ORIGINAL ARTICLE

ANTIBIOGRAM AND STRESS RESPONSE TO *Salmonella* INFECTION IN PHILIPPINE NATIVE CHICKENS

Lotis M. Balala, DVM, PhD [1], Bernadette C. Mendoza, DrPH[2],
Loinda R. Baldrias, DVM, PhD[3], Marilen P. Balolong, DrPH[4]
and Joseph S. Masangkay, DVM, PhD[3]

[1]College of Veterinary Medicine, Visayas State University, Visca, 6521-A Baybay City, Leyte; [2]Institute of Biological Sciences, University of the Philippines Los Baños, 4030 College, Laguna; [3]College of Veterinary Medicine, University of the Philippines Los Baños, 4030 College, Laguna; [4]Department of Biology, University of the Philippines Manila, 1000 Ermita, Manila, Philippines.

ABSTRACT

Free-range native chicken production is beset by multiple environmental threats. This study aimed to detect *Salmonella* from environmental samples and cloacal swabs of Philippine native chickens, characterize antimicrobial resistance pattern, and assess stress response to infection. *Salmonella* was detected through culture and PCR assay. The disk diffusion method was used to describe resistance pattern and differential leukocyte count to assess stress response. The overall detection rate of *Salmonella* was 8.9% (21/237), 2.11% (5/237), and 8.04% (16/199) of which were detected by culture and PCR, respectively. Isolates were 80% susceptible to amoxicillin-clavulanic acid and 60% to norfloxacin, cefaclor, and chloramphenicol. An intermediate to susceptible pattern (40%-80%) was demonstrated to ciprofloxacin, norfloxacin, and gentamicin. Antibiotic resistance was 80% in ampicillin, doxycycline, tetracycline, and trimethoprim-sulfamethoxazole; 60% in kanamycin and cefuroxime; and 40% in neomycin. Multidrug resistance was demonstrated by 80% of the isolates which had MAR indices ranging from 0.43-0.57. The H:L ratio was significantly high (p-value=0.005) during the highest detection rate at Day 150 indicating a stressful state. The study suggests the potential of native chickens as reservoir for multidrug-resistant *Salmonella* they possibly acquire from the environment. Attention should be given to the environmental conditions to which native chickens are raised.

Keywords: *Salmonella, multi-drug resistance, free-range native chickens, H:L ratio, MAR index*

INTRODUCTION

Recently, the demand for free-range chickens is rising due to the increasing health consciousness and food safety awareness of consumers across the globe. This preference gained wide acceptance as chickens are raised in friendly environment and without the use of antibiotics. But the ranging environment is a diverse ecosystem that may be a source of antibiotic resistant genes and a dispersal route for resistant pathogens. It is imperative that safety of the poultry products derived from this production system is investigated. Although highly adapted to environmental stress and apparently resistant to diseases, free-range chickens are not spared from the invasion of ubiquitous pathogens from their environment. *Salmonella* is one of the perennial pathogens that wreaks havoc on the production in poultry farms and in the food chain. Attacking chickens at their vulnerable age, *Salmonella* causes high mortality in young chicks that progresses into a carrier state for surviving adult chickens (Cosby *et al.*, 2015). Without the application of conventional

*FOR CORRESPONDENCE:

(e-mail address: balalalotis@vsu.edu.ph)

antibiotics, range chickens are easily invaded, unless alternative control measures against pathogens are in place. Aggravating this burden is the asymptomatic state of salmonellosis precluding early detection and thus, the need for regular monitoring.

Salmonella is one of the most important causes of bacterial foodborne zoonoses, with more than 2,600 nontyphoidal serotypes known to infect humans (Scallan *et al*., 2011). Despite stringent tolerance level, this organism persists in poultry and poultry products that serve as the key source of human salmonellosis (Swaggerty *et al*., 2019; Zhao *et al*., 2016). The importance of *Salmonella* as a public health threat not only lies in its capacity to cause infection but also in its wide range of antimicrobial resistance (Borges *et al*., 2019). The issue on resistance brings about great concern in the medical field because of the similarity of antimicrobials used in animal production and human medicine, compounding the problem of increased number of deaths and hospitalization costs due to salmonellosis (CDC, 2013). It is due to this fact that the World Health Organization (WHO) and the Office International des Epizooties (OIE) considered *Salmonella* a priority bacterium requiring active surveillance and monitoring in poultry and poultry products (Borges *et al*., 2019). Preliminary studies demonstrated the isolation of antibiotic-resistant *Salmonella* from free-ranging chickens in China (Zhao *et al*., 2016) and in nine (64%) of 14 free-range lots and 42 (31%) of 135 individual free-range birds in the US (Bailey and Cosby, 2005).

Monitoring of leukocyte counts is relevant in assessing stress response where heterophils and lymphocytes play a vital role in mounting immunity against bacterial infection. The heterophils are measured since they are the first among leukocytes to proliferate in circulation and arrive at the site of infection to engulf and destroy pathogens (Jain, 1993; Maxwell and Robertson, 2005). Lymphocytes, on the other hand, are the major cells involved in various immunological functions including antibody production (Tizard, 2009). Gross and Siegel (1983) proposed the concept of the heterophil:lymphocyte (H:L) ratio which measures the relative proportion of heterophils to lymphocytes during stress. The profile of H:L ratio in relation to the state of infection becomes a widely accepted reliable physiological indicator of stress responses in domestic fowl including those induced by *Salmonella* infection (Maxwell, 1993).

In the Philippines there is a dearth of Information regarding the antimicrobial resistance profile of *Salmonella* spp. in native chickens and stress response. This study investigated the shedding of *Salmonella* in free-range Philippine native chickens and characterized the sensitivity and resistance of the isolates to antibiotics. Through the resistance profile, MAR index was generated to ascertain severity of contamination. The leukocyte profile of the chickens predicts stress responses related to *Salmonella* infection. The profiling of resistance patterns and hematological parameters provides essential information in formulating strategic measures to control pathogens and improve management in free-range production systems.

MATERIALS AND METHODS

Study Site

The study was conducted at the Native Poultry Farm facility of the Institute of Animal Science (IAS), College of Agriculture and Food Science (CAFS), University of the Philippines Los Baños (UPLB) at Barangay Putho-Tuntungin, Los Baños, Laguna. This area is situated approximately 14.1556° N (longitude) and 121.2518° E (latitude) in the island of Luzon and is estimated to be 32.1 meters or 105.3 feet above mean sea level. The site was selected due to its proximity to the UPLB campus, the availability of the facility, and the relative distance from human settlements.

Experimental Design and General Description of the Study

A five-month longitudinal study was designed to detect the shedding of *Salmonella* spp. in 50 Banaba x Paroakan Philippine native chickens raised in a controlled, free-range environment. Day-old chicks were obtained from IAS-CAFS, UPLB, College, Laguna. The chicks were raised in pens constructed following the standard space requirement (0.5 ft2/chick) and brooded at night using a 1-watt incandescent bulb per chick. A ranging area was provided and secured with fence to allow at least eight hours of outdoor range access during the daytime. At 3-4 weeks, the chickens were allowed to roam around the farm during the day and kept in the pen at night. The chickens were given premium commercial feeds formulated for native chickens based on their nutritional requirement and *ad libitum* water. Vaccines and antibiotics were not administered except vitamin supplements during inclement weather. Biosecurity measures were made available in the study site including foot bath, control of animal or human entry inside the farm, and regular disinfection.

