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(Original Research)

Molecular Detection of Virulence Genes Associated with Salmonella Pathogenicity Islands (SPIs) 1 and 2 in *Salmonella enterica* Isolates from Poultry in Selected Areas of the Philippines

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Abstract

Background: Salmonellosis, particularly infections caused by *Salmonella enterica*, remains a major concern in poultry production and food safety. The disease poses significant economic losses due to mortality, reduced growth rates, and treatment costs, highlighting the need to characterize virulence gene patterns of Philippine isolates. This study aimed to molecularly confirm *S. enterica* isolates recovered from poultry-derived samples, particularly eggs, giblets, and cloacal swabs, collected from selected regions of the Philippines using 16S rRNA-targeted PCR, and to determine the presence and distribution of the *invA* (SPI-1) and *sseC* (SPI-2) virulence genes among these isolates. **Methods:** A total of 865 samples, including eggs, giblets, and cloacal swabs, were collected from Philippine poultry farms and wet markets. Standard microbiological procedures were employed: pre-enrichment in buffered peptone water, enrichment in Rappaport Vassiliadis broth, plating on xylose lysine deoxycholate agar, and purification on nutrient agar. Putative isolates were further analyzed using morphological and selected biochemical tests, followed by PCR targeting of *16S rRNA*, *invA*, and *sseC* genes. Amplicons were visualized by gel electrophoresis. **Results:** All 56 suspected isolates were confirmed as *S. enterica*. Among these, 69.6% carried both *invA* and *sseC*, 14.3% carried *invA* alone, 3.6% lacked both genes, and none harbored *sseC* alone. These genes are associated with pathogenicity islands SPI-1 (*invA*) and SPI-2 (*sseC*). **Conclusions:** The presence of virulence genes linked to SPI-1 and SPI-2 among Philippine *S. enterica* isolates highlights their role in pathogenesis. This provides baseline data on virulence gene prevalence in poultry-derived Salmonella and emphasizes the importance of continued surveillance to mitigate risks of salmonellosis from poultry-derived isolates.

Keywords

Salmonella enterica, Pathogenicity islands, Poultry, invA, sseC, virulence genes

1. Introduction

The Philippine Statistics Authority (PSA) reported that as of September 2023, the total chicken inventory reached 202.82 million birds. This figure represents a 1.1 percent increase compared to the year's inventory of 200.64 million birds. Of this total, native/improved chickens accounted for 43.0 percent, followed by broiler chickens at 34.7 percent, and layer chickens at 22.3 percent. The highest chicken inventory was noted in Central Luzon, with 35.80 million birds, followed by CALABARZON with 27.30 million birds, and Northern Mindanao with 26.74 million birds. Together, these regions represented 44.3 percent of the country's total chicken inventory during the review period. Per capita poultry consumption in the Philippines for 2024 is estimated at 14.91 kilograms [1].

Worldwide, non-typhoidal Salmonella is one of the leading bacterial causes of diarrhea, causing around 150 million illnesses and approximately 60,000 deaths each year [2]. Of the food poisoning cases with a known bacterial cause, it is the predominant pathogen, as evidenced by recorded cases from 2005 to June 2018 [3]. In this context, contaminated chicken meat can likely serve as a source of the pathogen for consumers of poultry products such as eggs, giblets, and meat.

The traditional classification system for Salmonella, based on Kauffmann-White (1934), utilizes O and H antigens to divide the organism into serovars. As of 2018, a total of 2,659 serovars of *Salmonella* spp. have been identified, with 1,586 of these belonging specifically to *S. enterica* subsp. *enterica* [4]. However, current taxonomy recognizes two species: *S. enterica* and *S. bongori*. Within *S. enterica*, there are six subspecies: *enterica, salamae, arizonae, diarizonae, houtenae*, and *indica*. Serovars and serogroups are contained within those taxonomic levels. These subspecies are further categorized into serogroups based on their antigenic characteristics [5,6]. A serovar (serotype) is a specific strain defined by its unique combination of surface antigens, while a serogroup is a broader category that clusters serovars sharing common O (somatic) antigen(s) (5). Almost all Salmonella organisms that cause disease in humans and domestic animals belong to *S. enterica* subspecies *enterica* [7]. Thus, reporting a strain's serovar (or serogroup) conveys antigenic identity used for surveillance and epidemiology, while species/subspecies indicate its broader genetic and evolutionary placement [4,5,6,7].

