control and Prevention (CDC 2022), which are caused by the consumption of raw vegetables.

Molecular Characterization of *Salmonella* spp.

Amplification of the invasion gene (*invA*) as reported in the study of Siringan and Torres (2019) indicated that onion leaf samples, which are normally consumed raw, can be a potential source of salmonellosis in the Philippines. Of the 98 onion leaf samples tested, 13 (13.3%) were shown to be positive for *Salmonella* spp., as indicated by the presence of the *invA* (Siringan and Torres 2019). This present study used the *spvC*, a plasmid-encoded virulence factor, as an additional marker for screening the virulence of *Salmonella* spp. Virulence plasmids have been found to be related to the survival and growth of the bacterium in host cells (Okamoto *et al.* 2009). The *spvC*, which is part of the *Salmonella* plasmid virulence (*spv*) operon, can be detected by PCR amplification in the *Salmonella* spp. isolates. In this study, none of the isolates (0/13) harbored the gene. It should be noted, however, that the non-detection of *spvC* does not indicate the absence of plasmids in the isolates, as the gene profile of plasmids may vary in different *Salmonella* serotypes (Derakhshandeh *et al.* 2013).

Serogrouping (O-typing) is a method commonly used to classify *Salmonella* strains into groups based on antigenic variability due to lipopolysaccharide moieties or O antigen present in the outer membrane (Grimont and Weill 2007). Approximately, 99% of *Salmonella* infections in humans and warm-blooded animals are caused by the strains within *Salmonella* serogroups A, B, C1, C2, D, and E (Popoff and le Minor 1997). Results of the O-typing conducted in this study – which investigated the above-mentioned serogroups – revealed that four (30.8%), three (23%), and four (30.8%) belonged to serogroups C1, C2, and E1, respectively. However, two (15.4%) isolates did not belong to any of the serogroups included in the study. On the other hand, serogroups A, B, and D were not detected from the isolates. The presence of the serogroups detected in this study is significant, as they pose health risks to humans and other warm-blooded animals (Popoff and le Minor 1997). Serogroup C detected in this study was reported to show a continuous increase in the frequency associated with human infections in the US from 1995–2011 and in Europe from 1995–2013 based on the data obtained from the US CDC and the European Surveillance System (Fuche *et al* 2016). In addition, *Salmonella* serogroup C1 serovars were reported to cause invasive disease in infants in Mali (Fuche *et al.* 2018). Moreover, serovars belonging to serogroups C1, C2, and E1 were reported to cause foodborne outbreaks resulting in illnesses and hospitalizations (Quiroz-Santiago *et al.* 2009; CDC 2022).

Aside from O-typing, H-typing was also done to determine the specific serovars of the *Salmonella* isolates (Grimont and Weill 2007). In this study, through multiplex

PCR assay using H antigen-based genes unique to four different *Salmonella enterica* serovars, the *Salmonella* spp. isolates were further characterized. These serovars – namely, *S. enterica* serovars Dublin, Typhimurium, Stanleyville, or Enteritidis – were of significant health risk to humans and warm-blooded animals (Agron *et al.* 2001; Levy *et al.* 2008; Tennant *et al.* 2010; CDC 2022). However, none of the 13 isolates belong to the four serovars examined based on H-typing. The *Salmonella* isolates in this study likely belong to other serotypes not targeted in this study. This is supported by the sequencing of the PCR products from O-typing, which revealed the presence of *S. enterica* serovars: Anatum, Newport, Weltevreden, and Tennessee. Serovar Anatum, detected in this study, was detected in vegetables (Quiroz-Santiago *et al.* 2009) and caused a foodborne outbreak in papaya in 2017 (CDC 2022). Recent outbreaks of serovar Newport infections linked to onions and papayas were reported in 2020 and 2017, respectively (CDC 2022).

Moreover, it should be noted that the serotypes obtained based on serogrouping and sequencing could be associated with the source of the sample where the isolate was obtained. All *S. enterica* subsp. *enterica* serovar Newport (serogroup C2) were isolated from onion leaves purchased from B Market. The *S. enterica* subsp. *enterica* serovar Weltevreden isolates (serogroup E1), on the other hand, were isolated from Dp Market. Finally, the *S. enterica* subsp. *enterica* serovar Tennessee (serogroup C1) and *S. enterica* subsp. *enterica* serovar Anatum (serogroup E1) were isolated from Cm Market. It should be noted that the *Salmonella* isolates used in this study were obtained from the previous project focusing on the incidence of the pathogen in fresh produce, and no *Salmonella* detection was done on water, soil, and the environment. No investigation was done to determine the source of *Salmonella*. Possibly, *Salmonella* contamination may have occurred during production, post-harvest production, transport, distribution, or retail of the produce.