Collection and Processing of Samples

Preliminary examination. Salmonella contamination in the flock and environment was ascertained before the start of the study. Boot sock field samples were obtained from the farm five days prior to stocking and on the day day-old chicks were brought to the farm. Feeds, water, and cloacal swab samples from day-old chicks were collected and analyzed at the first day of the experiment. Samples were analyzed by culture and PCR method at the Molecular Biology Laboratory of the College of Veterinary Medicine, UPLB, College, Laguna.

Collection of environmental samples. Collection of samples was done bi-weekly for five months. Field samples were collected by placing a pair of blue overshoes over boots, followed by a pair of boot socks. Sampling was done by walking through a "W" pattern (Peters and Laboski, 2013) in the farm premise to ensure that different sections were included. The boot socks were removed and placed inside a sealable polythene bag and brought to the lab. A 100 ml of phosphate-buffered saline (PBS) was added to the bag, shaken, and left to stand for 10 minutes. One mL sample aliquot was then used for bacterial isolation.

Twenty-five grams of feeds were collected from the feeding trough of chickens and were placed in sterile sealable plastic. The samples were pre-enriched in 225 ml buffered peptone water (BPW) and incubated at 37°C for 18 to 24 hours. One mL of the pre-enriched culture was then used for bacterial isolation.

Moreover, 20 mL of water samples from the birds' waterer were obtained in sterile plastic bottles and brought to the lab. Two mL of the sample was pre-enriched in 18 mL BPW and incubated at 37°C for 18 to 24 hours. One mL of the pre-enriched culture was then used for bacterial isolation.

Cloacal Swabs. To collect cloacal swabs, each chicken was restrained by holding it against the chest with its wings folded. A sterile cotton swab was gently inserted into its cloaca and rotated multiple times to ensure thorough staining with fecal material. The swabs were labeled correctly, placed inside ice-chilled containers and were transported to the lab. In the lab, each swab was directly inoculated onto brilliant green agar (BGA) and xylose lysine deoxycholate agar (XLD) and the tip of the swab was subsequently cut off and deposited into Rappaport Vassiliadis (RV) broth for enrichment. Growth from RV broth was then used for bacterial isolation.

Bacteriological Isolation of *Salmonella* spp.

A total of 180 cloacal swabs, 19 boot socks, 19 feeds, and 19 water samples were collected and analyzed for bacteriological isolation. The samples were pre-enriched in BPW, enriched in selenite broth and RV broth, and streaked into BGA and XLD. The culture media were aerobically incubated at 37 °C for 18-24 hours for pre-enrichment and selective media and 48 hours for enrichment (International Standard, 2015). Colonies resembling typical *Salmonella* were sub-cultured into fresh BGA and XLD plates until purified. Growth and biochemical reactions of the putative isolates were characterized in Mac Conkey agar, triple sugar iron agar, lysine decarboxylase broth, and urea broth. The results of these tests were then compared to the growth and reaction of NCTC 6017 *Salmonella* Abony (Medline Industries, Northfield, IL, USA) inoculated in the same media.

Serum Agglutination Test

Slide agglutination test using polyvalent O *Salmonella* antisera (Pro Lab Diagnostic Inc., ON, Canada) was carried out for phenotypic confirmation of the isolates. A bacterial suspension was emulsified in sterile saline solution and added with a drop of polyvalent O *Salmonella* antisera. Clump formation or agglutination of the mixture confirmed a positive reaction.

DNA Extraction and PCR Assay

DNA from boot socks and cloacal swabs were extracted using QIAamp Fast DNA Stool Mini Kit (Qiagen®, Germantown, MD, USA). One mL aliquot of boot sock from PBS was centrifuged at 13,000 rpm for seven minutes. The DNA from pellets was extracted using the kit following the manufacturer's protocol. Subsequently, the tip of the cloacal swab was deposited in 1 ml InhibitEX buffer and vortexed for one minute. After removing the swab, the suspension was heated for five minutes at 70°C, vortexed for 15 seconds, and centrifuged for another minute. About 200 µl of the supernatant was added into 15 µl of proteinase K with buffer AL, vortexed and heated at 70°C for 10 minutes. The resultant lysate was then centrifuged inside QIAamp spin column to purify the DNA. Finally, 200 µl of buffer ATE (10 mM Tris-Cl pH 8.3, 0.1 mM EDTA, 0.04% sodium-azide) was directly pipetted into the QIAamp membrane and centrifuged for one minute to elute the DNA. The DNA yield was quantified using NanoDrop™ (Thermo Fisher Scientific Inc., San Francisco, CA, USA).

A total volume of 25 µl PCR mixture containing 12.5 µl of GoTaq® Green Master Mix (Promega, Madison, WI, USA), 0.25 µl each of the invA primer (10 µM), 10 µl of nuclease-free water, and 2 µl of the DNA template was subjected to PCR. GoTaq® Green Master Mix consisted of 2X reaction buffer (pH, 8.5), 400µM dATP, 400µM dGTP, 400µM dCTP, 400µM dTTP, and 3mM MgCl2. Primer sequences according to Rahn *et al.* (1992) were used: invA-F, GTG AAA TTA TCG CCA CGT TCG GGC AA and invA-R, TCA TCG CAC CGT CAA AGG AAC C. The reaction conditions were set at 94°C for 7 minutes for initial denaturation, 94°C for one minute for denaturation, 53°C for two minutes for annealing, 72°C for three minutes for extension, and another 72°C for seven minutes for a final extension. Amplicons were analyzed in 2% agarose gel stained with GelRed® (Biotium, Fremont, CA, USA) set in gel electrophoresis at 120V/cm for 30-45 minutes. Amplified products were visualized in UV transilluminator (Vilber, Marne La Vallee, Ile-de-France, France) and considered *inv*A-positive when a 284 bp band is produced.

Antibiotic Disk Diffusion Assay and Multiple Antimicrobial Resistance Index

Bacterial suspension was prepared by inoculating four to five colonies of the purified culture in brain heart infusion broth and incubated at 37°C until a 0.5 McFarland turbidity standard was achieved. A volume of 0.1 ml of the culture was then swabbed evenly into dried Mueller Hinton agar using a sterile cotton swab. Fourteen antimicrobial disks were used and embedded onto the agar, seven disks per plate, at equal distance and incubated at 37°C for 16 to 18 hours. The antimicrobial disks consisted of ampicillin (10 µg), amoxicillin-clavulanic acid (10 µg), cefaclor (30 µg), cefuroxime (30 µg), chloramphenicol (30 µg), ciprofloxacin (5 µg), norfloxacin (10 µg), gentamicin (10 µg), streptomycin (10 µg), kanamycin (30 µg), neomycin (10 µg), doxycycline (30 µg), tetracycline (30 µg), and trimethoprim-sulphamethoxazole (1.25/23.75 µg) (Learn-Han et al., 2009). The zone of inhibition was measured (mm) and scored as sensitive, intermediate, and resistant following the Clinical Laboratory Standards Institute guidelines (CLSI, 2018).