Primers targeting the *16S rRNA* gene allow for more robust, reproducible, and accurate bacterial identification than that obtained by phenotypic testing [8]. However, if the goal is to differentiate species within a genus, other genes must be utilized; therefore, the study employed primers targeting the virulence genes.

Numerous virulence genes have been identified in Salmonella, contributing to its complex life cycle within infected birds and humans [9]. One of these is a bacterial secretion system known as the type III secretion system (T3SS), which is the most crucial virulence factor for *Salmonella* spp. [10]. This T3SS translocates effector proteins across bacterial and host membranes into the host cytosol, where they modulate signaling and cytoskeletal pathways to facilitate colonization and virulence [11]. The effector proteins disrupt various aspects of the host cell's physiology and immune response, thereby enhancing bacterial virulence [12].

Salmonella spp. has a chromosomal region where most T3SS genes are concentrated, known as Salmonella pathogenicity islands (SPIs), with 17 SPIs documented to date [13]. Among these SPIs, SPI-1 and SPI-2 are the most extensively studied and represent two key determinants of pathogenesis [14-16]. The SPI-1 secretion system enables bacterial invasion of epithelial cells [12], which then leads to inflammation of the intestinal epithelium and symptoms of diarrhea [9]. On the other hand, the principal role of SPI-2 is to promote the replication of intracellular bacteria found in the Salmonella-containing vacuoles (SCVs) [12]. The SPI-2 promotes intracellular survival and replication by modifying SCV thereby, interfering with host endosomal trafficking and vesicle fusion, and enabling systemic infection [15]. The SPI-1 and SPI-2 secretion systems facilitate Salmonella pathogenesis.

Comparative sequence analysis of 12 conserved chromosomal housekeeping genes and invasion-associated genes demonstrated that Group V (*S. bongori*) is strongly distinct from other Salmonella groups, supporting its status as a separate species [17]. Many of these genes, which are clustered in the SPIs, are responsible for virulence. However, *S. bongori* has reduced virulence as it carries a limited, ancestral set of virulence functions and subsequently elaborated this in a different direction than *S. enterica* [18].

The *invA* gene (also known as invasion A protein) and the *sseC* gene (Salmonella secreted protein C) are located in SPI-1 and SPI-2, respectively. Consequently, the presence of SPI-1 and SPI-2, along with their associated genes, serves as an indication of a virulent Salmonella [10]. The invasion-associated SPI-1, specifically *invA*, mediates epithelial cell invasion and is induced during the intestinal/epithelial contact phase, while the survival-associated SPI-2, namely *sseC*, contributes to intracellular survival and replication, induced following internalization in host cells [11]. Indeed, the presence of the virulence genes *invA* and *sseC* is necessary for Salmonella organisms to have an effect on both human and animal hosts, among other factors that depend on gene expression, regulatory networks, additional effectors, and host factors.

This study was henceforth aimed to molecularly confirm *Salmonella enterica* isolates recovered from poultry-derived samples (eggs, giblets, and cloacal swabs) collected from selected regions of the Philippines using 16S rRNA-targeted PCR, and to determine the presence and distribution of the *invA* (SPI-1) and *sseC* (SPI-2) virulence genes among these isolates.

2. Materials and Methods

2.1 Ethical Consideration

The research protocols were reviewed and approved by the Institutional Animal Care and Use Committees (IACUC) of the three participating institutions: Cavite State University with protocol number 2019-001, the University of Eastern Philippines with protocol number 2020-0001, and the University of the Philippines Los Baños with protocol number 2019-0027.