Antimicrobial Susceptibility Testing (AST)

AMR among *Salmonella* serotypes has become a serious problem worldwide (Chiu *et al.* 2002). Fluoroquinolones and cephalosporins are used in the treatment of infections caused by *Salmonella*, but cases of drug resistance to these agents have been reported since 1991 (Carattoli *et al.* 2002; Chiu *et al.* 2002). The occurrence of antimicrobial-resistant bacteria in foods, including fresh produce, has become a concern in public health worldwide (Kilonzo-Nthenge and Mukuna 2018).

This study used the VITEK®2 system, which includes antibiotics that are commonly used to treat *Salmonella* infections such as fluoroquinolones and cephalosporins. Results were in accordance with the findings of other studies on *Salmonella* spp. isolated from vegetables with resistance to several antibiotics. *Salmonella* spp. isolated from various vegetable samples in New Zealand were reported to be resistant to ampicillin, as well as vancomycin and penicillin (Wadamori *et al.* 2016). *Salmonella enterica* isolated from cabbage and lettuce samples in Ghana were reported to be resistant to one or more of the following antibiotics – ampicillin, chloramphenicol, ciprofloxacin, ceftriaxone, gentamicin, erythromycin, ofloxacin, trimethophrim/sulfamethoxazole, and tetracycline – some of which can be classified as multidrug-resistant (MDR) (Adzitey 2018). *Salmonella* spp. isolated from fruits and vegetables in India were also reported to be resistant to chloramphenicol, colistin, azithromycin, erythromycin, amikacin, streptomycin, doxycycline, tetracycline, co-trimoxazole, ampicillin, and amoxicillin-clavulanic acid (Verma *et al.* 2018). Results of this study showed that all the *Salmonella* spp. isolates (13/13) obtained from onion leaf samples were resistant to at least five antimicrobial agents, belonging to at least three antimicrobial categories, and were all MDR. All 13 isolates (100%) were resistant to cefuroxime and cefuroxime axetil (2nd generation cephalosporins). Twelve (12) isolates (92%) were resistant to cefoxitin (2nd generation cephalosporin), as well as amikacin and gentamicin (aminoglycosides), and 4/13 (31%) were resistant to ampicillin (penicillin) and trimethophrim/sulfamethoxazole (folate pathway inhibitors). Only one isolate (8%) showed resistance to ceftriaxone (3rd generation cephalosporin) and ciprofloxacin (fluoroquinolone). The study revealed that the antimicrobial profiles of the *Salmonella* spp. isolates were the same for those having the same serovars from the same source, except for two identified *S. enterica* subsp. *enterica* serovar, Anatum which showed different profiles from each other.

There are different mechanisms of AMR in *Salmonella* spp. Resistance to aminoglycoside antibiotics involves the enzymatic modification of the compound, which prevents the binding of the antibiotic to its ribosomal target. Some of the enzymes involved include acetyltransferases, phosphotransferases, and nucleotidyltransferases, which modify and inactivate the aminoglycoside, which confer resistance to gentamicin, tobramycin, kanamycin, and streptomycin (Ramirez and Tolmasky 2010). For β-lactam antibiotics, such as ampicillin, cephalosporins, and carbapenems, resistance is usually due to β-lactamases that are encoded by *bla* genes, whereas resistance to fluoroquinolones is linked to mutations in quinolone resistance-determining regions, which code for topoisomerase IV and gyrase (*gyrA* and *gyrB*) (Velge *et al.* 2005).

The presence of antimicrobial-resistant *Salmonella* spp. in fresh produce may be directly related to environmental contamination (Nair *et al.* 2018) and may have occurred

through the following sources: irrigation water, soil, fertilizers, and animal manure (Carstens *et al.* 2019). Horizontal gene transfer also plays a vital role in the spread of antibiotic-resistant *Salmonella* spp, wherein resistant genes acquired by plasmids, transposons, or integrons can transfer resistance to other strains or species (Nair *et al.* 2018). The presence of drug-resistant *Salmonella* spp. isolates from fresh produce may have an impact on the consumer's safety and treatment of infections. In particular, fluoroquinolones and extended-spectrum cephalosporin, including ceftriaxone, are used to treat invasive salmonellosis in adults and children, respectively (Nair *et al.* 2018).

Microbial Safety of Fresh Produce

Outbreaks linked to *Salmonella* have been reported, causing concerns about the safety of fresh produce (CDC 2022). The presence of these bacteria indicates contamination in farm operations, which may come from irrigation water, soil, animal manure, fertilizers, equipment, and workers (Carstens *et al.* 2019).

