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All communications should be addressed to:

The Editor-in-Chief
Philippine Journal of Veterinary Medicine
College of Veterinary Medicine
University of the Philippines Los Baños
Laguna, Philippines 4031
Telefax Nos. +63-49-536-2727, +63-49-536-2730
Email: pjvm1964@gmail.com, pjvm.uplb@up.edu.ph

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Volume 58 **No. 1** **January – July 2021**

CONTENTS

Original Articles

Research Notes

Case Report

ORIGINAL ARTICLE

ISOLATION AND UNIPLEX POLYMERASE CHAIN REACTION-BASED DETECTION OF *Salmonella* spp. IN NATIVE CHICKENS (*Gallus gallus domesticus* Linn.) FROM SELECTED LIVE BIRD MARKETS IN BATANGAS, PHILIPPINES

John Paul F. Galvez, DVM and Dennis V. Umali, DVM, PhD*

Department of Veterinary Clinical Sciences, College of Veterinary Medicine, University of the Philippines Los Baños, College, 4031, Laguna, Philippines

ABSTRACT

Salmonellosis continues to be a pressing problem in poultry. Currently, data gap exists between the distribution and prevalence of *Salmonella* spp. in live bird markets (LBM) in the Philippines. Hence, isolation and molecular detection of *Salmonella* in native chickens from four LBMs in Batangas, Philippines were performed. Conventional bacterial isolation and uniplex polymerase chain reaction (PCR)-based assay were utilized for detection of *Salmonella*. A total of 114 samples composed of 16 pooled cloacal swabs, 49 liver samples, and 49 caecum samples were used for bacterial isolation. For PCR, 38 pooled samples were utilized which comprised of 10 pooled HTT broth from liver, 10 pooled HTT broth from cecum, and 18 pooled cloacal swabs. Results showed that 1/16 (6.25%) of the cloacal swabs and 1/114 of the total samples (0.88%) were positive for Salmonella in bacterial isolation. In comparison, uniplex PCR showed a detection rate of 12/18 (66.67%) in pooled cloacal swabs and an average positivity detection rate of 31.57%. Among the LBMs, Lemery had the highest PCR-positivity rate which is 6/15 (40%) compared to 1/8 (12.5%) for Padre Garcia and 5/15 (33.33%) for Rosario. Routine surveillance for *Salmonella* contamination is essential in preventing foodborne diseases from poultry in the Philippines.

Keywords: *isolation, live bird market, uniplex PCR, Salmonella, swab*

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INTRODUCTION

Salmonellosis is a disease of significant public health and animal concern worldwide. In an epidemiological study conducted by the Centers for Disease Control and Prevention (CDC), most isolated serotypes are of animal origin (Galanis *et al.*, 2006). In the Philippines, data gap exists regarding the level of *Salmonella* contamination in poultry products especially in native chickens. Considering that chicken meat and egg are the main reservoirs of *Salmonella* infection in humans, continuous vigilance and awareness regarding *Salmonella* contamination in poultry is essential (Food and Agriculture Organization (FAO) and World Health Organization, 2002). Aside from its veterinary and public health significance, salmonellosis has a vital impact in agriculture and economy as well.

Salmonella spp. are facultative anaerobic, Gram-negative, non-spore forming bacteria. According to the FAO, *Salmonella* is taxonomically classified into two: *Salmonella enterica* and *S. bongori*. *Salmonella enterica* is considered the leading cause of foodborne illnesses in the world. Enterocytes and lymphoid tissues are the sites colonized by the bacteria resulting to bacteremia which makes the colonized organ to be *Salmonella* positive. The aforesaid organs are as follows: spleen, liver, bone marrow, ovary, and oviduct. For *Salmonella* testing, the conventional method such as bacterial isolation and biochemical test is considered time-consuming and labor intensive since it usually takes three to eleven days to perform the test. Bacterial culture isolation involves non-selective pre-enrichment, selective enrichment, and selective plating or use of differential media. In comparison, uniplex PCR which targets the *Salmonella* invasion gene (*invA)*

***FOR CORRESPONDENCE:**
(e-mail: dvumali@up.edu.ph)

proved to be reliable as the gene is unique with *Salmonella* and is more sensitive than bacterial culture and isolation. Furthermore, this gene acts as a protein encoder associated with the bacteria's pathogenesis. Consequently, *invA* is now considered in the international standard of *Salmonella* detection (Malorny *et al.*, 2003 as cited by Al-Khayat and Khammas, 2016).