2.2 Sample Collection

The sample size calculation was conducted according to Thompson's procedure [19]. A total of 865 samples were collected from poultry farms and wet markets across Regions 1, 2, 3, 4, 5, 6, 7, 8, 10, and 11, as well as from the National Capital Region and the Cordillera Administrative Region in the Philippines.

Stratified random sampling was used, in which the overall population was divided into strata (regions 1, 2, 3, 4, 5, 6, 7, 8, 10, 11, NCR, and CAR), followed by province, then cities/municipalities, and finally farms. Using 50% prevalence estimate which is the default prevalence to calculate for the largest possible sample size, accuracy at 5% and 95% level confidence.

2.3 Microbiological Methods

Sterile cotton-tipped applicator sticks were used to collect cloacal and organ/tissue samples from sick and necropsied poultry. The cloacal and organ/tissue samples were collected from individual birds. The protocols established by Kebede et al. [20] and Shirota *et al.* [21] for isolating *S. enterica* from swabs, organ/tissue, and eggshells were followed with slight modifications. The eggs were pooled with 10 eggs per pool. The swab samples were then aseptically placed in test tubes containing buffered peptone water (BPW) (Himedia®, HiMedia Laboratories, India). These tubes were accurately labeled and placed in an ice box for transportation to the laboratory for further isolation.

The samples submerged in BPW were incubated at 37 °C for 18 hours. Next, 0.1 mL of the broth culture was inoculated into 9.9 mL of Rappaport Vassiliadis (RV) broth (HiMedia®, HiMedia Laboratories, India) as an enrichment medium and incubated at 42 °C for 24 hours. Then, a loopful of the sample from the RV broth was streaked onto xylose lysine deoxycholate (XLD) agar (HiMedia®, HiMedia Laboratories, India) and incubated at 37 °C for 24 hours. Red colonies, with or without black centers, that developed on the XLD agar were presumed to be *S. enterica* serovars and were subsequently purified on nutrient agar (NA) plates using the quadrant streak plate method. The purified isolates were then transferred onto NA slants, incubated at 37 °C for 24 hours, and stored at 4 °C until use. All

procedures for isolation and handling of live bacteria were conducted in a Class II biosafety cabinet (Telstar Bio II Advance Plus®).

2.4 Morphological Observation and Biochemical Characterization

The presumptive *S. enterica* isolates were subjected to the Gram staining technique described by Cheesbrough [22]. As previously reported, all Gram-negative, rod-shaped organisms underwent standard biochemical methods [21,22] primarily oxidase test, catalase test, indole, methyl red, Voges-Proskauer, citrate utilization (IMViC) tests, and urease test. A reference strain of *S. enterica* was used as a positive control, and uninoculated media served as the negative control for all the tests mentioned above, allowing for comparison.

2.5 Genomic DNA Extraction

Isolates exhibiting typical biochemical characteristics of *Salmonella* spp. underwent genomic DNA extraction. Fifty-six bacterial isolates were inoculated onto XLD agar (HiMedia®, HiMedia Laboratories, India) for 24 hours. After incubation, three to five isolated colonies were selected and suspended in 1 mL of phosphate-buffered saline (PBS) (Thermo Fisher Scientific®, North Carolina, USA), and DNA extraction was performed using a commercial DNA extraction kit (DNeasy®, Qiagen, California, USA), adhering to the manufacturer's instructions.

2.6 Molecular Confirmation of *S. enterica* Isolates by Amplification of 16S rRNA and Virulence Genes Associated with SPI-1 and SPI-2

Polymerase chain reaction (PCR) targeting the bacterial *16S rRNA* gene was performed to confirm that the isolates were bacteria. Subsequently, the isolates that were confirmed to have the *16S rRNA* gene were subjected to PCR to detect the presence of the virulence genes of *S. enterica,* the *invA* and *sseC* genes.

Amplification of the target gene was performed using a commercial PCR kit (SapphireAmp® Fast PCR Master Mix, Takara Bio Inc., Shiga, Japan), along with forward and reverse primers and the extracted DNA template. Table 1 presents the sequences of the primers used, including those for the target gene and the reference. The samples were analyzed in a gradient thermocycler (SimpliAmp™ Thermal Cycler, Thermo Fisher Scientific®, North Carolina, USA). The PCR cycling conditions employed are outlined in Table 2.