Multidrug-resistant *Salmonella* in onion leaves pose public health risks; thus, it is imperative to address the microbial safety of fresh produce. In the Philippines, the DA has established Good Agricultural Practices (GAP) for Fruits and Vegetable Farming – Code of Practice (PNS/BAFS 49:2021). It aims to ensure the quality and safety of produce and is based on hazard analysis of critical control points and quality management principles (DA-BAFS 2021). Strict implementation and monitoring of the recommended hygienic practices for the production and primary processing of fresh produce listed in the standard should be undertaken.

CONCLUSION

In this study, detection of selected virulence genes, serogrouping, serotyping, and antibiotic resistance profiling of *Salmonella* spp. isolates were performed. Sequence analysis of selected *Salmonella* spp. isolates were also done to confirm serotypes. The existence of virulence genes in the isolates obtained from fresh produce indicates the presence of serotypes associated with these genes, as well as the potential of onions to serve as vehicles for food-associated infections, as these genes are responsible for the ability of the isolates to cause disease. The presence of drug-resistant *Salmonella* spp. in fresh produce has a significant impact on public health. Due to the antibiotic resistance of the pathogen, treatment of salmonellosis could be more difficult, resulting in prolonged illness, complications, suffering or death, and economic and emotional burden on families.

To date, this is the first study in the Philippines that established baseline data for the molecular features and antibiogram profiles of *Salmonella* spp. isolated in onion leaf samples collected in selected wet markets in Metro Manila, Philippines. Moreover, this study is the first report on the occurrence of four different *Salmonella* serovars (Newport, Weltevreden, Tennessee, and Anatum) from onion leaves, which are normally consumed raw. The data obtained in this study could be used as baseline information and a guide in the implementation of the GAP and monitoring programs of DA, as well as the formulation of laws and regulations that will address issues in fresh produce safety and hygienic practices in farm operations. Furthermore, the data on the AMR profiles of the 13 *Salmonella* spp. from onion leaves are significant contributions to the Antimicrobial Resistance Surveillance Program of the Philippines (https://arsp.com.ph). The data generated through this study could support the formulation of policies spearheaded by this national program, in line with the prevention and control of AMR emergence in the Philippines.

ACKNOWLEDGMENTS

This work was hosted by MRSL, NSRI, UPD and under the DABIOTECH-P1201 project titled "Incidence and Determination of Food-borne Pathogens in Vegetables: Towards the Development of Microbiological Standards for Product" funded by DA-BAR and DA-Biotech PIU.

REFERENCES

ADZITEY F. 2018. Antibiotic resistance *of Escherichia coli* and *Salmonella enterica* isolated from cabbage and lettuce samples in Tamale metropolis of Ghana. Food Contamination 5(7)._https://doi.org/10.1186/s40550-018-0068-z.

AGRON PG, WALKER RL, KINDE HS, SAWYER J, HAYES DC, WOLLARD J, ANDERSEN GL. 2001. Identification by subtractive hybridization of sequences specific for *Salmonella enterica* serotype Enteritidis. Appl Environ Microbiol 67(11): 4984–4991. https://doi.org/10.1128/AEM.67.11.4984-4991.2001

ANDREWS WH, JACOBSON A, HAMMACK TS. 2011. Bacteriological Analytical Manual Online: Chapter 5: *Salmonella*. Retrieved on 16 Jun 2014 from https://www.fda.gov/food/laboratory-methods-food/bam-chapter-5-salmonella

ANTUNES P, MOURAO J, CAMPOS J, PEXIE L. 2016. Salmonellosis: the role of poultry meat. Clin Microbiol

Infection 22(2): 110–121. https://doi.org/10.1016/j.cmi.2015.12.004.

AZANZA MPV, MEMBREBE BNQ, SANCHEZ RGR, ESTILO, EEC, DOLLETE GM, FELICIANO RJ, GARCIA NKA. 2019. Foodborne Disease Outbreaks in the Philippines (2005–2018). Philipp J Sci 148(2): 317–336.

