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# Molecular Characterization, Biochemical Isolation and Identification of Salmonella species from Poultry Liver and Fecal Samples at Dinajpur, Bangladesh

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## ABSTRACT

Poultry products are the main means of transmission for Salmonellosis, a significant foodborne infection in the world. The purpose of this work was to separate, biochemically identify, and molecularly describe Salmonellosis from samples of poultry feces and liver. A total of 150 samples (50 livers, 100 cloacal swabs) were collected from Dinajpur broiler farms and transferred to the Department of Microbiology, HSTU, Dinajpur, in an ice box with PBS by maintaining aseptic conditions. The samples were streaked in Nutrient Agar 37°C following incubation for 24 hour and sub-cultured on selective media MacConkey Agar at 37°C for 24 hours. Then Salmonella was identified in Gm staining technique. Then Colonies were streaked to S.S Agar(Salmonella Shigella Agar) and finally biochemical tests like Motility Indole Urease (MIU), Simmons citrate, Indole, Methyl Red, and Triple Sugar Iron (TSI) utilization were positive. Then Serological test was performed, followed by PCR-based confirmation including genomic DNA isolation and gel electrophoresis. Isolates confirmed as Salmonella were serotyped and further characterized by Polymerase Chain Reaction (PCR) targeting the invA genus-specific gene. The overall prevalence of Salmonella was 13.3 %, with a higher isolation rate from fecal samples (14%) than liver samples (12%).More poultry fecal and liver samples will be studied in future to carry out research.

**Keywords:** Biochemical identification, invA gene, poultry, PCR, Salmonellosis

# INTRODUCTION

Salmonella is a Gram-negative, facultatively anaerobic bacterium belonging to the family Enterobacteriaceae. It mostly contains two species: *Salmonella Enterica* and *Salmonella Bongori.* Over 2500 serotypes of the genus Salmonella have been identified globally to date, and many of them have the potential to infect both people and animals (Berhanu and Fulasa, 2020). Most human cases of salmonellosis are caused by *Salmonella enterica ser. Enteritidis (S. Enteritidis) and Salmonella enterica ser. Typhimurium (S. Typhimurium)* which are members of the non-typhoidal Salmonella group (NTS). Globally, non-typhoidal Salmonella is responsible for roughly 93 million cases of gastroenteritis and 155,000 fatalities annually (Majowicz *et al.,* 2010). Salmonellosis is a zoonotic food-borne enteric infection that has great economic value in the animal market, notably in the chicken business (Li *et al.,* 2020). Among animals that produce food, poultry is the primary reservoir for several non-typhoidal Salmonella (NTS) serotypes. Epidemiologically relevant NTS serotypes include *S. Typhimurium, S. Enteritidis, S. Heidelberg, and S. Newport.* In North America and Europe, *S. Enteritidis* dominated the egg-borne transmission of infection to humans, whereas *S. Typhimurium* was the predominant serovar linked with external egg contamination in Australia (Howard *et al.,* 2011). Poultry birds commonly operate as asymptomatic carriers of *S. enteritidis* with the bacteria colonizing the gastrointestinal tract and later infecting systemic areas such the liver, spleen, and ovaries (Gantois *et al.,* 2009). *Salmonella enterica* and *serovar Enteritidis (S. enteritidis)*, two of the approximately 2,600 serovars, have become the most common cause of human salmonellosis and are often linked to the consumption of contaminated poultry products, especially eggs and undercooked meat. Edible organs and, in the case of laying hens become contaminated as a result of this systemic invasion. Fecal shedding also adds to environmental contamination and horizontal transmission among flocks (Dunkley *et al.,* 2009). The rise of multi-drug resistant (MDR) Salmonella resistance to clinically important antimicrobial drugs hampers therapy and pro-phylaxis. This raises infection-related morbidity and mortality, while also raising healthcare and economic expenses (Gong *et al.,* 2013). Bio-film generation by bacteria such as Salmonella is a key virulence mechanism that aids evasion of human immunity and resists drug penetration. Consequently, there is a significant risk to both the food sector and public health (Harrell *et al.,* 2021).