The Philippine native chicken plays a significant role in the Philippine agriculture and economy as the bird is easily raised by the local farmers because of its meat and egg value as well as its income-generating use. In terms of production, the backyard poultry sector accounts for 54% of native chickens, 30% and 16% are broilers and layers, respectively (Philippine Statistics Office, 2017). Moreover, there is an observed increase in native or improved chicken population by 5.35%. Increased production of the native chickens in the poultry sector may imply an increase in demand of the consumption of native chicken meat, edible giblets, or egg. Hence, it is important to know the pathogens affecting the native chickens. This study was conducted to detect *Salmonella* spp. in native chickens using cloacal swabs, liver, and cecum as samples through bacterial isolation and uniplex PCR from selected live bird markets (LBM) in Batangas, Philippines. Data on *Salmonella* contamination in poultry, especially of native chickens in the Philippines, will be valuable in benchmarking studies and assessment of food safety so that more efficient strategies can be implemented in the improvement of quality of local chicken products for food processing and exports.

MATERIALS AND METHODS

Study population

Forty-nine (n=49) native chickens, regardless of sex and strain, were chosen from the three major native chicken-producing LBMs in Batangas in terms of the number of chickens sold per day. The LBM profile was obtained from Region IV-A Regional Animal Disease Diagnostic Laboratory, Bureau of Animal Industry. Out of 49 native chickens, 20 originated from Lemery, 15 from Padre Garcia, and 14 from Rosario. Aside from the selected native chickens that were brought to the laboratory, onsite cloacal swabs were also collected from other native chickens in the LBMs. A total of 10 birds were sampled onsite in Rosario, 15 birds from Lemery, and two birds from Tanauan, Batangas. The identity of the native chickens were confirmed based on the plumage pattern, plumage color, weight, comb, and ear lobes (Bondoc, 1998).

Sample Collection

All procedures performed in domestic chickens were approved by the Institutional Animal Care and Use Committee (IACUC) of the College of Veterinary Medicine, University of the Philippines Los Baños (UPLB).

All chickens were individually weighed and checked for any clinical signs of disease. The eyes, nares, ears, integument, footpad, and vent were examined for any lesions. Regardless of presence or absence of clinical signs, all native chickens were swabbed and the organs (liver and cecum) were harvested. Cloacal swabs were individually collected from (?) the birds using sterile cotton swabs. For bacterial isolation, samples were pooled at a maximum of five samples per pool per LBM. Swabs were immediately placed in 2 ml sterile normal saline solution. All chickens were euthanized humanely by cervical dislocation method. Approximately 5g of liver and left cecum were collected aseptically. After sample collection, tissue samples for PCR were homogenized manually using sterile mortar and pestle. Tissues for PCR were kept in separate air-tight plastic containers and stored at -20°C. Bacterial isolation was performed immediately after necropsy.

Bacterial Isolation and Identification

A total of 114 samples comprised of 49 liver, 49 left ceca, and 16 pooled cloacal swabs were utilized. Approximately 2 g of each individual tissues and 1 ml of each pooled cloacal swab were used as inoculums in 8 ml brain and heart infusion (BHI) broth (Eiken, Japan) and incubated at 37°C for 24 hours. Prior to inoculation onto BHI broth, swab tips were immersed onto a red-topped tube filled with 3 ml of 0.9% (w/v) normal saline solution (NSS) which served as a transport medium. The transport media were all enclosed inside a styrofoam box with ice gel packs to maintain the cold chain. On the other hand, organs were aseptically harvested during necropsy and were placed into a sterile Ziploc® individually. Afterwards, the organs were stored at -20°C which were eventually cut into tiny pieces before the pre-enrichment procedure. One milliliter of the BHI broth was then inoculated to 9 ml of Hajna Tetrathionate (HTT) (Eiken, Japan) broth with 0.2 ml potassium-iodide iodine solution and incubated at 42°C for 48 hours following manufacturer's recommendation. A loopful from HTT culture was then streaked onto a desoxychocolate hydrogen sulfide lactose (DHL) agar (Eiken) and incubated for 24 hours at 37ºC. *Salmonella* suspect colonies were identified by conventional biochemical tests using lysine decarboxylase (LD), sulphide indole

motility (SIM), Simmons citrate, Methyl Red-Voges Proskauer (MRVP), urease, and carbohydrate fermentation tests.

Uniplex Polymerase Chain Reaction

Approximately, 40 µl of the inoculated HTT broth for each type of samples were used for PCR. There was a total of 18 pooled cloacal swabs, 10 pooled HTT broths from the liver and 10 pooled HTT broths from the cecum thus, a total of 39 pooled samples (Tables 1 and 2). Using the pooled samples, molecular detection was performed through uniplex PCR. DNA extraction was performed using the QIAGEN® mini kit according to the manufacturer's recommendation. Uniplex PCR was performed to amplify a 284 bp *invA* gene fragment using SapphireAmp Fast PCR Master Mix (Takara Bio-Inc, Shiga, Japan) with a 25 µL/50 µl reaction volume, 0.2 µM forward and reverse primers, and an approximately 50-100 ng template. Primer sets used were 5'-GTG AAA TTA TCG CCA CGT TCG GGC AA-3' for the forward primer and 5'-TCA TCG CAC CGT CAA AGG AAC C-3' for the reverse primer. Thermocycling conditions were initial denaturation at 94°C for 5 minutes, followed by 35 cycles of denaturation at 94°C for 10 seconds, annealing at 55°C for 10 seconds, and extension at 72°C for 10 seconds. Final extension was carried out at 72°C for 10 minutes (Moussa *et al.,* 2010). Approximately, 5 µl of each PCR product was mixed with 1 µl loading buffer and was separated by electrophoresis in a 2% agarose gel with 0.25 µl/ml Gel Red® (Wako, USA) at 100 volts for 30 minutes and visualized using a UV transilluminator.

