Asia-Pacific Journal of Science and Technology: Volume: 30. Issue: 01. Article ID.: APST-30-01-03. Research Article

APST

Asia-Pacific Journal of Science and Technology

https://www.tci-thaijo.org/index.php/APST/index

Published by the Research Department,
Khon Kaen University, Thailand

Detection of multidrug-resistant *Salmonella* in native chickens by culture, polymerase chain reaction, and enzyme linked immunosorbent assay

Lotis M. Balala[1,*], Bernadette C. Mendoza[2], Loinda R. Baldrias[3], and Joseph S. Masangkay[3]

[1]College of Veterinary Medicine, Visayas State University, Visca, Baybay City, Leyte, Philippines
[2]Institute of Biological Sciences, University of the Philippines Los Baños, College, Laguna, Philippines
[3]College of Veterinary Medicine, University of the Philippines Los Baños, College, Laguna, Philippines
[*]Corresponding author: balalalotis@vsu.edu.ph

Received 13 November 2023
Revised 26 March 2024
Accepted 31 May 2024

Abstract

A compromise in biosecurity measures is a major determinant of pathogen emergence in free-range animals. This study aimed to detect antimicrobial-resistant *Salmonella* in free-range Philippine native chickens. Clinical and environmental samples were collected from a free-range farm with 50 Banaba x Paraoakan native chickens to analyze *Salmonella* using conventional culture, polymerase chain reaction (PCR), and enzyme-linked immunosorbent assay (ELISA). Antimicrobial resistance was confirmed through the agar disc diffusion method. *Salmonella* persisted in poultry samples from Day 10 to Day 150 with an overall detection rate of 8.9% (21/237). The earliest detection of *Salmonella* was on Day 10 by conventional culture and on Day 30 by PCR posing a detection rate of 2.11% (5/237) and 8.04% (16/199), respectively. ELISA detected seropositivity on Day 120 with an overall seroconversion rate of 60%. Antibiotic resistance of isolates was 80% in ampicillin, doxycycline, tetracycline, and trimethoprim-sulfamethoxazole; 60% in kanamycin and cefuroxime; and 40% in neomycin. Eighty percent (4/5) of the isolates demonstrated a potential multidrug resistance pattern. Multidrug resistant *Salmonella* persisted in free-range native chickens posing food quality and safety implications. Biosecurity and preharvest strategies should be improved to reduce the pathogen in free-range farming.

Keywords: Culture, ELISA, IgG, MDR, Native chickens, PCR, *Salmonella*

I. Introduction

In recent years, interest among poultry producers to engage in free-range production of native chickens has been expanding because of welfare concerns, less use of chemical products, and high-quality eggs and desirable meat flavor. With increasing consumers' preference for organic foods, this commodity has built up progressive market niches complementing commercial poultry. However, the growth of free-range production is marred by food safety concerns due to environmental issues involving foodborne pathogens. The range environment is a potential reservoir of various disease-causing agents from multiple external sources [1]. Among these are microbiological and parasitic pathogens that challenge the sustainability of free-range production systems and therefore should be given attention from the economic perspective. *Salmonella* is one of the major pathogens acquired from exposure to outdoor areas [2]. It is a perennial pathogen most difficult to control in poultry farms. With over 2600 serotypes, this intracellular, Gram-negative, motile bacterium survives broad environmental conditions [3]. It has become a significant concern prompting the imposition of stringent measures along different phases of the food production chain.

The persistence of *Salmonella* in its host has been a perpetual problem on farms due to the bacteria's ability to be transmitted vertically and horizontally. Accurate diagnosis is obliterated by the difficulty in detecting the pathogen because of the asymptomatic state of infected poultry and the intermittent shedding of the bacteria. The prevalence of *Salmonella* infection and environmental contamination in poultry farms have been diverse and sometimes contradictory depending on geographic state and managemental variations. Reports of multidrug-resistant *Salmonella* carriage are rising in free-range chickens due to limited sanitation and the impact of environmental

microbiota [4]. It underscores public health importance requiring surveillance and radical measures to ensure food safety. Three detection methods were combined to establish infection with antimicrobial-resistant *Salmonella* in free-range chickens. This undertaking has not been conducted in Philippine native chickens recently. Generated information is vital in refining farm practices in free-range production systems and strengthening quality control for free-range derived animal products.

2. Materials and methods

2.1 Study site and experimental design

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) located at Barangay Putho Tuntungin, Los Baños, Laguna, Philippines. The proximity of the farm, availability of the facility, and presence of a ranging area were among the considerations made in selecting the site.