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# MATERIALS AND METHODS

## Collection of samples

A total of 150 samples were collected as part of the study population during the September to December 2025 period in the Dinajpur district, namely in the vicinity of the Hajee Mohammad Danesh Science and Technology University (HSTU) University. Only 50 liver samples and 100 fecal(Cloacal swabs) samples were collected from the vicinity of Hajee Mohammad Danesh Science and Technology University in Dinajpur during the first stage of sample collection. The Phosphate Buffer Saline (PBS) Solution was made to keep the sample safe while it was being transported in a plastic zipper. For microbiological analysis, the samples were sent to the Department of Microbiology at HSTU, Dinajpur, in an ice box with PBS to ensure aseptic conditions.

## PBS Preparation

## Isolation and biochemical identification of bacteria

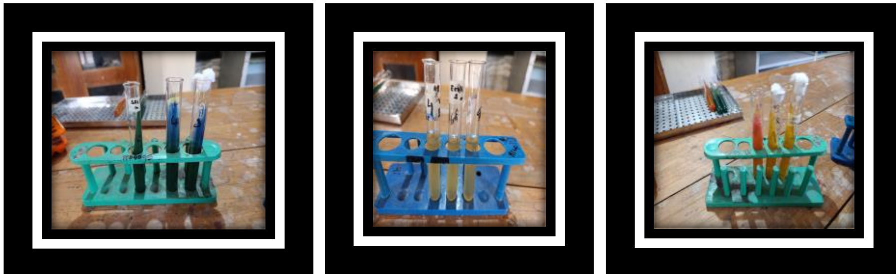
Samples were collected from broiler farms in Dinajpur and brought to the Department of Microbiology, HSTU, Dinajpur, in an aseptic ice box containing PBS. The obtained samples were prepared and cultured on nutrient agar at 37°C for 24 hours, after which samples indicating growth were selected for isolation and those without growth were rejected. The isolates were analyzed by morphology and staining to separate Gram-negative and Gram-positive bacteria; Gram-positive isolates were rejected while Gram-negative isolates were put to further culture on MacConkey agar. To isolate Salmonella species, a loopful of enriched solution was streaked in Mac-Conkey agar and incubated at 37°C for 24 hours.

**Table 1. PBS Preparation for poultry fecal and liver sample collection**

| Ingredients | Amount (per 1 L PBS) |
|---|---|
| NaCl | 8.0 g |
| KCL | 0.2 g |
| $Na_2HPO_4$ | 1.44 g |
| $KH_2PO_4$ | 0.24 g |
| Distilled Water | Up to 1 Liter |

. Presumptive Salmonella colonies showed as colorless or pale colonies on MacConkey agar and were subjected to Gram staining, which revealed Gram-negative rods. Before conducting biochemical assays including Motility Indole Urease (MIU), Simmons citrate, Indole, Methyl Red, and Triple Sugar Iron (TSI) utilization, colonies

were monitored and sub-cultured on selective media Salmonella Shigella (SS agar) at 37°C for 24 hours. Serological assays were used to establish the presence of bacteria, and then PCR-based confirmation using gel electrophoresis and genomic DNA extraction was carried out. Virulence genes will be identified in the future with more sample collections and antibiotic sensitivity tests will also be carried out using the disc diffusion method, leading to the final discovery of antibiotic resistance genes. Based on the colony morphology of the putative bacteria identified on the different selective agars, additional characterization was attempted utilizing staining techniques. Gram's staining was used to classify the isolates for the first time, confirming that they were Gram-negative bacilli. To detect their motility and flagellar arrangement, which is a major diagnostic feature, both flagellar staining using malachite green and the hanging drop slide technique were conducted. The positive isolates identified as Gram-negative bacilli were then selected for further inspection, ensuring that a pure culture from each was grown on appropriate selective media for future confirmation testing.