Determination of H:L Ratio

A differential leukocyte count was carried out to determine the H:L ratio at Days 10, 30, 120, and 150. Initially, a drop of fresh blood obtained from the uncoagulated blood of chicken was placed on one end of a clean glass slide to make a monolayer film of smear (Cotter, 2015). Air-dried slides were then immersed in a Diff-Quick™ set of stain. Leukocytes were identified based on the morphological characteristics described by Lucas and Jamroz (1961) and counted under oil immersion light microscopy. The H:L ratio according to Gross and Siegel (1983) was derived by dividing the relative heterophil counts (%) by the relative lymphocyte counts (%).

Ethical Considerations

The experiment and animal management protocols were compliant with the requirements of the Institutional Animal Care and Use Committee of UPLB with approval number CVM-2019-008.

Statistical Analysis

Descriptive statistics was used to analyze the data using Statistical Analysis Software. The detection rate was computed by dividing the number of samples positive to *Salmonella* by the total number of samples positive to *Salmonella*. Data with p value < 0.05 were considered significant.

RESULTS AND DISCUSSION

Detection of *Salmonella* in Chickens and Environmental Samples

Culture method. The preliminary culture of field samples and cloacal swabs from day-old chicks collected at Days 0 (five days before stocking) and 1, respectively, was negative of *Salmonella* suggesting that the environment and starting flock were initially free of *Salmonella* contamination at the start of the experiment. Succeeding collections detected five putative *Salmonella* isolates from the samples based on their phenotypic characteristics in culture media, biochemical, and serological reactions. *Salmonella* was detected from the cloacal swabs at Day 10 and 150, then from field samples at Day 44 and Day 150. Introduction of *Salmonella* in the farm assumes different paths including amplification by common vectors such as birds, flies, or rodents that are attracted to the feeds and fecal waste of chickens. These vectors acquire ubiquitous *Salmonella* from their environment and spread the organism to another place through their droppings and contaminated body surfaces (Craven *et al.*, 2000; Lapuz *et al.*, 2007; Leibana *et al.*, 2003; Wales *et al.*, 2010). Assuming that infection of chickens occurred at Day 10, *Salmonella* could be shed through the feces causing contamination in the farm. The detection rate of *Salmonella* by culture method was 2.11% (5/237) obtained from three (1.67%)

cloacal swabs and two (10.52%) field samples.

PCR Detection. A detection rate of 8.04% (16/199) was obtained by PCR (Fig. 1) from cloacal swabs at Day 30 and 150. This finding correlated with the detection of *Salmonella* by culture method confirming infection in chickens. PCR did not detect *Salmonella* from field samples corresponding to the results of other studies. Such finding could be due to various reasons. One is the presence of polysaccharides, phenolic, and metabolic compounds from environmental samples which are inhibitory to polymerase reaction (Farzan *et al.*, 2007; Schrank *et al.*, 2001). Secondly, the likelihood that persistent infection is present among the study flock. Persistent infection is a hallmark of salmonellosis characterized by intermittent shedding of very few bacteria (Kranker *et al.*, 2003) that could be missed during sampling.

Analysis of feed and water samples yielded negative results indicating absence of *Salmonella* from these samples throughout the entire duration of the study. Heat treatment during feed production and the inclusion of phytic acid in feeds are two factors that cause severe injury in *Salmonella* (Bohn *et al.*, 2008). When cultured, it will be challenging to revive the stressed cells even with use of enrichment media (Andrews, 1986). Clearance of water samples from *Salmonella* could be due to the chlorination of water sources supplying the farm. The standard range of residual chlorine (0.30–1.5 ppm) in treated water is known to suppress growth of many microorganisms (Magtibay *et al.*, 2015).

The detection of *Salmonella* in different samples by culture and PCR is summarized in Table 1. The overall detection rate derived from the combined methods was 8.9% (21/237). PCR was expected to have a higher detection rate because of its high sensitivity in amplifying the *inv*A genes (Rahn *et al.*, 1992).
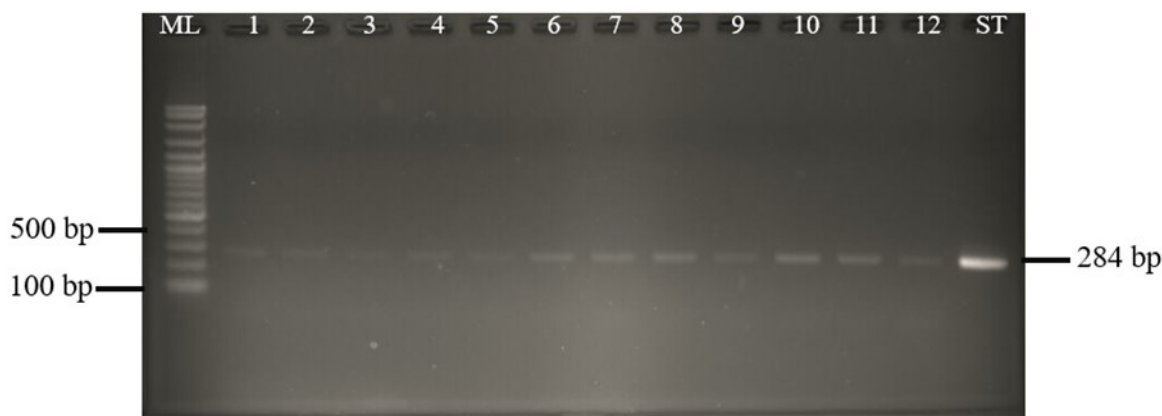

Figure 1. PCR amplicons obtained from cloacal swab samples at Day, Day 150. ML= molecular ladder, lanes 1-12 = cloacal swab samples, ST= *S. typhimurium*.

Table 1. Detection of *Salmonella* from environment and cloacal swab samples.

No.	Type of samples collected	Total no. of samples	*Salmonella* positive samples	
			Culture	PCR
1	Boot sock	19	2	0
2	Feeds	19	0	-
3	Water	19	0	-
4	Cloacal swabs	180	3	16
Total		237	2.11% (5/237)	8.04% (16/199)

Furthermore, an intermittent pattern of detection was observed in the samples at different sampling periods (Figure 2). Under this pattern, there were times when *Salmonella* was detected by culture method, other times by PCR assay in either sample. A particular bird could be positive in one sampling and negative in the subsequent samplings. Consistent infection, however, was observed in one bird (TB740) where *Salmonella* was detected in the cloacal swabs at Day 10 and at Day 150. This bird is believed to be one of the promoters of persistent infection among the flock. Such intermittent pattern has been an intriguing characteristic of persistent *Salmonella* infection allowing its maintenance in the host population (Gast and Holt, 1998). The same mechanism is thought to advance infection into the carrier state with the bacteria staying in low numbers for the bird's lifetime (Wigley, 2014).