A longitudinal study (150 days) was designed to detect and establish the persistence of *Salmonella* in a flock consisting of 50 one-day-old Banaba x Paroakan native chickens. Sample collection was done on Days 10, 30, 120, and 150. Initially, chicks were raised in a standard constructed pen with at least 0.5 ft^2/chick spaces and brooded using a 50-watt incandescent bulb. A clean sheet of paper was used as bedding material and changed daily. During the daytime, the chicks were provided at least eight hours of access to an outdoor range secured with a fence. At 3-4 weeks, the chickens were allowed extended range time during the day and only kept in a pen at night. The chickens were given *ad libitum* water and commercial feeds formulated for the different developmental stages of the native chickens. Medications were not administered, only during inclement weather that vitamins were supplemented via drinking water. Perimeter fence, foot bath, human and animal entry control, and regular disinfection were in place to ensure biosecurity.

Before commencing the study, a preliminary examination for *Salmonella* contamination in the flock and environment was carried out. Soil testing using boot sock sampling was conducted five days before loading and on the day the chicks were loaded on the farm. Feeds, water, and cloacal swab samples from day-old chicks were likewise examined on Day 1 of the experiment. *Salmonella* detection was performed using culture and polymerase chain reaction (PCR) methods at the Molecular Biology Laboratory of the College of Veterinary Medicine, UPLB, College, Laguna.

2.2 Collection and processing of samples

Clinical and environmental samples were collected bi-weekly for 150 days. Soil samples were obtained by walking through a "W" pattern on the farm while wearing a pair of blue overshoes over boots, followed by a pair of boot socks. Subsequently, the boot socks were removed, placed inside an air-free Ziploc bag, and brought to the lab. A 100 mL of sterile phosphate-buffered saline (PBS) was then added to the bag to disperse soil adhering to the socks and left to stand for 10 min. Subsequently, 1 mL of the sample was used for bacterial culture.

Feeds (25 g) and water (20 mL) were collected from the feeding and drinking trough of the chickens and placed in sterile containers. The samples were pre-enriched in buffered peptone water (BPW, Thermo Fisher, Singapore) at 1:10 dilution and incubated at 37ºC for 18 to 24 h. One mL of the pre-enriched culture was then used for bacterial isolation.

Cloacal swabs were obtained while securely restraining the chickens. While pressing the chicken gently against the chest, a commercial swab embedded in transport media was inserted into the cloaca and rotated multiple times. The swabs were brought to the lab in ice-chilled containers. Each swab was directly inoculated in Brilliant Green agar (Oxoid, UK) and Xylose Lysine Deoxycholate agar (Oxoid, UK), and the tip was subsequently cut off and deposited into Rappaport Vassiliadis (HiMedia, USA) broth for enrichment and isolation.

2.3 Bacteriological isolation and confirmation of Salmonella spp.

Within the duration of the study, 180 cloacal swabs, 19 boot socks, 19 feeds, and 19 water samples were analyzed for bacteriological isolation. Previously pre-enriched samples were subsequently used for bacterial isolation using the ISO-6579:2002 standard for *Salmonella* detection with minor modifications [5]. Candidate colonies displaying typical phenotypic characteristics of *Salmonella* were sub-cultured multiple times until purified. Putative isolates were confirmed through growth and biochemical reactions in Mac Conkey agar (BD BBL, USA), Triple Sugar Iron agar (HiMedia, USA), Lysine Decarboxylase broth (HiMedia, USA), and Urea broth (HiMedia, USA). Further confirmation was done through a slide agglutination test using polyvalent O *Salmonella* antisera (Pro Lab Diagnostic Inc., Canada). The results of each test were compared to the phenotypic reactions of *Salmonella* Abony (positive control) in the same tests.

2.4 DNA extraction and PCR amplification

Whole-cell DNA extraction was prepared using QIAamp Fast DNA Stool Mini Kit (Qiagen®, USA) as described in the manufacturer's protocol. PCR amplifications were prepared from constituting a 25 µl PCR mixture containing 12.5 µl of GoTaq® Green Master Mix (Promega, USA), 0.25 µl each of the invA primer (10 µM), 10 µl of nuclease-free water, and 2 µl of the DNA template. Primers were specific to the *invA* gene with sequences such as 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 1 minute for denaturation, 53°C for 2 minutes for annealing, 72°C for 3 minutes for extension, and another 72°C for 7 minutes for a final extension [6]. DNA yield was quantified using NanoDropTM (Thermo Fisher Scientific Inc., USA) and visualized in a UV transilluminator (Vilber, Marne La Vallee, Ile-de-France, France). Samples were considered *Salmonella*-positive upon the appearance of a 284 bp band reflecting the *invA* gene.