RESULTS AND DISCUSSION

This study detected the presence of *Salmonella* spp. in Philippine native chickens using conventional bacterial isolation and uniplex PCR from selected LBMs in Batangas, Philippines. Higher detection rate was observed in PCR assay (31.57%) as compared to conventional bacterial isolation (0.88%). The total positivity rate (0.88%) using conventional bacterial culture in this study was lower as compared to the study conducted by Baldrias and Capistrano (1997), which yielded an isolation rate of 20%. However, samples from the latter were obtained from dressed chickens in selected Metro Manila wet markets utilizing breast, inner thigh, and the perianal region by the swab method. Using cloacal swabs, an 8.3% isolation rate in dressed chickens and 20% in fresh chilled carcasses from selected wet markets in the Philippines were also reported previously (Capistrano, 1992; Martin, 1996; and Tacal, *et al.,*1972 as cited by Velasco, 1996). In addition, another study conducted using fresh chilled carcass and giblets as samples from dressed chickens at different poultry dressing plants yielded a higher isolation rate of 3% (Velasco, 1996). In other countries, a wide range of prevalence was noted in various broiler operations. The recorded prevalence of *Salmonella* spp. contamination in broilers was 42.3% in Korea, 72% in Thailand, 36.5% in Belgium, 35.5-47.7% in Austria, 52.2% in China, and 53.3% in Vietnam (Van *et al*., 2007; Yang *et al.*, 2011; and Kim *et al*., 2012); whereas in free range chickens, a prevalence of 12.7% was recorded using fecal swabs as samples in China (Zhao *et al.,* 2016).

The low isolation rate (6.25%) in cloacal swabs and extremely low occurrence (0.87%) of *Salmonella* in the LBM from Batangas using conventional bacterial isolation in this study can be due to the differences in the protocol used and types of samples analyzed (Halatsi *et al.*, 2006; Soria *et al.*, 2012; Ibrahim *et al*., 2014; and Langkabel *et al.*, 2014). Differences in bacterial isolation protocol can be considered a contributory factor among the variations in the isolation rates reported because of the dissimilar enrichment media used across studies. Dissimilarity in enrichment media used also denotes different nutritional contents for the bacteria which can also be applied in the selection of the transport medium. Differences across studies from which the samples were obtained can also be a contributory factor in isolation rate because the

Table 1. Sample type positivity rate profile of Salmonella from Philippine native chickens using conventional bacterial isolation

Sample	Total Number of Samples	Number of Positive *Salmonella* Isolates	Positivity Rate (%)
Sample Type			
Cloacal swabs	16	1	6.25
Liver	49	0	0
Caecum	49	0	0
Total	114	1	0.87

Table 2. Positivity rate profile of Salmonella from Philippine native chickens per origin using conventional bacterial isolation

Sample	Total Number of Samples	Number of Positive *Salmonella* Isolates	Positivity Rate (%)
Location			
Lemery	47	0	0
Padre Garcia	33	1	3.03
Rosario-Tanauan	34	0	0
Total	114	1	0.88

degree of environmental contamination varies across places. Type of the sample may also affect the isolation rate as certain tissues have higher chance of contamination as compared to other tissue samples or other types of samples such as swabs (NidaUllah *et al.,* 2016 and Shafini *et al.,* 2016).