To the best of the authors' knowledge, this study is the first to report detection of *Salmonella* spp. in free-range Banaba x Paroakan Philippine native chickens raised in a controlled farm environment. The detection rate (8.9%), however, is lower than the 12.7% detection rate in free-range broiler chickens in China (Zhao *et al.*, 2016) and 25% in conventional native chickens in South Korea (Park *et al.*, 2017) The variation could be attributed to the differences in the management practices and geographic location of the free-ranging farms (Corrêa *et al.*, 2018).
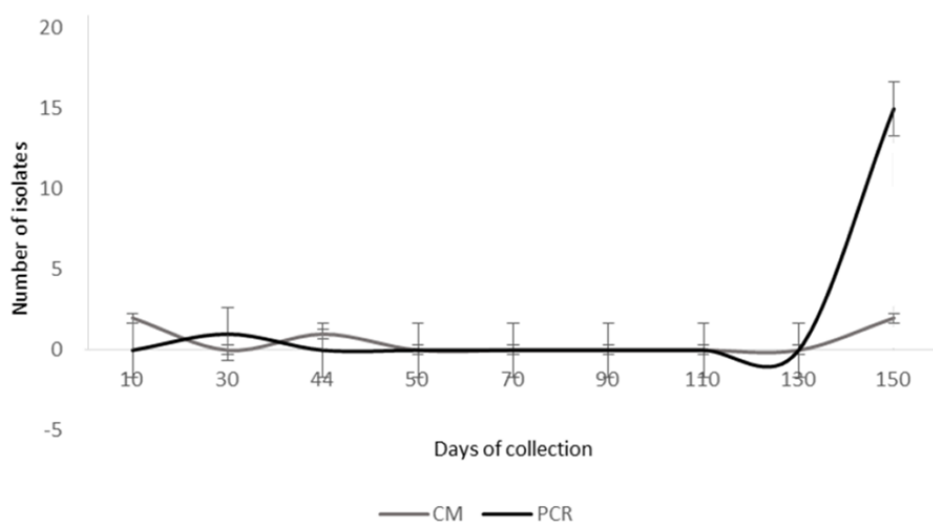

Figure 2. Intermittent detection of *Salmonella* from environment and cloacal swab samples of free-range native chickens by culture method (CM) and PCR.

Antibiotic Susceptibility Patterns of *Salmonella* Isolates

Figure 3 relates the susceptibility and resistance patterns of *Salmonella* isolates to 14 antimicrobials. Eighty percent of the isolates were susceptible to amoxicillin-clavulanic acid and 60% to norfloxacin, cefaclor, and chloramphenicol. The responsiveness of the organisms to these antibiotics implies that they can be used to control salmonellosis in free-range native chickens although resistance level should be taken into consideration. Intermediate susceptibility was demonstrated by 80% of the isolates to ciprofloxacin and gentamicin implying that they can be used to eliminate these isolates if they are present in body compartments (e.g., urinary tract) accessible to the antibiotic (Rodloff *et al.*, 2008) or if the dosing regimen or concentration is adjusted at the site of infection (EUCAST, 2021). Sensitivity of *Salmonella* to amoxicillin-clavulanic acid, tetracycline (Thung *et al.*, 2016) gentamicin (Abunna *et al.*, 2017; Balala *et al.*, 2006; Thung *et al.*, 2016), ciprofloxacin (Abunna *et al.*, 2017), and norfloxacin (Balala *et al.*, 2006) was reported in various studies. On the contrary, studies by Samanta *et al.* (2014) reported that *Salmonella* isolates from backyard chickens in West Bengal, India, were highly resistant to chloramphenicol ciprofloxacin, gentamicin, and norfloxacin.
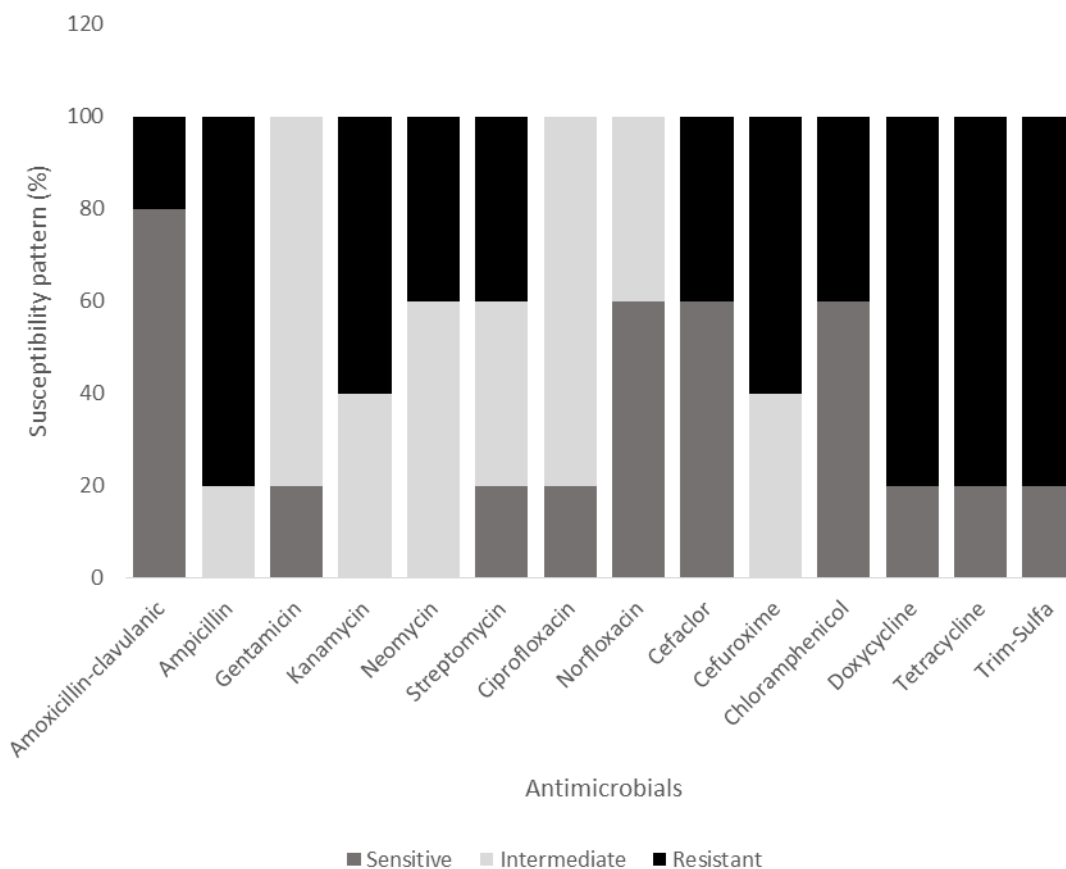
Figure 3. Antimicrobial susceptibility profile of *Salmonella* isolates from Philippine native chickens.