2.5 ELISA detection of anti-Salmonella IgG

The ELISA method described by Beal et al. [7] and Wigley et al. [8] was followed with some modifications. Briefly, blood samples were collected from the hearts of five chickens on Day 10, 30, 120, and 15 chickens on Day 150. After clotting for 1 hour, the blood was centrifuged at 1,000 rpm for 5-10 minutes to separate serum. Using a microtiter plate, 100 µL of soluble *Salmonella* antigen was added into the wells and incubated overnight at 4°C. The liquid was then pipetted out from the wells, followed by adding 300 µL of washing buffer into each well. The washing buffer was removed, and the washing step was repeated twice. All the liquid was entirely removed by blotting the plate dry on folded tissue paper. Subsequently, 100 µL of blocking buffer was added to each well and incubated in the dark for 1 hour at 37°C. After this, the wells were washed once with washing buffer and dried. About 100 µL of diluted chicken serum sample was added to each well and incubated for 1 hour at 37°C. Positive, negative, and blank samples were run simultaneously on each plate.

After incubation, the wells were washed with buffer three times, followed by the addition of alkaline phosphatase-conjugated to goat anti-chicken IgG (Sigma-Aldrich, Germany). The plates were then incubated for 1 hour at 37°C and washed five times with washing buffer afterward. Subsequently, 100 µL of p-nitrophenyl phosphate was added to each well and incubated in the dark for 30 minutes at room temperature. The reaction was stopped by the addition of 100 µL 3N sodium hydroxide. The absorbance of each well was measured using a microplate reader (Multiskan™ GO Microplate Spectrophotometer, Thermo Fischer Scientific Inc., Portugal) at a wavelength of 405 nm. The correct OD reading of the sample was calculated by subtracting the mean of blank readings from the OD readings of each well. In this study, the calculated cut-off value for a positive serum was 2.957.

2.6 AMR characterization of Salmonella isolates

Antimicrobial susceptibility testing of the isolates was based on Kirby-Bauer disc diffusion method [9] using commercial antibiotic discs (BD BBL, USA) including 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) [10]. A 0.5 McFarland turbidity standard bacterial suspension was prepared and inoculated into Mueller Hinton Agar. Antimicrobial disks were then embedded onto the agar and incubated at 37ºC for 16 to 18 hours. The zone of inhibition (mm) was measured and interpreted based on the CLSI guidelines [9]. Resistance of the isolates to at least one agent in at least three antimicrobial classes was used as the basis for establishing multidrug resistance [11].

2.7 Statistical analyses

Descriptive statistics were used to analyze the data using Statistical Analysis Software. The detection rate was computed by dividing the number of positive samples by the total number of samples tested for *Salmonella* multiplied by 100. Seroconversion was determined by dividing the number of blood samples with positive antibody titer over the total number of blood samples analyzed multiplied by 100. Cochran Q test was used to analyze significant differences in the detection rates of the three methods and Mc Nemar's test to measure the degree of agreement between the culture- and PCR-based methods and between culture and ELISA in detecting *Salmonella* spp. All data with p value < 0.05 were considered significant.

3. Results and discussion

Preliminary culture and PCR assay of soil samples and cloacal swabs from day-old chicks collected at Days 0 (five days before loading) and 1 (day of loading), respectively, turned out negative for *Salmonella*, suggesting that the environment and starting flock were initially free of *Salmonella* contamination. The earliest detection of *Salmonella* was on Day 10 by conventional culture and on Day 30 by PCR, with detection rates of 2.11% (5/237) and 8.04% (16/199), respectively (Table 1). ELISA detected seropositivity at Day 120 with an overall seroconversion rate of 60%.

Table 1 Detection and seroconversion rate (%) of *Salmonella* from environmental and chicken samples.

Types of samples collected	Number of samples tested (N=237)	Number of *Salmonella*-positive samples		Number of samples with positive antibody titer by ELISA
		Culture	PCR	
Boot sock	19	2	0	-
Feeds	19	0	-	-
Water	19	0	-	-
Cloacal swabs	180	3	16	-
Blood	30	-	-	18
Detection/seroconversion rate (%)		2.11 (5/237)	8.04 (16/199)	60 (18/30)

- Not tested

Figure 1 outlines the detection of *Salmonella* and seroconversion by the three methods at different sampling points within the 150-day observation period. The detection rates were mainly variable, and only on Day 150 did the three methods simultaneously confirm the presence of *Salmonella* in the samples. The data highlights the importance of using appropriate diagnostic tools in demonstrating the elusive *Salmonella*. Bacterial culture is the gold standard for *Salmonella* diagnosis [12, 13]. Still, the intermittence of shedding the bacteria and the asymptomatic state of infection require strategic and coordinated field-based diagnosis for free-range production. The intermittence of excretion was evident when *Salmonella* was detected in some chickens at a particular sampling time, but it was absent at subsequent samplings with the same chickens. *Salmonella* was sometimes present in the cloacal samples of some chickens but was lacking in the environmental samples; at other times, it was detected in the environmental samples but not in the cloacal swabs of the chickens. Specific anti-*Salmonella* antibodies were detected only in the latter part of the experiment, further confirming infection. With the bacteria at different sampling points, the use of various diagnostic methods was essential in establishing the persistence of *Salmonella* in the flock and environment. Understanding the biology of shedding and timing of detection are deemed critical to diagnosis as non-detection at one point could lead to inaction of the problem at hand.