CARATTOLI A, TOSINI F, GILES WP, RUPP ME, HINRICHS SH, ANGULO FJ, BARRETT TJ, FEY PD. 2002. Characterization of plasmids carrying CMY-2 from expanded-spectrum cephalosporin-resistant *Salmonella* strains isolated in the United States between 1996 and 1998. Antimicrob Agents Chemother 46(5): 1269–1272. https://doi.org/10.1128/AAC.46.5.1269-1272.2002

CARSTENS CK, SALAZAR JK, DARKOH C. 2019. Multistate Outbreaks of Foodborne Illness in the United States Associated with Fresh Produce from 2010 to 2017. Front Microbiol 10(2667). https://doi.org/10.3389/fmicb.2019.02667

[CDC] Centers for Disease Control and Prevention. 2022. Reports of Selected *Salmonella* Outbreak Investigations. Retrieved on 25 Feb 2022 from https://www.cdc.gov/salmonella/outbreaks.html

CHIU CH, OU JT. 1996. Rapid identification of *Salmonella* serovars in faeces by specific detection of virulence genes invA and spvC by an enrichment broth culture-Multiplex PCR combination assay. J Clin Microbiol 34(10): 2619–2622.

CHIU CH, WU TL, SU LH, CHU C, CHIA JH, KUO AJ, CHIEN MS, LIN TY. 2002. The emergence in Taiwan of fluoroquinolone resistance in *Salmonella enterica* serotype Choleraesuis. N Engl J Med 346: 413–419. https://doi.org/10.1056/NEJMoa012261

[DA-BAFS] Department of Agriculture–Bureau of Agriculture and Fisheries Standards. 2021. Good Agricultural Practices (GAP) for Fruits and Vegetable Farming – Code of Practice (PNS/BAFS 49:2021). 31p.

DERAKHSHANDEH A, FIROUZI R, KHOSHBAKHT. 2013. Association of Three Plasmid-encoded *spv* Genes among Different *Salmonella* Serotypes Isolated from Different Origins. Indian J Microbiol 53(1): 106-110. https://doi.org/10.1007/s12088-012-0316-5

FUCHE FJ, SEN S, JONES JA, NKEZE J, PERMALA-BOOTH J, TAPIA MD, SOW SO, TAMBOURA B, TOURE AA, ONWUCHEKWA U, SYLLA M, KOTLOFF, KL TENNANT SM. 2018. Characterization of Invasive *Salmonella* Serogroup C1 Infections in Mali. Am J Trop Med Hyg 98(2): 589–594. https://doi.org/10.4269/ajtmh.17-0508

FUCHE FJ, SOW O, SIMON R, TENNANT SM. 2016. *Salmonella* Serogroup C: Current Status of Vaccines and Why They Are Needed. Clin Vaccine Immunol 23(9): 737–745. https://doi.org/10.1128/CVI.00243-16

GREISEN K, LOEFFELHOLZ M, PUROHIT A, LEONG D. 1994. PCR primers and probes for the 16S rRNA gene of most species of pathogenic bacteria, including bacteria found in cerebrospinal fluid. J Clin Microbiol 32: 335–351.

GRIMONT PAD, WEILL FX. 2007. Antigenic formulae of the *Salmonella* serovars. 9th edition. WHO Collaborating Center for Reference and Research on *Salmonella*, Paris, France: Institut Pasteur.

HERRERA-LEON S, MCQUISTON JR, USERA MA, FIELDS PA, GARAIZAR J, ECHEITA MA. 2004. Multiplex PCR for distinguishing the most common phase-1 flagellar antigens of *Salmonella* spp. J Clin Microbiol 42(6): 2581–2586. https://doi.org/10.1128/JCM.42.6.2581-2586.2004.

HIROSE K, ITOH K, NAKAJIMA H, KURAZONO T, YAMAGUCHI M, MORIYA K, EZAKI T, KAWAMURA Y, TAMURA K, WATANABE H. 2002. Selective amplification of *tyv* (*rfbE*), *prt* (*rfbS*), *viaB*, and *fliC* genes by multiplex PCR for identification of *Salmonella enterica* serovars Typhi and Paratyphi A. J Clin Microbiol 40(2): 633– 636. https://doi.org/10.1128/JCM.40.2.633-636.2002.

HONG Y, LIU T, LEE MD, HOFACRE CL, MAIER M, WHITE DG, AYERS S, WANG L, BERGHAUS R, MAURER JJ. 2008. Rapid screening *of Salmonella enterica* serovars Enteritidis, Hadar, Heidelberg, and Typhimurium using a serologically-correlative allelotyping PCR targeting the O and H antigen alleles. BMC Microbiol 8(178). https://doi.org/10.1186/1471-2180-8-178

KILONZO-NTHENGE A, MUKUNA W. 2018. *Salmonella* and Antimicrobial Resistance in Fresh Produce. In: *Salmonella* – a Re-emerging Pathogen. Mascellino MT ed. IntechOpen. Retrieved on 02 Sep 2021 from https://www.intechopen.com/chapters/58719