Table 1. Primer sequences used in the study.

PRIMER	SEQUENCE (5' TO 3')	TARGET GENE	AMPLICON SIZE	REFERENCE
F- *S. enterica* 16S rRNA	TGTTGTGGTTAATAACCGCA	*S. enterica 16S rRNA*	572	*Nyabundi et al.* (2017) [23]
R- *S. enterica* 16S rRNA	CACAAATCCATCTCTGGA			
F-invA	GTGAAATTATCGCCACGTTCGGGCAA	*invA*	284	*Salehi et al.* (2005) [24]
R-invA	TCATCGCACCGTCAAAGGAACC			
F-sseC	TATGGTAGGTGCAGGGGAAG	*sseC*	121	*Fazl et al.* (2013) [25]
R-sseC	CTCATTCGCCATAGCCATTT			

Table 2. Thermal profiles of the PCR protocols used in the study.

Target Gene	Initial Denaturation	Denaturation	Annealing	Extension	Cycles	Final Extension
16S rRNA	95˚C, 3 min	95˚C, 2 min	55˚C, 30 s	72˚C, 1 min	35	72˚C, 10 min
invA	94˚C, 1 min	94˚C, 1 min	64˚C, 30 s	72˚C, 30 s	35	72˚C, 5 min
sseC	95˚C, 5 min	95˚C, 1 min	50˚C, 1 min	72˚C, 1 min	35	72˚C, 5 min

PCR amplicons were analyzed through electrophoresis using a 1.2% (w/v) agarose gel (Vivantis®, Malaysia) in 1X Tris Acetate EDTA buffer (Vivantis®, Malaysia), which included GelRed® (Biotium, CA, USA) for staining. A molecular weight standard (Vivantis®, Malaysia) was used to estimate the size of the amplified fragments. Genomic DNA from a known *S. enterica* strain and sterile distilled water served as positive and negative controls in each assay, respectively. Electrophoresis was performed at 120 volts for 35 minutes using a Mupid® system (USA), and the results were visualized with a gel documentation system (GelOne®, Cleaver Scientific Ltd., United Kingdom).

3. Results

From the 865 poultry and market samples, 56 or 6.5%, were identified as putative *S. enterica* isolates which appeared as red colonies with or without black centers, or as completely black colonies, on XLD agar (Himedia®), and were gram-negative rod-shaped bacteria. The results in the biochemical tests were as follows: oxidase negative, catalase positive, indole negative, methyl red positive, Voges-Proskauer negative, citrate utilization positive (IMViC) or - + - +, and urease negative. These isolates were subsequently confirmed using 16S rRNA primers. These samples originated from various regions in the Philippines, with 62.5% coming from Cavite (Table 3).

Table 4 shows the percentage of positive results for the putative Salmonella isolates for the 16S rRNA gene and the SPI-1 and SPI-2 virulence genes of interest. Among the 56 *S. enterica* isolates, 69.6% (39/56) possessed *invA* and *sseC*, 14.3% (8/56) harbored *invA* alone (Fig. 1), 3.6% (2/56) lacked both virulence genes, and none of the isolates harbored *sseC* alone.

Table 3. Geographical location of the putative Salmonella isolates (n=56) and type of samples collected.

Geographical location	%	Type of sample
National Capital Region	25	Eggs
Pampanga (Region III)	7.1	Broiler chicks
Cavite (Region IV)	62.5	Broiler and poultry giblets
Laguna (Region IV)	3.6	Breeder
Iloilo (Region VI)	1.8	Broiler chicks

Table 4. Percent positive of the putative Salmonella isolates for the *16S rRNA*, and the SPI-1 and SPI-2 virulence genes of interest.