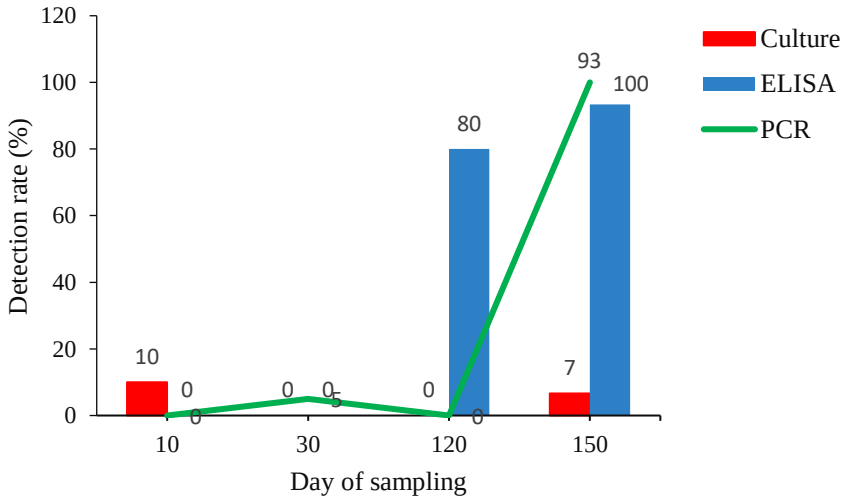

Figure 1 Detection and seroconversion rates (%) of *Salmonella* from samples during the 150-day study.

The detection of *Salmonella* on Day 10 marked the initial stage of infection, originating from a few birds that became the source of environmental contamination. This was later confirmed by detecting *Salmonella* in the soil on Days 44 and 150. Although PCR could be more sensitive than the culture method, non-detection may be due to the shedding of very few cells characteristic of persistent infection and the presence of substances inhibitory to PCR from clinical and environmental samples [14]. It is imminent for seroconversion among infected birds to occur at the latter stage as the activation and stimulation process of the immune system takes time to develop.

Cochran's Q test measured the differences in the detection rates of the three methods and revealed significant differences (p=0.000, X2=24.400) in the proportion of chickens positive for *Salmonella* (Table 2). Moreover, the three tests simultaneously detected *Salmonella* at Day 150, with the highest detection rate demonstrated by PCR (100%), followed by ELISA (86.68%) and conventional culture method (10.53%). A pairwise comparison test

Table 2 Results of Cochran's Q test in determining significant differences in the *Salmonella* detection rates in free-range native chickens using conventional culture, polymerase chain reaction (PCR) and enzyme-linked immunosorbent assay (ELISA).

Tests	N	Mean	Standard deviation	Minimum	Maximum	Cochran's Q test	*p*-value
Conventional culture (CS)	15	0.07	0.258	0	1	24.400	0.000
PCR (CS)	15	1.00	0.000	1	1		
ELISA	15	0.93	0.258	0	1		

Significant p value < 0.05
N – sample size, CS – cloacal swab

was used to determine the efficacy and agreement of conventional culture, PCR, and ELISA in detecting *Salmonella* during this stage. The McNemar test indicated significant differences in the detection rates between the conventional culture and PCR (p = 0.000) and between conventional culture and ELISA (p =0.001). However, there were no significant differences in the detection rates between PCR and ELISA (p =1.000), indicating strong agreement between these two methods (Table 3).

Table 3 Pairwise comparisons of the efficacy of *Salmonella* detection rates in free-range native chickens on Day 150 using conventional culture, polymerase chain reaction (PCR) and enzyme-linked immunosorbent assay (ELISA).

Pairs of detection methods compared	Culture (CS) and PCR (CS)	Culture (CS) and ELISA	PCR (CS) and ELISA
N	15	15	15
Exact Sig. (2-tailed)	.000[a]	.001[a]	1.000[a]

[a]Binomial distribution used. N – sample size, CS – cloacal swab

The lowest detection rate by the conventional culture method concurred with previous studies [15, 16]. The apparent advantage of PCR is due to the specificity of its target DNA which can be detected in either dead or live organisms. Conversely, ELISA is a highly sensitive assay that detects specific antibodies once an infection is established. Bacterial culture, however, depends on the resuscitation of injured cells after enrichment and the interaction of *Salmonella* with many competitive organisms [17]. Under stressful conditions, several microorganisms enter starvation mode of metabolism, making them viable but nonculturable in solid media.

