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ORIGINAL ARTICLE

Molecular Characterization of *Salmonella* Isolates Circulating in the Layer Farms of Bangladesh

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Abstract

Background: *Salmonella* infections are a major concern for the layer poultry industry in Bangladesh and pose a significant public health risk due to their zoonotic potential. Effective control depends on accurate isolation and molecular identification of circulating strains. Therefore, this study aimed to isolate and molecularly characterize *Salmonella* strains circulating in layer farms in Bangladesh.

Methods: The study was conducted at Krishibid Farm, Valuka Upazila, Mymensingh district, and in the Department of Pathology, Faculty of Veterinary Science, Bangladesh Agricultural University, Mymensingh. Cloacal swab samples were collected from layer chickens. Isolation was performed using standard bacteriological culture techniques. DNA was extracted from cultured isolates, quantified, and subjected to PCR targeting the *invA* and ITS genes. Amplified products were purified using a gel extraction method and subsequently sequenced. Sequence data were analyzed using BLAST, and phylogenetic relationships were assessed through phylogenetic tree construction.

Results: PCR amplification identified *Salmonella enteritidis* and *Salmonella gallinarum* in 32 out of 100 field samples. Sequence analysis and BLAST comparison demonstrated 100% homology with corresponding sequences available in GenBank. Alignment of nucleotide and deduced amino acid sequences revealed no mutations relative to reference strains. Five representative isolates were selected for phylogenetic analysis. The constructed phylogenetic tree showed that the isolates clustered in a monophyletic group with global reference strains.

Conclusion: The molecular identification and characterization of circulating Salmonella strains provide essential information for controlling zoonotic transmission, particularly of Salmonella enteritidis, to humans. These findings also support efforts to improve biosecurity and facilitate the production and potential export of Salmonella-free poultry meat and eggs.

Keywords: *Salmonella,* invA and ITS gene, PCR, sequencing, phylogenetic analysis.

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Introduction

Bangladesh's poultry sector plays a vital role in national economic growth by generating employment opportunities and contributing substantially to income generation, poverty reduction, and improved human nutrition through the supply of meat and eggs (Islam, 2005; Saleque and Ansarey, 2020). Despite this progress, the industry faces multiple constraints that limit its full potential. These include infectious diseases, poor husbandry practices, low productivity, indiscriminate use of antibiotics, unstable market conditions, high input costs with comparatively low product prices, inadequate vaccination coverage, and feed shortages (Haque *et al*., 1991; Jabbar *et al*., 2011; Islam *et al*., 2014).

Among infectious diseases, Salmonella infections are of particular importance in the layer industry of Bangladesh due to their significant economic and public health implications. Salmonellosis is a bacterial disease affecting poultry of all ages and both sexes. It is associated with high morbidity and mortality in chicks and reduced egg production in layers, resulting in considerable economic losses. Moreover, Salmonella spp. are major food-borne pathogens, posing a substantial zoonotic risk to humans. Infection may occur through the handling, transport, or consumption of contaminated or undercooked poultry meat and eggs (Bailey and Cosby, 2003; Kimura *et al*., 2004). In many countries, particularly in developing regions, salmonellosis is recognized as a leading food-borne disease (Soultos *et al*., 2003; Carraminana *et al*., 2004).

Ensuring Salmonella-free poultry products is essential for protecting public health and for accessing international export markets. Molecular techniques such as polymerase chain reaction (PCR) have become reliable tools for rapid and specific identification of Salmonella isolates (Malorny *et al*., 2003). Primers targeting conserved genes such as invA and the internal transcribed spacer (ITS) region are widely used for molecular confirmation (Oliveira *et al*., 2003).

In Bangladesh, layer chickens are typically reared for extended production cycles of up to approximately 100 weeks. However, routine vaccination programs against Salmonella are not widely implemented, and a standardized national strategy for Salmonella control in poultry remains limited. Given the growing potential for poultry product export, systematic isolation, identification, and molecular characterization of *Salmonella* circulating in layer farms are essential. Such investigations provide critical epidemiological data necessary for disease control, public health protection, and strengthening the competitiveness of Bangladesh's poultry sector in international markets.