Antibiotic resistance was 80% to ampicillin, doxycycline, tetracycline, and trimethoprim-sulfamethoxazole; 60% to kanamycin and cefuroxime; and 40% to neomycin. This profile is common and has been described in several studies. In the Philippines, *Salmonella* resistant to ampicillin, tetracycline, and trimethoprim-sulfamethoxazole were recovered from poultry and poultry products (Balala *et al.*, 2006; Bautista and Mendoza, 2016; Elumba *et al.*, 2018). Resistance to tetracycline, kanamycin (Abunna *et al.*, 2017), and ampicillin (Castro-Vargas *et al.*, 2020; El-Sharkawy *et al.*, 2017; Zhao *et al.*, 2016) by *Salmonella* isolated from free-range and broiler chickens was also reported in previous studies conducted abroad. This would indicate the wide range of antimicrobial resistant salmonellae from poultry that could be associated with the broad use and misuse of antibiotics in production (Mehdi *et al.*, 2018). The location, differences in production system and antibiotic usage, and distribution of the antibiotic genes in the environment (soil and water) are believed to be responsible for the variation of resistance patterns across different countries (Karabasanavar *et al.*, 2020 Samanta *et al.*, 2014; Zhao *et al.*, 2016).

Resistance to quinolones (ciprofloxacin and norfloxacin) and gentamicin was not observed in the study confirming results of other works (Khan *et al.*, 2015; Karabasanavar *et al.*, 2020; Thung *et al.*, 2016). Ciprofloxacin and norfloxacin are members of the quinolone group which have broad-spectrum activity against Gram-positive and Gram-negative bacteria (Pham *et al.*, 2019). Quinolones enter the bacteria through porins and disrupt the topoisomerase IV and DNA complex, inhibiting bacterial nucleic acid synthesis (Castro-Vargas *et al.*, 2020; Pham *et al.*, 2019). When resistance developed against the first-line agents such as ampicillin, chloramphenicol, and trimethoprim-sulfamethoxazole, quinolones were considered as one of the drugs of choice for treating salmonellosis (typhoid and paratyphoid) in humans (Pham *et al.*, 2019; WHO, 2019). Ciprofloxacin is also the drug of choice for salmonellosis of poultry origin (Karabasanavar *et al.*, 2020). Recently, however, resistance to these antibiotics has been demonstrated by *Salmonella* (Castro-Vargas *et al.*, 2020; Pham *et al.*, 2019; Samanta *et al.*, 2014).

Aminoglycosides like gentamicin are broad-spectrum antibiotics found to be effective against serious bacterial pathogens but they do not have a significant bactericide effect on intracellular bacteria mostly due to their reduced permeability through the eukaryotic cell membrane (Menashe *et al.*, 2008) However, in vitro results showed efficacy of gentamicin and amikacin against MDR *S. enterica* serovar Typhi infection at very low concentrations (2 µg/ml) after 6 h incubation (Mandal *et al.*, 2009). In 1989, the MDR *S. enterica* serovar Typhi responsible for the enteric fever epidemic in Kolkata, India showed 100% sensitivity to gentamicin with subsequent 60% clinical response of patients receiving gentamicin treatment (Anand *et al.*, 1990). It would appear that instead of binding to the bacterial ribosome and interfering with protein synthesis (Magnet and Blanchard, 2005) gentamicin affects intracellular pathogen in other ways unelucidated.

The free-range flock used in the study was provided an antibiotic-free diet and management.

But despite this fact, multidrug-resistance (MDR) was exhibited by 80% (4/5) of the *Salmonella* isolates (Table 2). Specifically, isolates from cloacal swabs were resistant to eight antibiotics (AMCECCXMDOKNSXTTE) belonging to five classes of antimicrobials while a field sample isolate was MDR to five antibiotics (AMCDOSSXTTE) belonging to four classes of antimicrobials. The MAR indices of the MDR isolates ranged from 0.43-0.57 indicative of the exposure of the isolates to several antibiotics. *Salmonella* isolates with a high MAR index (>0.20) constitute a high-risk source of contamination (Krumperman, 1983) and therefore implies a human health concern. Also isolated was the low risk (MAR index = 0.14) environmental isolate which exhibited resistance to two antibiotics (AMCCEC) from two classes of antimicrobials. Moreover, between chicken and the environmental isolates, the former demonstrated greater resistance rates (71%) than the latter (57%).

Table 2. Antibiotic resistance profile and MAR indices of *Salmonella* isolates obtained from cloacal swab of native chickens and environmental samples.

Isolate No.	Source	Antibiotic Resistance Profile	MAR Index
1	Cloacal swab	AMCECCXMDOKNSXTTE	0.57
2	Cloacal swab	AMCECCXMDOKNSXTTE	0.57
3	Cloacal swab	AMCDOKSSXTTE	0.50
4	Boot sock	AMCCEC	0.14
5	Boot sock	AMCDOSSXTTE	0.43

AM-Ampicillin; AMC- Amoxicillin-clavulanic acid; C-Chloramphenicol; CEC-Cefaclor; CXM-Cefuroxime; DO-Doxycycline; K-Kanamycin; N-Neomycin; S-Streptomycin; SXT-Trimethoprim-sulfamethoxazole; TE-Tetracycline

H:L Ratio

After the detection of *Salmonella* at Day 10, a gradual increase in heterophils was recorded throughout the observation periods, although a significant difference was only observed at Day 150 (p-value=0.010). This could indicate that the infection at Day 10 had initiated an acute inflammation that triggered an enhanced production of heterophils by the innate immune system to eliminate pathogens. Like their mammalian counterpart neutrophils, heterophils are the first to proliferate in the circulation and arrive at the site of infection to engulf and destroy pathogens (Jain, 1993; Maxwell and Robertson, 2005). Conversely, a descending trend was observed in lymphocytes since Day 10 with significant decrease at Day 150 (p-value=0.015). The relative decrease could be due to redistribution of lymphocytes from the blood into

secondary lymphoid organs where they are sequestered and activated to produce antibodies (Dhabhar, 2002; Chung *et al.*, 1986). It appears in the study that the H:L ratio pattern was the inverse relationship between heterophil and lymphocyte. This heterophilia-lymphopenia profile corroborated the findings of other workers who evaluated stress response in poultry due to *Salmonella* infection (Gross and Siegel, 1983; Al-Murrani *et al.*, 2002). It is indicative of the inverse effect of infection in the numbers of heterophils and lymphocytes defining the composite measure of stress response caused by different physiological stressors (Davis *et al.*, 2008).