Although flock infection may have been established as early as Day 10, detection by any method was not always attainable due to the intermittent excretion of *Salmonella* in the feces. This intermittence primarily affected the timing of isolating the bacteria failing to isolate the organism during sampling. It should also be noted that intermittently shed serovars, Pullorum, and Gallinarum, are usually excreted in low numbers and may not be picked up during collection [8]. This would suggest that a negative culture does not always imply the absence of *Salmonella* from the flock. Intermittent shedding also makes results of bacteriology and serology challenging to compare since birds may not be shedding *Salmonella* during sampling but have detectable IgG due to an earlier infection. This makes ELISA an advantageous test since it is not dependent on whether the bird is shedding the bacteria at the time of sampling.

The detection of *Salmonella* at varying ages using different methods became a valuable tool in assessing the persistent and intermittent nature of shedding the bacteria through the feces and developing seroconversion against the infection. Several studies have described these characteristics and helped design control strategies against *Salmonella* in free-range chickens. It led investigators to evaluate the possible factors responsible for introducing *Salmonella* on the farm at a specific period, consider the persistent nature of *Salmonella* in proposing effective means to boost gut immunity, and find ways to achieve a substantial biosecurity measure.

The antimicrobial resistance pattern of the isolates to the 14 antimicrobials is summarized in Table 4. Isolates were 80% susceptible to amoxicillin-clavulanic acid and 60 % to norfloxacin, cefaclor, and chloramphenicol. Intermediate susceptibility was exhibited to ciprofloxacin (80%), gentamicin (80%), and neomycin (60%). The response suggests these antibiotics can treat *Salmonella* infection in free-range native chickens. Adjustment of dosing concentration at the site of infection is required for antibiotics with intermediate action [18]. *Salmonella* is susceptible to amoxicillin-clavulanic acid, gentamicin [19, 20, 21], ciprofloxacin [21], and norfloxacin [19] in various studies. A recent study, however, reported that *Salmonella* isolates from backyard chickens in West Bengal, India were highly resistant to ciprofloxacin, gentamicin, and norfloxacin [22].

Antimicrobial resistance was 80% to ampicillin, doxycycline, tetracycline, and trimethoprim-sulfamethoxazole; 60% to kanamycin and cefuroxime; and 40% to neomycin. This observation is consistent with several studies and has also been demonstrated by *Salmonella* isolated from poultry products in the Philippines. For instance, *Salmonella* isolates recovered from poultry carcasses were resistant to ampicillin, tetracycline, and trimethoprim-sulfamethoxazole [19, 23]. Resistance of *Salmonella* isolated from free range and broiler chickens to tetracycline, kanamycin [21], and ampicillin [24, 25, 26] is consistent with previous studies conducted abroad.

Table 4 Antimicrobial susceptibility profile of *Salmonella* isolates from free-range native chickens.

Class of antibiotics	Antibiotics (concentration in ug)	Susceptibility pattern*		
		Sensitive (%)	Intermediate (%)	Resistant (%)
Tetracycline	Doxycycline (30ug)	20 (1/5)	0 (0)	80 (4/5)
	Tetracycline (30ug)	20 (1/5)	0 (0)	80 (4/5)
Penicillin	Ampicillin (10ug)	0 (0)	20 (1/5)	80 (4/5)
	Amoxicillin-Clavulanic acid (10ug)	80 (4/5)	0 (0)	20 (1/5)
Aminoglycosides	Streptomycin (10ug)	20 (1/5)	40 (2/5)	40 (2/5)
	Gentamicin (10ug)	20 (1/5)	80 (4/5)	0 (0)
	Kanamycin (30ug)	0 (0)	40 (2/5)	60 (3/5)
	Neomycin (10ug)	0 (0)	60 (3/5)	40 (2/5)
Quinolones	Ciprofloxacin (5ug)	20 (1/5)	80 (4/5)	0 (0)
	Norfloxacin (10ug)	60 (3/5)	40 (2/5)	0 (0)
Cephem	Cefaclor (30ug)	60 (3/5)	0 (0)	40 (2/5)
	Cefuroxime (30ug)	0 (0)	40 (2/5)	60 (3/5)
Phenicol	Chloramphenicol (30ug)	60 (3/5)	0 (0)	40 (2/5)
Folate pathway inhibitor	Trimethoprim-Sulfamethoxazole (1.25/23.75ug)	20 (1/5)	0 (0)	80 (4/5)

*CLSI 2018

Quinolones (ciprofloxacin and norfloxacin) and gentamicin were effective in inhibiting the isolates. Quinolones have been one of the drugs of choice for the treatment of salmonellosis (typhoid and paratyphoid) in humans after resistance has been developed against first-line agents such as ampicillin, chloramphenicol, and trimethoprim-sulfamethoxazole [27]. Ciprofloxacin is the drug of choice for salmonellosis of poultry origin [28]. Gentamicin, on the other hand, is a broad-spectrum antibiotic that is effective against serious bacterial pathogens but does not have a significant bactericide effect on intracellular bacteria like *Salmonella* [29]. In vitro studies by Mandal et al. [30] showed the efficacy of gentamicin and amikacin against multidrug-resistant (MDR) *S. enterica* serovar Typhi infection at deficient concentrations (2 μg/mL) after 6 h of incubation. The mechanism, however, was not clearly defined.