Genes	Number Tested	Number Positive	% Positive (95% CI)
16S rRNA	56	56	100.0 (93.6, 100.0)
invA	56	48	85.7 (74.6, 93.0)
sseC	56	39	69.6 (57.0, 81.6)

4. Discussion

Salmonellae are widespread bacteria present in animals, humans, and the environment, promoting transmission and cross-contamination [20]. Janda and Abbott emphasized the routine use of *16S rRNA* gene sequences as a key tool in bacterial taxonomy and phylogeny [26]. It serves as a valuable complement to routine clinical microbiology [27]. The *16S rRNA* gene is the region of DNA that is most frequently used to classify bacteria because it is conserved [28]. The degree of conservation arises from its critical role in cell function [29]. No gene has demonstrated as broad applicability across all taxonomic groups as the 16S rRNA gene, which can be problematic. Thus, Clarridge noted that if the goal is to differentiate species within a particular genus, a better gene than the 16S rRNA gene may be found for identifying species [8], which is why in this study, both the *invA* and *sseC* genes were also targeted.

All putative isolates were confirmed as *S. enterica* by PCR targeting the 16S rRNA gene, and their virulence potential was further characterized by PCR detection of the invA and sseC genes. However, it has been reported that validated primers targeting the *invA* and *16S rRNA* genes may yield false-positive signals with isolates of *Citrobacter* spp., *E. coli*, and *Serratia* spp. [30], prompting the development of alternative assays targeting the *ttrA/C* genes to distinguish *S. enterica* from non-Salmonella isolates. Building on these observations, future studies on local poultry strains could integrate more advanced synthetic DNA tools [31,32], alongside whole-genome sequencing and BLAST-based comparative analysis, to further refine the detection and characterization of *S. enterica* populations in the Philippines [33].

The *invA* gene promotes and controls the initial phase of Salmonella pathogenesis. It is the most common and clinically significant genetic marker for Salmonella serovars [34]. The use of *invA* in PCR for *Salmonella* spp. was first proposed by Rahn *et al.* in 1992 and is employed to differentiate Salmonella from other bacteria. It serves as a common molecular target for Salmonella-specific detection methods [35]. The most prevalent serovars associated with human salmonellosis worldwide are *S.* Typhimurium and *S.* Enteritidis, and both commonly carry the *invA* virulence gene [36].

Numerous studies from abroad have demonstrated the utility of *invA* as a target gene for Salmonella detection in various animal and environmental samples. The *invA* gene plays a crucial role not only in Salmonella detection but also in the processes of colonization and invasion within the intestinal epithelium of free-range chickens [37]. It serves as a dependable PCR target for Salmonella detection in different samples worldwide, as evidenced by Sunar *et al.* (2010) where the *invA* gene exhibited a highly specific alignment with sequences from Salmonella species found in compost samples [38]. The *invA* virulence gene was identified in milkfish samples in Indonesia [39]. In one study, the *invA* gene was found in 100% of Salmonella isolates from poultry farms [40].

Eight isolates (14.3%) that tested positive for the *invA* gene alone were identified in eggs (50%), cloacal swabs (37.5%), and giblets (12.5%). Additionally, 14 (25%) of the *S. enterica* isolates were sourced from eggs, and among these, 71.4% exhibited both virulence genes, while 28.6% had *invA* only. A report has indicated that phylogenetic analysis of the *invA* gene sequences from their Salmonella isolates in human and egg samples clustered together, suggesting a common source of infection [36]. Their results emphasized that chickens are a significant source of Salmonella and that humans primarily acquire the infection from these sources [41]. A local study also evaluated the presence of the *invA* gene and other genes related to SPI-1 in *S. enterica* isolates from swine and poultry in the Philippines, highlighting the potential risk these animal isolates pose to humans [33].

Additionally, giblets can harbor Salmonella when macrophages ingest the bacteria but fail to eliminate it, allowing systemic infection [42]. A study found an overall detection rate of 29% of *S.* Typhimurium in giblets from retail chickens sold in Egypt [43].