Figures 4 shows the progressive increase of the H:L ratio towards Day 150 with slight inversion at Day 120. The H:L ratio was significantly highest during Day 150
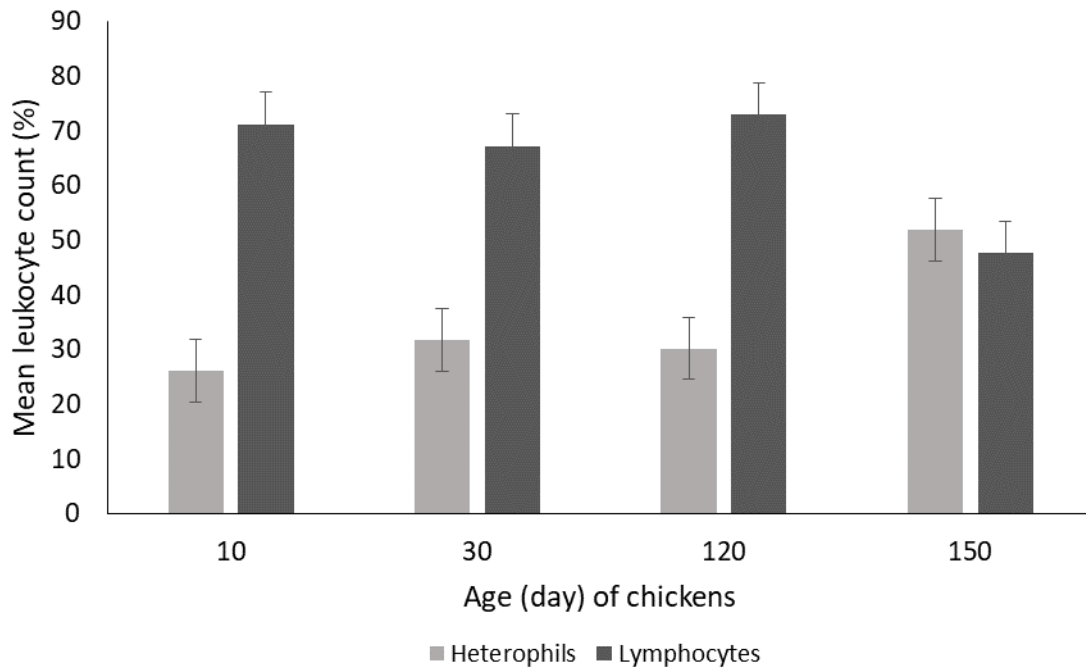
Figure 4. Increasing proportions of the heterophils and lymphocytes in free-range native chickens at Day 10, 30, 120, and 150

(p-value=0.005) coinciding with the highest detection rate of *Salmonella* in native chickens. This increase clearly indicated a stressful state as more chickens tested positive to *Salmonella* infection at Day 150.

Moreover, at Day 150 when the female native chickens were expected to reach sexual maturity, the H:L ratio between female and male chickens was compared to determine if sex is a contributor of stress in infected chickens. Table 3 shows that the mean H:L ratio in female chickens was higher than that of the male, however, differences were not statistically significant (p-value=0.133). The results suggest that all of the *Salmonella*-positive chickens, whether male or female, had similar burden of stress due to the infection. This finding contradicts the study of Wigley *et al.* (2005), which reported that female native chickens were more predisposed to stress burden associated with the recrudescence of infection during reproductive maturity as puberty hormone lowers the resistance to diseases. Videla *et al.* (2020), on the other hand, found out that male chickens had higher H:L ratio than female

and were more susceptible to the harmful pathogens because of the increasing concentration of stress response mediators in these animals.

It is inferred that the native chickens acquired MDR salmonellae from the environment as may be introduced by loitering vectors such as bird feeders, flies, and rodents. The findings of the study implicate the free-range native chickens as potential reservoir for MDR *Salmonella* that can be excreted into the environment and reach human food chain. *Salmonella* infection induces immune stress as bacterial invasion and colonization in the intestines trigger an inflammatory response. Altogether, physiological stressors produce adverse impact on the birds by negatively affecting energy usage and feed intake resulting to reduced overall performance (Liu *et al.*, 2014; Gomes *et al.*, 2014). Both issues on antimicrobial resistance and stress response warrant closer attention that should be addressed for the free-range chicken production system.

Table 3. Mean H:L ratio of female and male free-range native chickens taken at Day 150.

| Sex | Number | Mean %± SE of H and L | | H:L ratio |
		H	L	(Mean±SE)
Female	7	51.43 ±3.38	46.43±3.52	1.17±0.15
Male	8	43.38±3.75	55.00±3.83	0.85±0.13

SE- Standard error; H-heterophils; L-lymphocytes

Free-range native chickens can harbor persistent MDR *Salmonella* that make them a public health threat. A biosecurity breach allows entry of the pathogen into the farm via amplifier vectors that also facilitate spread of ARGs in the environment. The findings highlighted the importance of tighten biosecurity measures designed for free-range native chicken production. In developing a range farm, complexity of the soil microbial structure, and presence of resistance genes should be taken into consideration. Innovative ways to prime the bird's resistance against enteric pathogens should also be introduced. Although, *Salmonella* positivity was established in this study, it is recommended that a comprehensive analysis of bacterial contamination should be carried out within the farm source of chicks including farm premise, parent stock, and common vectors that frequented the farm. Isolation of *Salmonella* from internal tissues of day-old-chicks is deemed important to increase certainty of starting with a *Salmonella*-free flock. It is also essential to serotype *Salmonella* isolates for future tracing and epidemiological studies.

ACKNOWLEDGMENT

STATEMENT ON CONFLICT OF INTEREST

The authors have no competing interests to declare.

AUTHOR'S CONTRIBUTION

BCM and LRB conceptualized and designed the research. LMB drafted the manuscript, analyzed the samples and collected the data. MPB and JSM conceptualized the methodology and interpreted the data.

REFERENCES

Abunna SF, Bedasa M, Beyene T, Ayana D, Mamo B and Duguma R. 2017. *Salmonella*: Isolation and antimicrobial susceptibility tests on isolates collected from poultry farms in and around Modjo, Central Oromia, and Ethiopia. *Journal of Animal and Poultry Sciences* 5(2): 21-35.

Al-Murrani WK, Al-Rawi IK and Raof NM. 2002. Genetic resistance to *Salmonella typhimurium* in two lines of chickens selected as resistant and sensitive on the basis of heterophil/lymphocyte ratio. *British Poultry Science* 43:4 501-507.

Anand AC, Kataria VK, Singh W and Chatterjee SK. 1990. Epidemic multiresistant enteric fever in Eastern India. *The Lancet* 335 (8685): 352.

Andrews WH. 1986. Resuscitation of injured *Salmonella* spp. and coliforms from foods. *Journal of Food Protection* 49(1): 62-75.

Bailey JS and Cosby DE. 2005. *Salmonella* prevalence in free-range and certified organic chickens. *Journal of Food Protection* 68(11): 2451-2453.

Balala LM, Rovira HG, Vizmanos MFC, Bernardo FAEM and Divina BP. 2006. Isolation, identification and antibiotic sensitivity testing of *Salmonella* spp. in chickens. *Philippine Journal of Veterinary Medicine* 43(2):63-70.

Bautista VALM and Mendoza BC. 2016. Multiple drug resistance profile of *Salmonella enterica* subsp. *enterica* serovar Kentucky obtained from apparently healthy layer chickens in San Jose, Batangas, Philippines. *Philippine Journal of Veterinary Medicine 53(1): 17-25.*

Beshiru A, Igbinosa IH and Igbinosa EO. 2019. Prevalence of antimicrobial resistance and virulence gene elements of *Salmonella* serovars from ready-to-eat (RTE) shrimps. *Frontiers in Microbiology* 10: 1613.

Bohn L, Meyer AS and Rasmussen SK. 2008. Phytate: impact on environment and human nutrition. A challenge for molecular breeding. Journal of Zhejiang University *Science B* 9(3): 165-191.

Borges KA, Furian TQ, Souza SN, Salle CTP, Moraes HLS and Nascimento VP. 2019. Antimicrobial resistance and molecular characterization of *Salmonella enterica* serotypes isolated from poultry sources in Brazil. *Brazilian Journal of Poultry Science* 21(1): 1-8.

Castro-Vargas RE, Herrera-Sánchez MP, Rodríguez-Hernández R and Rondón-Barragán IS. 2020. Antibiotic resistance in *Salmonella* spp. isolated from poultry: A global overview.