Furthermore, despite the absence of antibiotics in the management of native chickens, MDR profiling (Table 5) revealed that all (100%) isolates from cloacal swabs were multidrug-resistant and exhibited resistance to at most eight antibiotics (AMCECCXMDOKNSXTTE) belonging to five classes of antimicrobials. Fifty percent of the isolates from boot sock samples exhibited multidrug resistance to five antibiotics (AMCDOSSXTTE) belonging to four classes of antimicrobials. These results indicate a wide use and misuse of antibiotics in poultry, creating selective pressure for bacteria to develop resistance. Most environmental isolates were more susceptible to most aminoglycosides, quinolones, and cefaclor than the poultry isolates, which were susceptible only to amoxicillin-clavulanic acid, and quinolones.

Table 5 Multidrug resistance profile of *Salmonella* isolates from environmental and cloacal swab samples.

Source of isolates	Maximum number of antibiotics exhibiting resistance	Resistant antibiotics	Resistance rate (%)	Multidrug resistance rate (%)
Cloacal swabs (n=3)	8	AMCECCXMDOKNSXTTE	100	100
Boot socks (n=2)	5	AMCDOSSXTTE	100	50

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

The absence of *Salmonella* was assumed based on the PCR and culture analysis of cloacal swabs and environmental samples at the beginning of the study. Although examination of the parent flock was beyond the scope of the research, testing the internal organs for *Salmonella* colonization in the starting flock could enhance the screening for *Salmonella*-negative flock. The liver is a site of persistent infection that provides relevant information. *Salmonella* is vertically transmitted and colonizes internal organs such as the spleen, liver, and cecum. Horizontal transmission through a contaminated environment mostly affects young birds, which succumb to infection with as few as 100 *Salmonella* cells [31]. Infection of the experimental flock is perceived to be introduced by biological vectors, including flies, rodents, and synanthropic birds which amplify the spread of *Salmonella* on the farm. These vectors are attracted to visit animal farms because of the availability of food and water [32]. The mobility of rodents is responsible for the spread of *Salmonella* from flock to flock and across nearby farms. Farms with high-density rodent populations have been consistently associated with *Salmonella* Enteritidis

contamination [33]. Like other animal hosts, mice can maintain asymptomatic infection and shed *Salmonella* intermittently through their feces [34]. Airborne transmission of *Salmonella* through dust particles or contaminated aerosols is another perpetrator. Contaminated dust samples re-introduce salmonellae in poultry houses even after cleaning and disinfection. This makes the airborne movement of *Salmonella* a crucial route for disseminating infection within flocks [35]. Transmission in different manners progresses to persistent, latent infection, causing birds to intermittently shed bacteria in their feces throughout their productive lifetime [36]. Stressful conditions such as drastic changes in climate, heat stress, and the peak of reproductive hormones should be considered promoters of recrudescent infection. The isolation of AMR *Salmonella* in the farm possibly occurred either through biological vectors or the bacteria's spontaneous acquisition of antibiotic resistance genes (ARGs) from the environmental resistome linked and intertwined in the ecosystem [37]. Increased human use and misuse of antibiotics in food production should be regulated to prevent the dissemination of ARGs in the environment.

4. Conclusion

The study unveiled the presence of multidrug-resistant salmonellae in free-range native chickens and their environment. These findings seriously affect the sustainability of free-range production systems and the safety of their products. The detection requires a well-planned and well-coordinated diagnostic strategy to time the excretion of the elusive *Salmonella*. The pros and cons of each diagnostic method should be considered. Even though the bacterial culture is the gold standard method, its detection is slow and time-consuming. The molecular technique is rapid but insensitive to low organism carriage, and the serologic test has limited sensitivity and specificity. Understanding the biology of *Salmonella* and the complexity of the environment is therefore necessary in designing preharvest strategies for early detection and reduction of *Salmonella* contamination. Nonetheless, efforts should delve into stringent biosecurity measures to ensure food safety and the quality of poultry products derived from free-range chickens.

5. Ethical Approval

The experimental protocol was reviewed and approved by the Animal Care and Use Committee of UPLB with reference number CVM-2019-008.