**Picture 1. (Simmons Citrate-Positive) (Indole -Negative) (MIU) -Positive)**

The Gram-negative isolates underwent a thorough battery of biochemical assays for final identification after the isolation and staining processes. Fructose, galactose, glucose, sucrose, and mannose were used to evaluate the isolates' capacity to ferment carbohydrates. Motility Indole Urease (MIU), Simmons citrate, Indole, Methyl Red, and Triple Sugar Iron (TSI) utilization were among the routine enterobacterial tests carried out. The specific combination of observed reactions typically positive for glucose fermentation (with gas), MR, TSI (alkaline slant/acid butt with H2S), citrate utilization, and ornithine decarboxylase, but negative for VP, indole, and urease provided a conclusive biochemical profile consistent with *Salmonella* species. Following biochemical identification, the putative *Salmonella* isolates were verified serologically using a slide agglutination test. One drop of the isolate's overnight broth culture and one drop of Polyvalent O antisera, which contains antibodies against *Salmonella's* common somatic (O) antigens, were combined on a clean glass slide for

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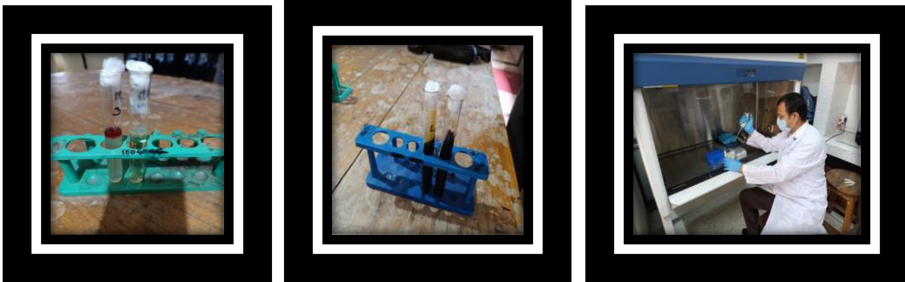
this process. After that, the slide was gently rocked for up to two minutes, and any apparent agglutination; the clumping of bacterial cells indicated a successful antigen-antibody response. Before moving on to more precise serotyping to identify the *Salmonellosis,* a positive result with the polyvalent antisera offered serological evidence that the isolate belonged to the genus *Salmonella*.



**Picture 2. (Methyl Red-Positive) (TSI-Positive) (PCR-DNA Extraction)**

**Molecular characterization of virulence genes**

To enable molecular characterization, bacterial genomic DNA was extracted from the pure cultures after serological confirmation. Two procedures were employed: a standard boiling method and a commercial kit protocol conducted according to the manufacturer's instructions. For the boiling procedure, 1.5 mL of a pure bacterial culture was transferred to a micro-centrifuge tube and pelleted. The pellet was then resuspended in 100 µL of nuclease-free distilled water. The suspension was exposed to boiling in a hot water bath at 100°C for 10 minutes to lyse the bacterial cells and denature proteins. Immediately after boiling, the tube was transported to an ice box for 30 minutes to induce a cold shock, which aids in precipitating cellular debris. The lysate was then centrifuged at 10,000 rpm for 10 minutes to separate the cellular fragments from the soluble DNA. After the genomic DNA was extracted, the supernatant was carefully collected and put into a new microcentrifuge tube to be stored at -20°C until further PCR analysis. A modified boiling cell approach was used to extract the genomic DNA from *Salmonella* isolates (Pui *et al.,* 2011). Cultured Salmonella colonies were injected into 1 mL of Luria-Bertani broth and incubated at 37◦C for 24 h. After centrifuging the bacterial culture for three minutes at 15,000 x g, the supernatant was disposed of. The pellet was resuspended in 500 µL of nuclease-free water, heated at 100◦C for 10 min, and then rapidly cooled to 4◦C for 10 min. Following this, the samples were centrifuged at 15,000 x g for 3 min. After that, the genomic DNA-containing supernatant was moved to a new Eppendorf tube and kept at 20°C until PCR amplification. Using spectrophotometry (Quawell, UV-Vis