Veterinary World 13(10): 2070-2084.

Center for Disease Control and Prevention (CDC). 2013. Antibiotic resistance threats in the United States. https://www.cdc.gov/drugresistance/pdf/ar-threats-2013-508.pdf. Accessed 15 January 2021.

Chung H, Samlowsk WE and Doyner RA. 1986 Modification of the immune system by glucocorticoids: alteration of the tissue localization properties of circulating lymphocytes. *Cell Immunology* 101: 571–585.

Clinical Laboratory Standards Institute (CLSI). 2018. Performance standards for antimicrobial susceptibility testing. 28th ed. CLSI supplement M100. Wayne, PA: Clinical and Laboratory Standards Institute.

Corrêa IMO, Pereira LQ, Silva IGO, Altarugio R, Smaniotto BD, Silva TM, Okamoto AS and Andreatti Filho RL. 2018. Comparison of three diagnostic methods for *Salmonella enterica serovars* detection in chicken rinse. *Pesquisa Veterinaria Brasileira* 38(7): 1300-1306.

Cosby DE, Cox NA, Harrison MA, Wilson JL, Buhr RJ and Fedorka-Cray PJ. 2015. *Salmonella* and antimicrobial resistance in broilers: A review. *Journal of Applied Poultry Research* 24(3): 408-426.

Cotter PF. 2015. Animal well-being and behavior: Are peripheral Mott cells an indication of stress or inefficient immunity? *Poultry Science* 94:1433–1438.

Craven SE, Stern NJ, Line E, Bailey JS, Cox NA and Fedorka-Cray P. 2000. Determination of the incidence of *Salmonella* spp., *Campylobacter jejuni*, and *Clostridium perfringens* in wild birds near broiler chicken houses by sampling intestinal droppings. *Avian Diseases* 44(3): 715-720.

Cui L, Liu Q, Jiang Z, Song Y, Yi, Qui J, Hao G and Sun S. 2021. Characteristics of *Salmonella* from Chinese native chicken breeds fed on conventional or antibiotic-free diets. *Frontiers of Veterinary Science* 8: 607491.

Davis AK, Maney DL and Maerz JC. 2008. The use of leukocyte profiles to measure stress in vertebrates: a review for ecologists. *Functional Ecology* 760-772.

Davis R and Brown PD. 2016. Multiple antibiotic resistance index, fitness and virulence potential in respiratory *Pseudomonas aeruginosa* from Jamaica. *Journal of*

Medical Microbiology 65: 261-271.

Dhabbar ES. 2002. A hassle a day may keep the doctor away: Stress and the augmentation of immune reaction. *Integrative and Comparative Biology* 42: 556-564.

El-Sharkawy H, Tahoun A, El-Gohary AEA, El-Abasy M, El-Khayat F, Gillespie T, Kitade Y, Hafez HM, Neubauer H and El-Adawy H. 2017. Epidemiological, molecular characterization and antibiotic resistance of *Salmonella enterica* serovars isolated from chicken farms in Egypt. *Gut Pathogens* 9: 8.

Elumba ZS, Allera MLM and Taganas RRR. 2018. Occurrence and antibiotic sensitivity of *Escherichia coli* and *Salmonella* spp. in retail chicken meat at selected markets in Valencia City, Bukidnon, Philippines. *Asian Journal of Biological Life Sciences* 7 (2): 53-58.

EUCAST (2021). New definitions of S, I and R from 2019. https://www.eucast.org/newsiandr/. Accessed 06 March 2021.

Farzan A, Friendship RM and Dewey CE. 2007. Evaluation of enzyme-linked immunosorbent assay (ELISA) tests and culture for determining *Salmonella* status of a pig herd. *Epidemiology of Infections* 135: 238-244.

Gast RK and Holt PS. 1998. Persistence of *Salmonella* enteritidis from one day of age until maturity in experimentally infected layer chickens sensitivity of young chickens to *Salmonella* typhimurium var. copenhagen and *S.* infantis infection and the preventive effect of cultured intestinal microflora. *Poultry Science* 77:1759-1762.

Gomes AVS, Quinteiro-Filho WM, Ribeiro A, Ferraz -De-Paula V, Pinheiro ML, Baskeville E, Akamine AT, Astolfi-Ferreira CS, Ferreira AJP and Palermo-Neto J. 2014. Overcrowding stress decreases macrophage activity and increases *Salmonella* Enteritidis invasion in broiler chickens. *Avian Pathology* 43: 82 -90.

Gross WB and Siegel HS. 1983. Evaluation of the heterophil/lymphocyte ratio as a measure of stress in chickens. *Avian Diseases* 27(4): 972-979.

International Standard. Microbiology of food and animal feeding stuffs — Horizontal method for the detection of *Salmonella* spp. 4th edition. Switzerland: ISO Office; 2002.

Jain NC. 1993. Essentials of veterinary hematology. Blackwell Publishing,

Philadelphia, PA.

Karabasanavar NS, Madhavaprasad CB, Gopalakrishna SA, Hiremath J, Patil GS and Barbuddhe SB. 2020. Prevalence of *Salmonella* serotypes *S.* Enteritidis and *S.* Typhimurium in poultry and poultry products. *Journal of Food Safety* 40(6).

Khan JA, Mir IA, Soni SS and Maherchandani S. 2015. Antibiogram and multiple antibiotic resistance index of *Salmonella enterica* isolates from poultry. Journal of Pure and *Applied Microbiology* 9(3).

Kranker S, Alban L, Boes J, Dahl J. 2003. Longitudinal study of *Salmonella* enterica serotype Typhimurium infection in three Danish farrow-to-finish swine herds. *Journal of Clinical Microbiology* 41 (6): 2282-2288.

Krumperman PH. 1983. Multiple antibiotic resistance indexing of *Escherichia coli* to identify high-risk sources of fecal contamination of foods. *Applied Environmental Microbiology* 46: 165–170.

Lapuz RRSP, Tani H, Sasai K, Shirota K, Katoh H and Baba E. 2007. An epidemiological analysis of *Salmonella* enteritidis contamination in a rat-infested chicken layer farm, an egg processing facility, and liquid egg samples by pulsed-field gel electrophoresis. *Journal of Veterinary Medical Science* 69(6): 649-652.

Learn-Han L, Yoke-Kqueen C, Shiran MS, Sabrina S, Noor Zaleha AS, Sim JH, Chai-Hoon K and Son R. 2009. Molecular characterization and antimicrobial resistance profiling of *Salmonella enterica* subsp. *enterica* isolated from 'Selom' (*Oenanthe stolonifera*). *International Food Research Journal* 16: 191-202.

Leibana E, Garci-Migura L, Clouting C, Clifton-Hadley FA, Breslin M and Davies RH. 2003. Molecular fingerprinting evidence of the contribution of wildlife vectors in the maintenance of *Salmonella* Enteritidis infection in layer farms. *Journal of Applied Microbiology* 94:1024-1029.

Liu L, Qin D, Wang X, Feng Y, Yang X and Yao J. 2014. Effect of immune stress on growth performance and energy metabolism in broiler chickens. *Food and Agricultural Immunology* 26:194-203.