6. Acknowledgment

This study is funded by the Department of Agriculture-Newton Project, Department of Science and Technology-Accelerated Science and Technology Human Resource Development Program, and the Philippine Council for Agriculture, Aquatic and Natural Resources Research and Development. The authors acknowledged technical support of Dr. Marilen P. Balolong (†), Dr. Reuel Marte, Ezra Salamat, Trisha Nicole Agulto, and Armando Anono (†).

7. Conflict of Interest

The authors have no conflict of interest to declare.

8. References

[1] Ricke SC, Rothrock MJ Jr. Gastrointestinal microbiomes of broilers and layer hens in alternative production systems. Poult Sci. 2020;99(2):660–669.

[2] Mollenhorst H, van Woudenbergh CJ, Bokkers EGM, de Boer IJM. Risk factors for *Salmonella enteritidis* infections in laying hens. Poult Sci. 2005;84(8):1308–1313.

[3] Cui L, Liu Q, Jiang Z, Song Y, Yi S, Qiu J, et al. Characteristics of *Salmonella* from Chinese native chicken breeds fed on conventional or antibiotic-free diets. Front Vet Sci. 2021;8:607491.

[4] Hedman HD, Vasco KA, Zhang L. A Review of Antimicrobial Resistance in Poultry Farming within Low-Resource Settings. Animals. 2020;10(8):1264.

[5] International Standard. Microbiology of food and animal feeding stuffs — Horizontal method for the detection of *Salmonella* spp. 4th ed. ISO Office, Switzerland; 2002.

[6] Rahn K, De Grandis SA, Clarke RC, McEwen SA, Galán JE, Ginocchio C, et al. Amplification of an invA gene sequence of *Salmonella typhimurium* by polymerase chain reaction as a specific method of detection of *Salmonella*. Mol Cell Probes. 1992;6(4):271-279.

[7] Beal RK, Powers C, Wigley P, Barrow PA, Smith AL. Temporal dynamics of the cellular, humoral and cytokine responses in chickens during primary and secondary infection with *Salmonella enterica* serovar Typhimurium. Avian Pathol. 2004;33(1):25-33.

[8] Wigley P, Hulme SC, Powers C, Beal RK, Berchieri A Jr, Smith A, et al. 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. Infect Immun. 2005;73(5):2986-2990.

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

[10] Learn-Han L, Yoke-Kqueen C, Shiran MS, Sabrina S, Noor Zaleha AS, Sim JH, et al. Molecular characterization and antimicrobial resistance profiling of *Salmonella enterica* subsp. *enterica* isolated from 'Selom' (*Oenanthe stolonifera*). Int Food Res. J. 2009;16(2):191-202.

[11] Sweeney MT, Lubbers BV, Schwarz S, Watts JL. Applying definitions for multidrug resistance, extensive drug resistance and pandrug resistance to clinically significant livestock and companion animal bacterial pathogens. J Antimicrob Chemother. 2018;73:1460–1463.

[12] Sullivan G, Guo X, Tokman JI, Roof S, Trmcic A, Baker RC, et al. Extended enrichment procedures can be used to define false-negative probabilities for cultural gold standard methods for *Salmonella* detection, facilitating comparisons between gold standard and alternative methods. J Food Prot. 2020;83(6):1030-1037.

[13] Wang M, Zhang Y, Tian F, Liu X, Du S, Ren G. Overview of rapid detection methods for *Salmonella* in foods: progress and challenges. Foods. 2021;10(10):2402.

[14] Schrank IS, Mores MAZ, Costa JLA, Frazzon APG, Soncini R, Schrank A, et al. Influence of enrichment media and application of a PCR based method to detect *Salmonella* in poultry industry products and clinical samples. Vet Microbiol. 2010;82(1):45–53.

[15] Oliveira GH, Berchieri A Jr, Montassier HJ. Chicken serologic response to *Salmonella enterica* serotype Typhimurium assessed by ELISA. Braz J Poult Sci. 2006;8(1):51-54.

[16] Soria MC, Soria MA, Bueno DJ. Comparison of 2 culture methods and PCR assays for *Salmonella* detection in poultry feces. Poult Sci. 2012;91(3):616-626.

[17] Carli KT, Unal GB, Gaiier V, Eyigor A. Detection of salmonellae in chicken feces by a combination of tetrathionate broth enrichment, capillary PCR, and capillary gel electrophoresis. J Clin Microbiol. 2001;39(5): 1871-1876.

[18] European Committee on Antimicrobial Susceptibility Testing (EUCAST). New definitions of S, I and R from 2019 [Internet]. 2021 [cited 2022 September 20] Available from https://www.eucast.org/newsiandr/.

[19] Balala LM, Rovira HG, Vizmanos MFC, Bernardo FAEM, Divina BP. Isolation, identification and antibiotic sensitivity testing of *Salmonella* spp. in chickens. Philipp J Vet Med. 2006;43(2): 63-70.