Meterials and Methods

Study Area

The study was conducted at Krishibid Farm, Valuka Upazila, Mymensingh district, Bangladesh. Laboratory analyses were performed at the Department of Pathology, Faculty of Veterinary Science, Bangladesh Agricultural University (BAU), Mymensingh.

Sample Collection

A total of approximately 1,000 layer chickens were included in the study. Cloacal swab samples were collected at seven different age points: 16, 18, 30, 42, 54, 66, and 78 weeks. For each time point, 160 cloacal swabs were collected randomly, representing about 2% of live birds. Sampling at 16 weeks represented the pre-vaccination period, while the remaining ages corresponded to post-vaccination periods to monitor Salmonella shedding over time.

Swabs were collected aseptically, immediately inoculated into Tetrathionate Broth (TTB) for selective enrichment, and transported in a cool box with ice packs to the Department of Pathology, BAU. Upon arrival, samples were stored at −20 °C until bacteriological and molecular analyses.

Oligonucleotide Primers

Two sets of primers were used for PCR amplification of the invA and internal transcribed spacer (ITS) genes of Salmonella isolates (Table 1).

For *Salmonella enteritidis* (invA gene):

Forward (invAF): 5′-GTGAAATTATCGCCACGTTCGGGCAA-3′

Reverse (invAR): 5′-TCATCGCACCGTCAAAGGAACC-3′

For *Salmonella gallinarum* (ITS gene):

Forward (ITSF): 5′-TATAGCCCCATCGTGTAGTCAGAAC-3′

Reverse (ITSR): 5′-TGCGGCTGGATCACCTCCTT-3′

These primers were originally described by Rahn *et al.* (1992) and are widely used for molecular identification of Salmonella species.

Table 1: Oligonucleotide primers with their fragment length used for the amplification of *invA* and *ITS* genes of *Salmonella enteritidis* and *Salmonella gallinarum*

Primers	Gene	Primer Sequence (5′-3′)	Amplicon Size	Reference
*inv*AF	*inv*A	GTGAAATTATCGCCACGTTCGGGCAA	284bp	Rahn
*inv*AR	*inv*A	TCATCGCACCGTCAAAGGAACC		*et. al.*,
ITSF	*ITS*	TATAGCCCCATCGTGTAGTCAGAAC	312bp	1992
ITSR	*ITS*	TGCGGCTGGATCACCTCCTT		

DNA Visualization, Quantification, and Storage

PCR products and DNA samples were visualized using a transilluminator, AlphaImager HP® (Cell BioSciences Pty Ltd., Australia). DNA concentration and purity were measured with a spectrophotometer (BioSpec-nano®, Shimadzu Corporation, Japan). Nuclease-free water was used for blank measurements, and 1 µl of each DNA sample was measured in a 0.185 mm pathlength chamber. DNA concentration was expressed in ng/µl, and purity as the OD260/OD280 ratio. Samples with OD values ranging from 1.7 to 2.0 were considered sufficiently pure. High-concentration DNA was diluted to 350 ng/µl, while samples with ~200 ng/µl were also used for downstream applications. DNA was stored at −20 °C until PCR amplification.

PCR Amplification of Extracted DNA

PCR was performed on DNA extracted from 100 isolates, of which 32 were PCR-positive. The invA gene (284 bp) and ITS gene (312 bp) were amplified using the primers described above. PCR reactions were prepared in 50 µl volumes using Dream® Taq Green 2X Master Mix (Thermo Scientific, USA) in a biosafety cabinet to avoid contamination. Each reaction contained: 25 µl of 2X Master Mix, 1 µl of each primer (20 pmol/µl; final concentration 0.4 µM), 200–350 ng template DNA and nuclease-free water to a final volume of 50 µl (Table 2). PCR amplification was performed on a ProFlex™ PCR System (Applied Biosystems, USA) using a previously standardized thermal cycling profile. Positive and negative controls, along with a DNA ladder, were included in each run. Amplification was confirmed by gel electrophoresis.