The use of *invA* gene in the molecular detection of *Salmonella* spp. is well supported by several studies as this gene is present in all *Salmonella* spp. up to the serovar level (Singer *et al.,* 2006; Moussa *et al.,* 2010 and Al-Khayat & Khammas, 2016). Several studies have used HTT broth which involves the inoculation of the broth from *Salmonella*-identified colonies. Studies on the use of HTT broth in PCR are limited but involve almost the same procedures. (Makino *et al*., 1999; Carli *et al*., 2001; Eyigor *et al*., 2001, 2007). Using uniplex PCR, *Salmonella* spp. was confirmed to be present in 12 out of 38 pooled cloacal swab samples (31.57%)(Tables 3 and 4). Six out of fifteen (40%) of pooled cloacal swabs from Lemery were positive from *Salmonella* using uniplex PCR followed by five out of fifteen pooled cloacal swabs (33.33%) from Rosario-Tanauan that were *Salmonella* positive using uniplex PCR as well. On the contrary, Padre Garcia had the highest positivity rate profile using bacterial isolation which yielded 1 out of 33 (3.03%) *Salmonella* positive samples. Only one sample tested positive in Padre Garcia (12.5%), which was the same sample that was positive in conventional bacterial isolation. The positive detection rate (31.57%) for pooled samples is comparable to other molecular studies which utilizes PCR. A comparable multiplex PCR-based assay which utilizes cloacal swabs yielded a positive detection rate of 25% for *Salmonella* spp. (Paião *et al*., 2013). This is in contrast with a study conducted by Moussa *et al*. (2010) in which the detection rate is lower. Similarly, an even lower positivity rate of 2.74% was obtained using poultry meat as samples in a study in Brazil (De Freitas *et al*., 2010). Differences in the tissue and swab samples and the method of collection can be accounted for as different parts of the chicken have different levels of contamination.

A higher positivity rate profile in PCR versus conventional bacterial isolation was observed which can be due to higher sensitivity and specificity of molecular detection. This implies that the bacterial DNA can be detected even at low levels. In comparison with bacterial isolation, detection is based from the exclusion principle in which the bacteria of interest compete for food with other existing microorganisms. On the

Table 3. Sample type detection rate of *Salmonella* from Philippine native chickens using uniplex PCR

Sample	Total Number of Samples	Number of Positive Samples	PCR Detection Rate (%)
Sample Type			
Pooled cloacal swab	18	12	66.67
Pooled Liver	10	0	0
Pooled Caecum	10	0	0
Total	38	12	31.57

Table 4. Detection rate of *Salmonella* from Philippine native chickens per origin using uniplex PCR

Sample	Total Number of Samples	Number of Positive Samples	PCR Detection Rate (%)
Location			
Lemery	15	6	40
Padre Garcia	8	1	12.55
Rosario	15	5	33.33
Total	38	12	31.57

contrary, molecular detection via PCR still detects bacterial DNA even if the bacterial cell is dead. Among the tissue samples used in molecular detection, cloacal swabs only garnered a positive detection rate (66.67%). *Salmonella* contamination in a live bird market is more likely to occur due to environmental contamination of poultry droppings because *Salmonella* is mainly transmitted via the feco-oral route. The positive PCR detection rateobtained in the study is higher as compared to studies conducted by Al-Abadi and Mayah (2012) and Moraes *et al.* (2016), which yielded 19% and 15.3%, respectively. Cloacal swabs obtained by Al-Abadi and Al-Mayah (2012) were from broilers and layers from wet markets in Iraq. On the contrary, Moraes *et al*. (2016), collected samples from commercial layer farms in Brazil. Non-detection of *Salmonella* spp. in liver and intestine is in conjunction with the study conducted in a commercial layer farm in Japan (Lapuz *et al.*, 2012). The differences across positive rate profile can also be attributed to the variations in the protocols used in molecular detection, the type of chicken used in the studies, and the environmental setting. The possible reason for higher *Salmonella*-contamination in this study can be due to the hygienic practices in LBMs. Compared to wet markets and layer farms, poultry droppings are more commonly seen in LBMs since cleaning is not frequently performed as compared to a farm. In a wet market, birds are usually sold as carcasses and are already eviscerated (Bondoc, 1998). Hence, a decrease in the possible contamination can be noted. Moreover, environmental shedding of *Salmonella* may occur at a higher level in LBMs where most stalls are make-shift. In Rosario and Lemery, the LBMs are not enclosed in a structure which makes cleaning more difficult. In comparison, biosecurity measures in a farm setting are laid down. Among the three major LBMs studied, Lemery had the highest detection rate (40%) using uniplex PCR assay. As of this writing, no study has been stablished with the detection of *Salmonella* spp. from LBMs in Batangas. Hence, no comparable studies on LBMs can be cited. Analyzing the LBM profile, it can be hypothesized that higher PCR positive detection rate in Lemery is due to the higher volume of native chickens being sold in the market.

In summary, this study investigated the presence of *Salmonella* spp. in native chickens from LBMs in Batangas, Philippines. It was observed that cloacal swabs had the highest isolations rates both in conventional bacterial isolation and uniplex PCR. The highest occurrence of *Salmonella* among the studied birds was in Lemery. Assessment of *Salmonella* contamination in native chickens from the LBM is essential as a preventive measure of disease surveillance and assessment of food safety in the country.

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STATEMENT ON COMPETING INTEREST

The authors have no competing interests to declare.

AUTHOR'S CONTRIBUTION

DV Umali conceived and designed the study. DV Umali and JP Galvez performed the experiments and analyzed the data. JP Galvez and DV Umali wrote the manuscript. All authors read and approved the final version.

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