Spectrophotometer Q5000) at 260 and 280 nm, the concentration and purity of the DNA extracted from the Salmonella culture were measured; acceptable ratios ranged from 1.6 to 2. One virulence gene *(invA)* was amplified by PCR using a Bio-Rad T100TM Thermal Cycler (Bio-Rad, USA). Five microliters of Master Mix (Invitrogen), one microliter of forward primer, one microliter of reverse primer, one microliter of DNA template, and two microliters of nuclease-free water (Ambion, REF: AM9932) were all included in each 10-microliter reaction mixture. PCR was performed to amplify the virulence genes of *Salmonella*. The Primer *InvA* -F(5'-CGGTGGTTTTAAGCGTACTCTT-3') and *InvA* -R (5' CGAATATGCTCCACAAGGTTA-3'). The cycling conditions included pre-denaturation at 95◦C for 5 min, 95◦C for 3o second, 60◦C for 30 seconds, 72◦C for 60 second with 35 cycles, 72◦C for 7 minutes and 4◦C with infinite. 1.5% agarose gels made in 0.5X TBE (Tris-Borate-EDTA) with 2 µl ethidium bromide (EtBr) were used to separate the amplified PCR products. A 1000 bp DNA ladder was loaded with microliters of PCR product combined with loading dye. DNA bands were visible under

a Ultra Violet (UV) transilluminator (Platinum Q9, Uvitec Cambridge) after the gel electrophoresis was carried out at 90 V for 60 minutes. In order to use agarose gel electrophoresis to analyze PCR products, 0.75 grams of agarose powder were dissolved in 5 milliliters of Tris-Acetate-EDTA Buffer (TAE) buffer and 45 milliliters of distilled water to create a 1.5% gel solution. The mixture was then heated in a microwave until the agarose was completely dissolved. In Micro wave at 30 second interval the solution was mixed by shaking with hands for four times at similar time interval. After a little cooling, a DNA-intercalating dye was added, and the mixture is then poured into a casting tray using a comb to create wells. Once the gel solidified, it was placed into an electrophoresis chamber filled with TAE buffer that submerges the gel, and the PCR products, mixed with a loading dye, were carefully pipetted into the wells alongside a DNA ladder for size comparison. The lid was secured, and an electric current was applied, causing the negatively charged DNA fragments to migrate through the gel matrix towards the positive anode; smaller fragments move faster and travel farther than larger ones. Once the dye front moved far enough, the power was switched off, the gel was examined under a UV lamp, and the band pattern that result was examined to verify the size and existence of the amplified DNA targets from the initial PCR reaction.

**Statistical analysis**

The collected data were processed and analyzed with Microsoft Excel.

# RESULTS AND DISCUSSION

## Identification of bacteria by cultural, morphological and bio-chemical properties

This study analyzed 150 samples (100 fecal, 50 liver) for *Salmonella* using culture on MacConkey and Salmonella Shigella agar followed by biochemical confirmation. While initial growth on MacConkey agar suggested 33 positives, biochemical tests, the definitive method, confirmed only 20 samples (14 fecal and 6 liver) as true positives. The final overall prevalence from this step-by-step confirmation procedure was 13.3%, with a slightly higher confirmed rate in fecal samples (14%) than in liver samples (12%).

**Table 2. Table of Fecal and Liver Samples**

| Category | Total Samples | Positive Samples on Culture in MacConkey Agar | Positive Samples on Culture in Salmonella Shigella Agar | Confirmed by Biochemical Test | Prevalence % |
|---|---|---|---|---|---|
| Fecal (Cloacal swabs) | 100 | 22 | 17 | 14 | 14% |
| Liver | 50 | 11 | 8 | 6 | 12 % |
| Total | 150 | 33 | 25 | 20 | 13.3% |

This table illustrates the variability of culture-based screening by describing a rigorous diagnostic procedure in which 150 samples were cultured on selective media. MacConkey agar initially suggested 33 positives, but the more selective Salmonella Shigella agar only detected 25. Only 20 of these presumed isolates were conclusively identified as true *Salmonella* by biochemical confirmation, which was a crucial step that effectively eliminated over one-third of the initial candidates as false positives and highlighted the need for confirmatory testing. This multi-stage verification ultimately yielded a true, confirmed overall prevalence of 13.3%, with a slightly higher rate in fecal samples (14%) than in liver samples (12%), reflecting the expected biological distribution of the pathogen.