Purification of PCR Products

PCR products were re-amplified in 100 µl total volume (50 µl × 2) for purification. Five microliters of each sample were checked via electrophoresis to confirm the presence of desired bands. The remaining amplified DNA was purified using the PureLink® Quick Gel Extraction Kit (Invitrogen™, USA; catalog no. K2100-12) according to the manufacturer's protocol. Purified DNA concentrations ranged from 20 to 80 ng/µl with OD260/280 values of 1.7–2.0, suitable for sequencing.

Sequencing of Purified PCR Amplicons

Purified PCR products were sent to 1st BASE Sdn Bhd, Malaysia, for commercial sequencing. For each sample, 30 µl DNA was prepared (15 µl for forward primer reaction and 15 µl for reverse primer reaction). Primer volumes of 50 µl each (forward and reverse) were also included. Samples and primers were labeled and sealed with parafilm and sent via the company's local representative.

Sequence Editing, Analysis, and Phylogenetic Analysis

Raw sequence data were checked and edited using BioEdit® software. Consensus sequences were generated and saved in FASTA format. Sequences were compared against NCBI GenBank entries using BLAST to assess homology. Homologous sequences were selected based on location, year, and identity for phylogenetic analysis.

Multiple sequence alignments were performed using Clustal W, and phylogenetic trees were constructed using the neighbor-joining method in MEGA-X software (Kumar *et al.*, 2018). Node stability was tested with 1,000 bootstrap replications (Felsenstein, 1985). Evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura *et al.*, 2004), measured as the number of base substitutions per site. Phylogenetic trees were analyzed to determine the evolutionary relationships between the isolates and global Salmonella sequences from GenBank.

Table 2: Composition of 50µl reaction mixture used for PCR amplification of template DNA (tDNA)

Component	Volume/ Reaction
PCR Mastermix,2x	25µl
Primer forward (20pmol/µl)	1µl
Primer reverse (20pmol/µl)	1µl
Template DNA (200-350ng/µl)	1µl
Nuclease-free water	22µl
Total Volume	50 µl

Results

Following the culture-based identification, the Salmonella isolates were confirmed by PCR amplification of genomic DNA. Genomic DNA was extracted from 100 locally isolated organisms as described in the Materials and Methods section. Out of these, 32 isolates tested positive by PCR.

PCR amplification using gene-specific primers successfully amplified the invA and ITS genes, producing fragments of 284 bp and 312 bp, respectively (Figures 1 and 2). The presence of a 284 bp amplicon indicated Salmonella enteritidis, whereas a 312 bp amplicon corresponded to *Salmonella gallinarum* (Figures 1 and 2).

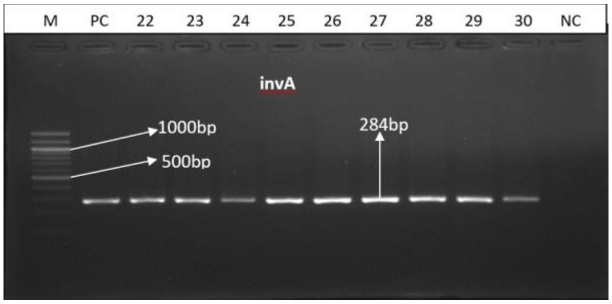


Figure 1: Detection of *Salmonella enteritidis* from experimental birds by PCR. Electrophoresis on 1.5% agarose gel showing 284 bp invA gene amplicons in lanes 22–30. PC: positive control showing expected 284 bp band; NC: negative control with no amplification; M: 100 bp DNA ladder (molecular weight marker).