Lucas AM, and Jamroz C. 1961. Atlas of avian hematology. US Department of Agriculture. Washington, DC, Agriculture Monograph 25.

Magnet S and Blanchard JS. 2005. Molecular insights into aminoglycoside action and resistance. *Chemical Reviews* 105(2): 477-498.

Magtibay B, Anarna MS and Fernando A. 2015. An assessment of drinking-water quality post-Haiyan. *Western Pacific Surveillance and Response Journal* 6(1): 48-52.

Mandal S, Manda MD and Pal NK. 2009. In vitro activity of gentamicin and amikacin against *Salmonella enterica* serovar Typhi: a search for a treatment regimen for typhoid fever. *Eastern Mediterranean Health Journal* 15(2): 264-268.

Maxwell M. 1993. Avian blood leucocyte responses to stress. *World's Poultry Science Journal* 49 (1): 34-43.

Maxwell MH and Robertson GW. 1998. The avian heterophil leucocyte: a review. *World's Poultry Science Journal* 54: 155-178.

McEachran AD, Blackwell BR, Hanson JD, Wooten KJ, Mayer GD, Cox SB and Smith PN. 2015. Antibiotics, bacteria, and antibiotic resistance genes: aerial transport from cattle feed yards via particulate matter. *Environmental Health Perspective* 123(4): 337.

Mehdi Y, Létourneau-Montminy M, Gaucher M, Chorfi Y, Suresh G, Rouissi T, Brar SK, Cote C, Ramirez AA and Godbout S. 2018. Use of antibiotics in broiler production: Global impacts and alternatives. *Animal Nutrition* 4(2): 170-178.

Menashe O, Kaganskaya E, Baasov T and Yaron S. 2008. Aminoglycosides affect intracellular *Salmonella enterica* serovars *typhimurium* and *virchow*. *Antimicrobial Agents and Chemotherapy* 52(3): 920-926.

Park JH, Kim HS, Yim JH, Kim YJ, Kim DH, Chon JW, Kim H, Om AS and Seo KH. 2017. Comparison of the isolation rates and characteristics of *Salmonella* isolated from antibiotic-free and conventional chicken meat samples. *Poultry Science* 96: 2831-2838.

Peters JB and Laboski CAM. 2013. Sampling soils for testing. https://lacrosse.extension.wisc.edu/files/2020/09/sampling-soils-for-farm-fields.pdf. Accessed 20 August 2022.

Pham T, Ziora ZM and Blaskovich M. 2019. Quinolone antibiotics. *MedChemComm* 10 (10): 1719-1739.

Pruden A, Larsson DG, Amézquita A, Collignon P, Brandt KK, Graham DW, Lazorchak JM, Suzuki S, Silley P, Snape JR, Topp E, Zhang T and Zhu YG. 2013. Management

options for reducing the release of antibiotics and antibiotic resistance genes to the environment. *Environmental Health Perspectives* 121(8): 878-885.

Rahn K, De Grandis SA, Clarke RC, McEwen SA, Galán JE, Ginocchio C, Curtiss R and Gyles CL. 1992. Amplification of an invA gene sequence of *Salmonella typhimurium* by polymerase chain reaction as a specific method of detection of *Salmonella*. *Molecular Cell Probes* 6(4): 271-279.

Rodloff A, Bauer T, Ewig S, Kujath P and Müller E. 2008. Susceptible, intermediate, and resistant - the intensity of antibiotic action. *Deutsches Arzteblatt International* 105(39): 657-662.

Samanta I, Joardar SN, Das PK, Sar SK, Bandyopadhyay S, Dutta TK and Sarkar U. 2014. Prevalence and antibiotic resistance profiles of *Salmonella* serotypes isolated from backyard poultry flocks in West Bengal, India. *Journal pf Applied Poultry Research* 23: 536-545.

Scallan E, Hoekstra RM, Angulo FJ, Tauxe RV, Widdowson MA, Roy SL, Jones JL and Griffin PM. 2011. Foodborne illness acquired in the United States—major pathogens. *Emerging Infectious Diseases* 17(1): 7-15.

Schrank IS, Mores MA, Costa JL, Frazzon AP, Soncini R, Schrank A, Vainstein MH and Silva SC. 2001. Influence of enrichment media and application of a PCR based method to detect *Salmonella* in poultry industry products and clinical samples. *Veterinary Microbiology* 82: 45-53.

Song J, Rensing C, Holm PE, Virta M and Brandt KK. 2017. Comparison of metals and tetracycline as selective agents for development of tetracycline resistant bacterial communities in agricultural soil. *Environmental Science and Technology* 51: 3040-3047.

Swaggerty Cl, Callaway TR, Kogut MH, Piva A and Grilli E. 2019. Modulation of the immune response to improve health and reduce foodborne pathogens in poultry. *Microorganisms* 7: 65.

Sweeney MT, Lubbers BV, Schwarz S and Watts JL. 2018. Applying definitions for multidrug resistance, extensive drug resistance and pandrug resistance to clinically significant livestock and companion animal bacterial pathogens. *Journal of Antimicrobial and Chemotherapy* 73: 1460-1463.

Thung TY, Mahyudin NA, Basri DF, Radzi WM, Nakaguchi Y, Nishibuchi M and Radu S.2016. Prevalence and antibiotic resistance of *Salmonella* Enteritidis and *Salmonella* Typhimurium in raw chicken meat at retail markets in Malaysia. *Poultry Science* 95(8): 1888-1893.

Videla EA, Giayetto O, Fernández ME, Chacana PA, Marín RH and Nazar FN. 2020. Immediate and transgenerational effects of thymol supplementation, inactivated *Salmonella* and chronic heat stress on representative immune variables of Japanese quail. *Scientific Reports* 10: 18152.

Wales AD, Carrique-Mas JJ, Rankin M, Bell B, Thind BB and Davies RH. 2010. Review of the carriage of zoonotic bacteria by arthropods, with special reference to *Salmonella* in mites, flies and litter beetles. *Zoonoses Public Health* 57(5): 299-314.

World Health Organization (WHO). 2019. Critically important antimicrobials for human medicine, 6th revision. Geneva: World Health Organization.

Wigley P. 2014. *Salmonella enterica* in the chicken: How it has helped our understanding of immunology in a non-biomedical model species. *Frontiers in Microbiology* 5: 482.

Wigley P, Hulme SC, Powers C, Beal RK, Berchieri A Jr., Smith A and Barrow P. 2005. Infection of the reproductive tract and eggs with *Salmonella enterica* serovar Pullorum in the chicken is associated with suppression of cellular immunity at sexual maturity. *Infection and Immunity* 73(5): 2986-2990.

Xiong W, Wang M, Dai J, Sun Y and Zeng Z. 2018. Application of manure containing tetracyclines slowed down the dissipation of tet resistance genes and caused changes in the composition of soil bacteria. *Ecotoxicology and Environmental Safety* 147: 455-660.

Zhao X, Gao Y, Ye C, Yang L, Wang T and Chang W. 2016. Prevalence and characteristics of *Salmonella* isolated from free-range chickens in Shandong province, China. *BioMed Research International* 8183931.