[20] Thung TY, Mahyudin NA, Basri DF, Radzi WM, Nakaguchi Y, Nishibuchi M, et al. Prevalence and antibiotic resistance of *Salmonella* Enteritidis and *Salmonella* Typhimurium in raw chicken meat at retail markets in Malaysia. Poult Sci. 2016;95(8):1888-1893.

[21] Abunna SF, Bedasa M, Beyene T, Ayana D, Mamo B, Duguma, R. *Salmonella*: Isolation and antimicrobial susceptibility tests on isolates collected from poultry farms in and around Modjo, Central Oromia, and Ethiopia. J animal poult sci. 2017;5(2):21-35.

[22] Samanta I, Joardar SN, Das PK, Sar SK, Bandyopadhyay S, Dutta TK, et al. Prevalence and antibiotic resistance profiles of *Salmonella* serotypes isolated from backyard poultry flocks in West Bengal, India. J Appl Poult Res. 2014;23:536–545.

[23] Bautista VALM, Mendoza BC. Multiple drug resistance profile of *Salmonella enterica* subsp. *enterica* serovar Kentucky obtained from apparently healthy layer chickens in San Jose, Batangas, Philipp J Vet Med. 2016:53(1):17-25.

[24] Castro-Vargas RE, Herrera-Sánchez MP, Rodríguez-Hernández R, Rondón-Barragán IS. Antibiotic resistance in *Salmonella* spp. isolated from poultry: A global overview. Vet World. 2020;13(10):2070-2084.

[25] El-Sharkawy H, Tahoun A, El-Gohary AEA, El-Abasy M, El-Khayat F, Gillespie T, et al. Epidemiological, molecular characterization and antibiotic resistance of *Salmonella enterica* serovars isolated from chicken farms in Egypt. Gut Pathog. 2017;9(1):8.

[26] Zhao X, Gao Y, Ye C, Yang L, Wang T, Chang W. Prevalence and characteristics of *Salmonella* isolated from free-range chickens in Shandong province, China. Biomed Res Int. 2016;2016(1):8183931.

[27] Pham T, Ziora ZM, Blaskovich M. Quinolone antibiotics. Medchemcomm. 2019;10(10):1719–1739.

[28] Karabasanavar NS, Madhavaprasad CB, Gopalakrishna SA, Hiremath J, Patil GS, Barbuddhe SB. Prevalence of *Salmonella* serotypes *S*. Enteritidis and *S*. Typhimurium in poultry and poultry products. J Food Saf. 2020;40(6):e12852.

[29] Menashe O, Kaganskaya E, Baasov T, Yaron S. Aminoglycosides affect intracellular *Salmonella enterica* serovars *typhimurium* and *virchow*. Antimicrob Agents Chemother. 2008;52(3):920–926.

[30] Mandal S, Manda MD, Pal NK. In vitro activity of gentamicin and amikacin against *Salmonella enterica* serovar Typhi: a search for a treatment regimen for typhoid fever. East Mediterr Health J. 2009;15(2):264-268.

[31] Van Immerseel F, de Buck J, Pasmans IF, Bohez L, Boyen F, Haesbrouck F, et al. Intermittent long-term shedding and induction of carrier birds after infection of chickens early posthatch with low or high dose of *Salmonella enteritidis*. Poult Sci. 2004;83(11):1911-1916.

[32] Umali DV, Lapuz RRSP, Suzuki T, Shirota J, Katoh H. Transmission and shedding patterns of *Salmonella* in naturally infectied captive wild roof rats (*Rattus rattus*) from a *Salmonella*-contaminated farm. Avian Dis. 2012;56:288-294.

[33] Henzler DJ, Opitz HM, Saeed AM. Role of rodents in the epidemiology of *Salmonella* enterica serovar enteritidis and other *Salmonella* serovars in poultry farms. *Salmonella* enterica serovar enteritidis in humans and animals: Epidemiology, pathogenesis and control. Ames (USA): Iowa State University Press; 1999. p. 443.

[34] Maciel BM, Sriranganathan N, Romano CC, dos Santos TF, Dias JC, Gross E, et al. Infection cycle of *Salmonella enterica* serovar Enteritidis in latent carrier mice. Can J Microbiol. 2012;58:1389-1395.

[35] Gast RK, Mitchell BW, Holt PS. Airborne transmission of *Salmonella enteritidis* infection between groups of chicks in controlled-environment isolation cabinets. Avian Dis. 1998;42(2):315-320.

[36] McWhorter AR, Chousalkar K. A long-term efficacy trial of a live, attenuated *Salmonella typhimurium* vaccine in layer hens. Front Microbiol. 2018;9:1380.

[37] Zhuang M, Achmon Y, Cao Y, Liang X, Chen L, Wang H, et al. Distribution of antibiotic resistance genes in the environment. Environ Pollut. 2021;285:117402.