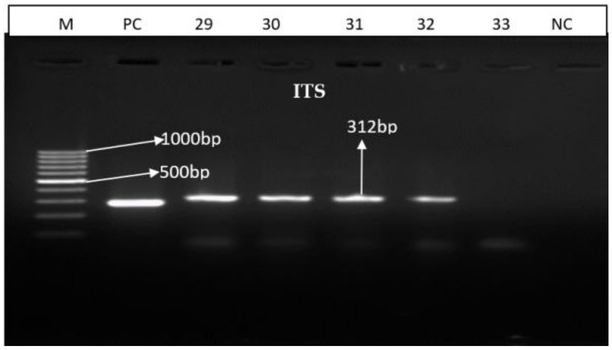
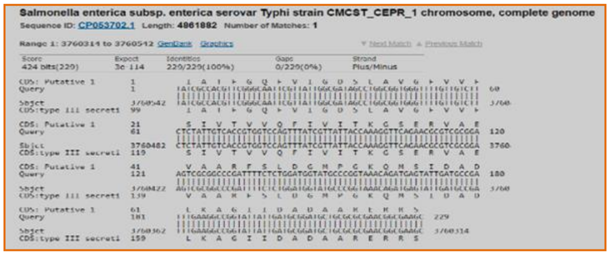


Figure 2: Detection of *Salmonella gallinarum* from experimental birds by PCR. Electrophoresis on 1.5% agarose gel showing 312 bp ITS gene amplicons in lanes 29–33. PC: positive control showing expected 312 bp band; NC: negative control with no amplification; M: 100 bp DNA ladder (molecular weight marker).

Sequence Alignment and Phylogenetic Analysis

BLAST analysis of the consensus sequences for each gene revealed 100% homology with corresponding sequences available in GenBank. Multiple sequence alignment showed no nucleotide substitutions compared to the reference sequences (Figures 3 and 4). Similarly, analysis of the coding regions revealed no changes at the amino acid level, indicating that the sequences encode functional type III secretion systems of *Salmonella enteritidis* and *Salmonella gallinarum*. Sequences with high similarity to the local isolates were selected from GenBank based on year, geographic location, and sequence identity. These sequences, along with the study isolates, were used to construct phylogenetic trees. The resulting phylogenetic analysis demonstrated that the isolates formed a monophyletic group with the selected reference sequences (Figure 5), confirming their evolutionary relationship.

The sequences generated in this study were submitted to GenBank and assigned accession numbers (Table 3).

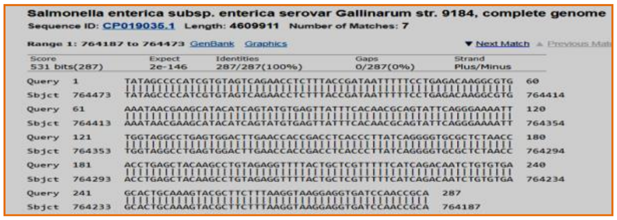


```
Salmonella enterica subsp. enterica serovar Typhi strain CMCST_CEPR_1 chromosome, complete genome
Sequence ID: CP053702.1  Length: 4861882  Number of Matches: 1

Range 1: 3760314 to 3760512 GenBank  Graphics                    ▼ Next Match  ▲ Previous Match

Score            Expect     Identities        Gaps          Strand
424 bits(229)    3e-114     229/229(100%)     0/229(0%)     Plus/Minus
```