The bar chart (Figure 1) illustrates a multi-stage diagnostic process for detecting *Salmonella* in 150 samples (100 fecal, 50 liver). The most presumptive positives (33

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total) were found in the initial culture on MacConkey agar. These were further refined by the more selective Salmonella Shigella agar (25 total), and biochemical tests ultimately produced 20 true positives. The overall prevalence was 13.3% as a result of this stepwise confirmation. Fecal samples had a marginally higher confirmed infection rate (14%) than liver samples (12%).

**Picture 3. Picture showing *Salmonella* Positive in S.S. Agar**

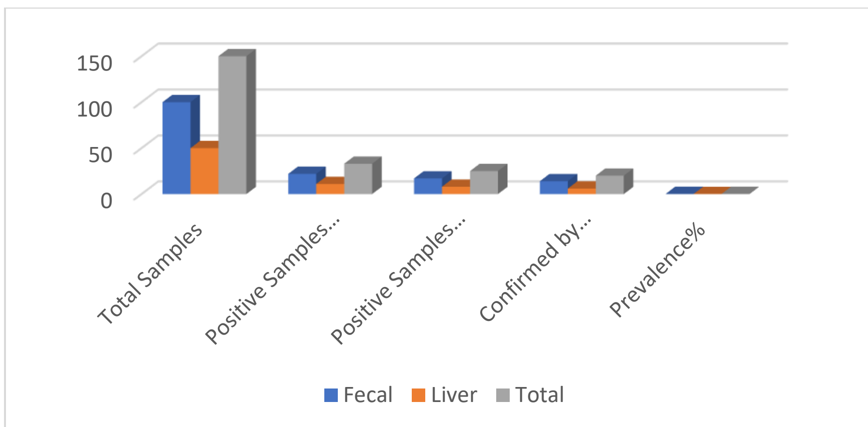



**Figure 1. Bar showing Liver and Fecal positive samples**

**Molecular Confirmation by PCR**

The isolation results from poultry fecal and liver samples were graphically compared in the chart, which indicates that culture on MacConkey Agar produced the greatest number of positive samples for both sample types, followed by Salmonella Shigella Agar and biochemical confirmation produced the most conclusive but lowest count. These confirmed cases used to calculate the final prevalence percentage, which shows the true infection rate lower than the initial culture-positive results.

Only few samples seem to be better for PCR as *Salmonella* appear as transparent or translucent, colorless colonies, often with a black center on Salmonella Shigella Agar. PCR was performed however it gave bands not in convincing way. The PCR enable to find the expected band however it might be due to low sample size for PCR or denaturation and annealing might have significantly affect the outcome of a PCR test for *Salmonella* in poultry samples, and problems with these steps are a common reason for non-clear bands especially when dealing with complex sample matrices. In order to obtain positive *Salmonella* genes in PCR as well as specific *Salmonella species* as specified in the experiment, more broiler samples from different regions will be gathered in the future, and numerous PCRs will be carried out following positive biochemical reactions. For the molecular detection of *Salmonella* in poultry fecal samples via PCR, the *invA* gene primers would be gold-standard tool, targeting a conserved sequence essential for the bacterium's invasion of host cells.