The overall detection rate of *invA* in this study is 85.7% (48/56), while the detection rate for *sseC* is 69.6% (39/56). Theoretically, isolates that contain *invA* but lack *sseC* can still be virulent because *invA* enables the organism to invade eukaryotic cells [44]. None of the isolates found to possess the 16S rRNA gene were only positive for the *sseC* gene. The *sseC* gene allows the organism

to affect biological processes and transport proteins directly into the host cell cytoplasm [45].

The SPI-1 encodes the first type III secretion system (SPI-1 T3SS) that Salmonella uses to facilitate the infiltration of non-phagocytic cells [46]. In contrast, SPI-2 encodes a second type III secretion system (SPI-2 T3SS) that enables evasion of NADPH oxidase–mediated oxidative defenses by preventing oxidase localization to Salmonella-containing vacuoles [47]. The *sseC* gene is crucial for establishing systemic infection [48], and SseC, together with SseD, facilitates bacterial translocation [49].

Dual targeting of the *invA* and *sseC* genes was conducted because Salmonella virulence is regulated by a complex network [44]. Esquivel-Hernandez *et al.* (2018) confirmed that dual detection yields robust and artifact-free amplicons [50].

The presence of virulence genes in Salmonella is associated with salmonellosis in humans [51]. Mutations in these genes lead to decreased virulence [7]. Serovars pathogenic to poultry include *S.* Pullorum and *S.* Gallinarum, while zoonotic ones include *S.* Typhimurium and *S.* Enteritidis [52]. Garcia *et al.* reported *S.* Enteritidis in eggs and broilers [53].

Two isolates (3.6%) that lacked both virulence genes may represent reduced virulence strains. Other virulence factors include plasmids, flagella, capsules, and alternative SPIs [48]. The *spv* gene, plasmid-encoded, is one example [54].

Molecular analyses of Salmonella virulence factors *invA* and *sseC* shed light on its diversity as well as the regulation of pathogenicity. These findings, together with ecological and epidemiological information, are crucial for developing control measures at the farm and community level. Therefore, effective biosecurity measures, including vaccination, must consider the complexity of gene expression, host-pathogen interaction, and environmental factors.

5. Conclusions

Determining the presence of *invA* and *sseC* genes, which are key virulence factors associated with SPI-1 and SPI-2 in *S. enterica*, is essential for evaluating the organism's virulence potential. Additionally, it has been found that poultry, including eggs and giblets from various regions in the Philippines, harbors potentially virulent strains of *S. enterica*. As the *invA* and *sseC* genes contribute to regulatory networks underlying Salmonella pathogenicity in poultry, their detection could help in the development of diagnostic approaches to improve prevention, treatment and control strategies of Salmonella infections in these animals, ultimately benefiting the poultry-consuming public in the long term.

Availability of Data and Materials

All data are presented in the manuscript.

Author Contributions:

Conceptualization – M.C.R.D.C., G.A.C., and D.V.U.; Methodology – M.C.R.D.C., G.A.C., Y.R.M.T., R.D.D., M.L.G.A., G.M.R.G. and D.V.U.; Investigation – M.C.R.D.C., G.A.C., Y.R.M.T., R.D.D., M.L.G.A., G.M.R.G., and D.V.U.; Writing – Original Draft, M.C.R.D.C., G.A.C., Y.R.M.T., M.L.G.A., and D.V.U.; Writing – Review & Editing, M.C.R.D.C.; Funding Acquisition – M.C.R.D.C., G.A.C., and D.V.U.; Resources – M.C.R.D.C., G.A.C., R.D.D., and D.V.U.; Supervision – M.C.R.D.C., G.A.C., and D.V.U.; Project Administration – M.C.R.D.C., G.A.C., and D.V.U.

Ethics Approval and Consent to Participate

All animal procedures were reviewed and approved by the Institutional Animal Care and Use Committees (IACUC) of Cavite State University, the University of Eastern Philippines, and the University of the Philippines Los Baños, and permissions were obtained from farm owners/market authorities prior to sample collection; no human participants were involved.

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Conflict of Interest

The authors declare no conflict of interest.

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