Figure 3: Alignment of nucleotide sequences of *invA* gene of *Salmonella enteritidis* showed 100% homology with the available sequences of the GenBank. No changes were observed in the nucleotide level and amino acid level.



```
Salmonella enterica subsp. enterica serovar Gallinarum str. 9184, complete genome
Sequence ID: CP019035.1  Length: 4609911  Number of Matches: 7

Range 1: 764187 to 764473 GenBank  Graphics                    ▼ Next Match  ▲ Previous Mat

Score            Expect     Identities        Gaps          Strand
531 bits(287)    2e-146     287/287(100%)     0/287(0%)     Plus/Minus

Query  1       TATAGCCCCATCGTGTAGTCAGAACCTCTTTACCGATAATTTTTCCTGAGACAAGGCGTG  60
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  764473  TATAGCCCCATCGTGTAGTCAGAACCTCTTTACCGATAATTTTTCCTGAGACAAGGCGTG  764414

Query  61      AAATAACGAAGCATACATCAGTATGTGAGTTATTTCACAACGCAGTATTCAGGGAAAATT  120
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  764413  AAATAACGAAGCATACATCAGTATGTGAGTTATTTCACAACGCAGTATTCAGGGAAAATT  764354

Query  121     TGGTAGGCCTGAGTGGACTTGAACCACCGACCTCACCCTTATCAGGGGTGCGCTCTAACC  180
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  764353  TGGTAGGCCTGAGTGGACTTGAACCACCGACCTCACCCTTATCAGGGGTGCGCTCTAACC  764294

Query  181     ACCTGAGCTACAAGCCTGTAGAGGTTTTACTGCTCGTTTTTCATCAGACAATCTGTGTGA  240
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  764293  ACCTGAGCTACAAGCCTGTAGAGGTTTTACTGCTCGTTTTTCATCAGACAATCTGTGTGA  764234

Query  241     GCACTGCAAAGTACGCTTCTTTAAGGTAAGGAGGTGATCCAACCGCA  287
               |||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  764233  GCACTGCAAAGTACGCTTCTTTAAGGTAAGGAGGTGATCCAACCGCA  764187
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Figure 4: Alignment of nucleotide sequence of ITS gene of *Salmonella enterica* serover *gallinarum* strain showed 100% homology with the GenBank sequences. No changes were observed in the nucleotide level and amino acid level

Phylogenetic Analysis

Phylogenetic analysis revealed that the isolated strain kbd-01L (GenBank accession number MZ956130) clustered closely with *Salmonella enterica* strain 392869-2 from the USA. The local isolates kbd-02L (MZ893332), kbd-03L (MZ893333), kbd-04L (MZ893334), and kbd-05L (MZ893335) showed close evolutionary relationships with *Salmonella enterica* subsp. *enterica* serovar *gallinarum* strain 11AlxB from Egypt, Salmonella enterica subsp. enterica strain SE124 from China, *Salmonella enterica* subsp. *enterica* strain SCMCST CEPR1 from India, and *Salmonella enterica* subsp. *enterica* strain CVM 30155 from the USA, respectively.

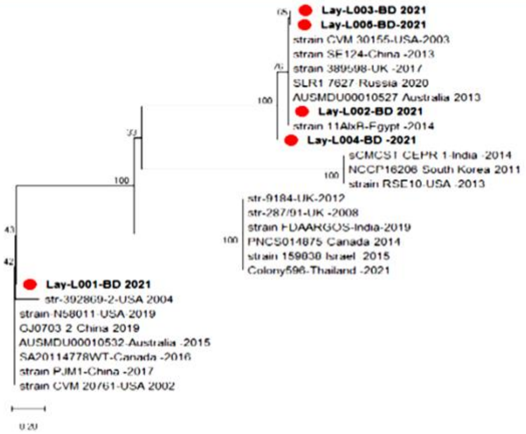

Figure 5: Phylogenetic tree of Salmonella isolates based on invA and ITS gene sequences. The tree includes 22 nucleotide sequences, comprising locally isolated strains and reference sequences from GenBank, and was constructed using the Neighbor-Joining method with 1,000 bootstrap replicates. All local Salmonella isolates formed a monophyletic group. Evolutionary analyses were performed using MEGA-X software.

Table 3: List of isolates and strain with the corresponding identification and accession numbers on GenBank for sequence analysis

Sl. No.	Strain ID	Strain Name	Host	Accession No.
1	kbd-01L	*Salmonella enterica*	Layer	MZ956130
2	kbd-02L	*Salmonella enterica* subsp. *Enterica* serovar Gallinarum	Layer	MZ893332
3	kbd-03L	*Salmonella enteric* subsp. *enterica*	Layer	MZ893333
4	kbd-04L	*Salmonella enteric* subsp. *enterica*	Layer	MZ893334
5	kbd-05L	*Salmonella enteric* subsp. *enterica*	Layer	MZ893335

Discussion

Isolation and identification of Salmonella traditionally rely on culture using selective media followed by biochemical characterization (Haider *et al.*, 2003). Although effective, these conventional methods are time-consuming and labor-intensive, particularly when large numbers of samples are processed. DNA extraction of Salmonella strains in the present study was performed using the boiling method (Murugkar *et al.*, 2003), a simple and cost-effective approach among the various available DNA extraction techniques (Forbes, 2003).