LEVY H, DIALLO S, TENNANT SM, LIVIO S, SOW SO, TAPIA M, FIELDS PI, MIKOLEIT M, TAMBOURA V, KOTLOFF KL, LAGOS R, NATARO JP, GALEN JE, LEVINE MM. 2008. PCR method to identify *Salmonella enterica* serovars Typhi, Paratyphi A, and Paratyphi B among *Salmonella* isolates from the blood of patients with clinical enteric fever. J Clin Microbiol 46(5): 1861–1866. https://doi.org/10.1128/JCM.00109-08

NAIR DVT, VENKITANARAYANAN K, JOHNY AK. 2018. Antibiotic-resistant *Salmonella* in the Food Sup-

ply and the Potential Role of Antibiotic Alternatives for Control. Foods 7(10): 167. https://doi.org/10.3390/foods7100167

OKAMOTO AS, FILHO RLA, ROCHA TS, MENCONI A, MARIETTO-GONÇALVES GA. 2009. Relation Between the *SpvC* and *InvA* Virulence Genes and am of *Salmonella enterica* Serotype Enteritidis Isolated from Avian Material. Int J Poult Sci 8(6): 579–582. https://doi.org/10.3923/ijps.2009.579.582

POPOFF MY, LE MINOR L. 1997. Antigenic formulas of the *Salmonella* serovars, 7th revision. World Health Organization Collaborating Centre for Reference and Research on *Salmonella*, Paris, France: Pasteur Institute.

QUIROZ-SANTIAGO C, RODAS-SUAREZ OR, VASQUEZ Q CR, FERNANDEZ FJ, QUINONES-RAMIREZ EI, VASQUEZ-SALINAS C. 2009. Prevalence of *Salmonella* in Vegetables from Mexico. J Food Prot 72(6): 1279–1282. https://doi.org/10.4315/0362-028x-72.6.1279

RAHN K, DE GRANDIS SA, CLARKE RC, CURTISS R, GYLES CL. 1992. Amplification of an invA gene sequence of *Salmonella typhimurium* by polymerase chain reaction as a specific method of detection of *Salmonella.* Mol Cell Probes 6(4): 271–279. https://doi.org/10.1016/0890-8508(92)90002-f

RAMIREZ MS, TOLMASKY ME. 2010. Aminoglycoside modifying enzymes. Drug Resist Updat 13(6): 151–171. https://doi.org/10.1016/j.drup.2010.08.003

SHANMUGASAMY M, VELAYUTHAM T, RAJESWAR J. 2011. *InvA* gene specific PCR for detection of *Salmonella* from broilers. Vet World 4(12): 562–564. https://doi.org/10.5455/vetworld.2011.562-564

SIRINGAN MAT, TORRES GAV. 2019. Terminal Report on the "Incidence and Determination of Food-borne Pathogens in Vegetables: Towards the Development of Microbiological Standards for Produce" (DABIOTECH-P1201). Department of Agriculture–Bureau of Agricultural Research (DA-BAR) and DA–Biotechnology Program Implementing Unit (DA–Biotech PIU).

TENNANT SM, DIALLO S, LEVY H, LIVIO S, SOW SO, TAPIA M, FIELDS PI, MIKOLEIT M, TAMBOURA B, KOTLOFF KL, NATARO JP, GALEN JE. 2010. Identification by PCR of Non-typhoidal *Salmonella enterica* Serovars Associated with Invasive Infections among Febrile Patients in Mali. PLoS Negl Trop Dis 4(3): 621. https://doi.org/10.1371/journal.pntd.0000621

VELGE P, CLOECKAERT A, BARROW P. 2005. Emergence of *Salmonella* epidemics: the problems related to *Salmonella enterica* serotype Enteritidis and multiple antibiotic resistance in other major serotypes. Vet Res 36(3): 267–288. https://doi.org/10.1051/vetres:2005005

VERMA P, SAHARAN VV, SINGH AP. 2018. Phenotypic and virulence traits of Escherichia coli and *Salmonella* strains isolated from vegetables and fruits from India. J Appl Microbiol 125(1): 270–278. https://doi.org/10.1111/jam.13754

VITAL PG, DIMASUAY KG, WIDMER KW, RIVERA WL. 2014. Microbiological quality of fresh produce from open air markets and supermarkets in the Philippines. Scientific World Journal [219534]. https://doi.org/10.1155/2014/219534

WADAMORI Y, FAM F, HUSSAIN MA, GOONERATNE R. 2016. Microbiological risk assessment and antibiotic resistance profiling of fresh produce from different soil enrichment systems: a preliminary study. Cogent Food and Agriculture 2(1): 1274281. https://doi.org/10.1080/23311932.2016.1274281