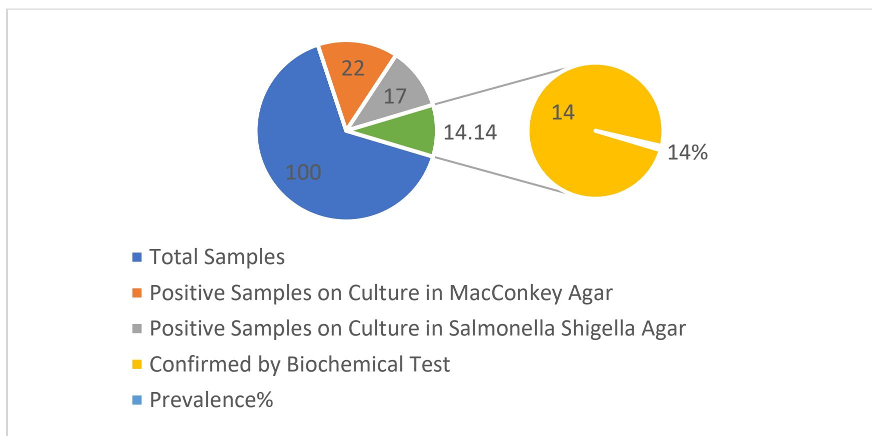
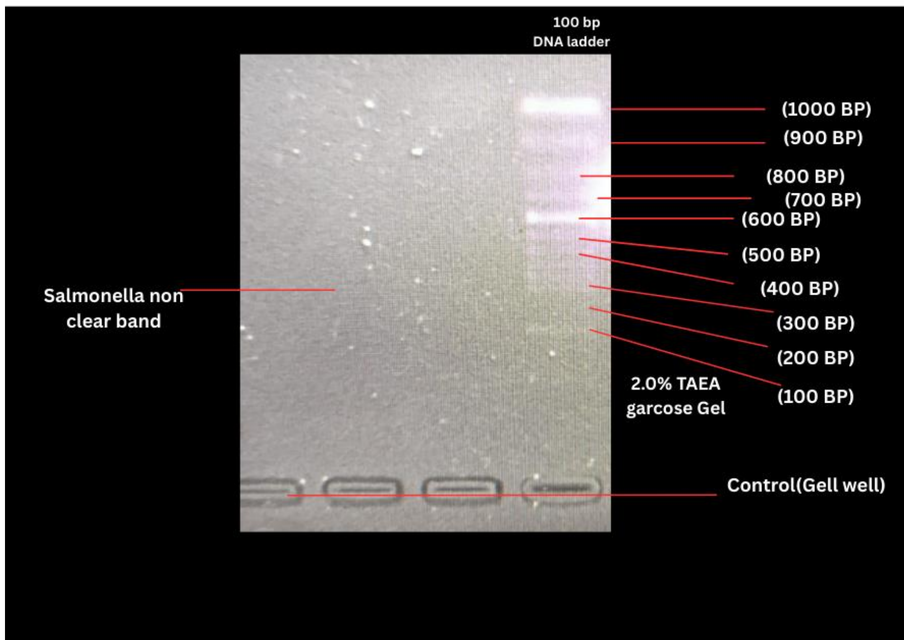



**Figure 2. Pie chart showing positive Fecal and liver sample**

The prevalence of *Salmonella* found in this study is in close agreement with reported rates as reported by (Shehata 2019) in Pakistani hatcheries found to be (12.5%) and Egyptian researcher (Barac *et al.,* 2024) reported in hatcheries to be (10%) prevalence. The 13.3% prevalence of *Salmonella* observed in this study is consistent with international reports placing it within a well-documented global range for poultry. Ferreira (2021) reported 12.8% of *Salmonella* Prevalence in Brazilian broiler flocks. Kuan (2017) reported 14.2% prevalence rate in Malaysian markets. India Kumar *et al.,* (2020) and Wangy *et al.,* (2018) reported nearly identical figures of *Salmonella* Prevalence rate as 13.5% and 13.8%, respectively. This prevalence rate of 11-16% is further supported by research from Odonkor *et al.,* (2018) in Nigeria (15.0%), Padungtod *et al.,* (2006) in Thailand (11.4%), Rahimi *et al.,* (2014) in Iran (14.1%), Islam *et al.,* (2021) in Bangladesh (16.3%), and finally Wasyl (2012) reported in Poland (11.8%) in Prevalence. All this prevalence from this literature supports the result of Prevalence in this Experiment. The challenge of *Salmonella* contamination is a persistent and widespread issue in the poultry industry across diverse geographic and production systems.




**Picture 4. PCR for Identification of Salmonellosis**

# CONCLUSION

In the biochemical and laboratory test it showed positive and even in Salmonella Shigella Agar it showed positive result for the fecal samples. This study successfully isolated *Salmonella* from poultry samples with an overall prevalence of 13.3%, but highlighted a critical methodological discrepancy where biochemically confirmed However; isolates failed to yield more convincing positive PCR results with their band for the *invA* gene. The bands seem to be non-convincing. This might be potentially attributable to the small number of samples processed by PCR or technical issues during the denaturation and annealing steps that can lead to such results and underscores the challenges in molecular characterization from complex matrices like poultry feces and liver. In future more sample sizes with various regions would be collected so, that might give a convincing positive result of PCR .

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