Polymerase chain reaction (PCR) has emerged as a powerful tool in microbiological diagnosis due to its rapidity, sensitivity, and specificity (Malorny *et al.*, 2003). PCR-based assays can also facilitate differential diagnosis between closely related serovars such as *Salmonella gallinarum* and *Salmonella pullorum* (Park *et al.*, 2001; Karine *et al.*, 2002). Compared to traditional phenotyping and serotyping methods, PCR offers a faster and more reliable alternative for identification at both genus and serovar levels.

In particular, PCR targeting the invA gene is widely recognized as a rapid and specific method for detecting Salmonella at the genus level (Osman *et al.*, 2010). The invA gene encodes a protein located in the inner membrane of the bacterium and is essential for invasion of host epithelial cells (Darwin and Miller, 1999). Because the invA gene contains highly conserved sequences across Salmonella species (Galan and Curtiss, 1991), it serves as a reliable molecular marker. Shi *et al.* (2012) further reported that the invA gene is present and functional in most, if not all, virulent Salmonella strains. Several studies have successfully detected Salmonella isolates from poultry using invA-specific primers (Hoorfar *et al.*, 1992; Oliveira *et al.*, 2002; Oliveira *et al.*, 2003; Ammar *et al.*, 2016).

In the present study, *Salmonella gallinarum* isolates were enriched in Tetrathionate Broth (TTB), and extracted DNA was amplified using invA-specific primers. A 284 bp amplicon was observed following electrophoresis on 1.5% agarose gel, consistent with previously reported findings (Hoorfar *et al.*, 1992; Oliveira *et al.*, 2003). Sequence alignment of the amplified invA and ITS gene fragments revealed 100% homology with corresponding Salmonella sequences available in GenBank. These findings are consistent with the observations of Shi *et al.* (2012), although Jarvik *et al.* (2010) reported slightly lower similarity (98%) among certain Salmonella strains.

Phylogenetic analysis demonstrated that the local isolates (kbd-01L, kbd-02L, kbd-03L, kbd-04L, and kbd-05L) clustered closely with *Salmonella enterica* subsp. *enterica* strains, including *S. gallinarum*, reported from the USA, Egypt, China, and India. The isolates formed a monophyletic group with global reference strains, indicating a high degree of genetic conservation and close evolutionary relationships.

Overall, the molecular findings of this study confirm the reliability of PCR targeting the invA and ITS genes for accurate identification and characterization of Salmonella isolates from poultry. The observed genetic homogeneity with international strains highlights the conserved nature of these virulence-associated genes and underscores the importance of molecular surveillance for effective disease control.

Funding

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Data Availability Statement

The data used to support the findings of this study are available from the corresponding author upon reasonable request.

Conflict of Interest

The authors declare no conflict of interest

Conclusion

Salmonella infections are a significant concern in the layer industry of Bangladesh and can cause substantial economic losses. In this study, Salmonella isolates from a layer farm were successfully identified and characterized using PCR, sequencing, and phylogenetic analysis. PCR amplification of the invA and ITS genes confirmed the presence of *Salmonella enteritidis* and *Salmonella gallinarum*, and sequence analysis showed 100% homology with reference strains in GenBank. Phylogenetic analysis demonstrated that the isolates formed a monophyletic group with global Salmonella strains, indicating high genetic conservation. These results confirm that molecular methods provide an accurate and reliable approach for the identification and characterization of Salmonella isolates. The findings support the use of such techniques for disease surveillance in poultry and provide baseline data that can inform strategies to reduce Salmonella infection in layer flocks.

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