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Biochemical and Molecular Characterization of *Escherichia coli* from Poultry Intestine and Feces in Dinajpur, Bangladesh

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ABSTRACT

Escherichia coli (E. coli) in poultry presents a significant zoonotic concern, as certain pathogenic strains particularly ETEC (Enterotoxigenic Escherichia coli) can be transmitted from poultry to humans through direct contact, environmental contamination, or the food chain, especially via undercooked poultry meat or contaminated produce. The objective of this study was to separate, biochemically identify, and molecularly characterize E. coli from samples of poultry feces and intestine. A total of 150 samples (50 intestinal, 100 feces) were collected from Dinajpur broiler farms and transported to the Department of Microbiology, HSTU, Dinajpur, in an ice box with PBS while maintaining aseptic conditions. In Nutrient Agar Colonies were observed and further sub cultured on MacConkey agar and then on selective media Eosin Methylene Blue Agar (EMB agar) at 37°C for 24 hours before undergoing biochemical tests such as Motility indole Urease (MIU), Simmons citrate, Indole, Methyl Red and Triple sugar Iron (TSI) utilization. Bacterial confirmation was performed followed by PCR-based confirmation including genomic DNA isolation and gel electrophoresis. Isolates confirmed as E. coli were serotyped and further characterized by Polymerase Chain Reaction (PCR) targeting the E. coli primer gene. The overall prevalence of E. coli was 69.33 %, with a higher isolation rate from fecal samples (73%) than intestine samples (62%). There weren't many samples used for PCR. PCR produced bands that were positive during the analysis. In the future, more intestinal and fecal samples from different areas will be examined for research purposes.

Keywords: Biochemical, Identification*, E. coli,* ETEC, PCR, Poultry

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INTRODUCTION

The Enterobacteriaceae family includes the ubiquitous gram-negative bacterium *Escherichia coli,* which is frequently found in the environment and in poultry's normal intestinal flora. Although the majority of strains are not harmful, certain virulent strains, referred to as Avian Pathogenic *E. coli* (APEC), can result in colibacillosis, a serious economic illness. When the host's defenses are weakened by co-infections, immunosuppression, or environmental stressors, APEC usually functions as an opportunistic pathogen, causing localized or systemic infections (such as cellulitis, septicemia, or omphalitis). Because of resistance issues and possible zoonotic connections between some APEC and human extra-intestinal pathogenic *E. coli* (Ex PEC), control depends on biosecurity, good management, and vaccination. Antimicrobial use is also cautious (Percival and Williams, 2014; Islam *et al*., 2023). While many strains are harmless, some particularly Avian Pathogenic *E. coli* (APEC) can cause severe disease in birds, leading to disorders such as colibacillosis, which affects the respiratory and systemic systems and leads in economic losses owing to mortality and reduced production. Additionally, zoonotic *E. Coli* strains, especially those resistant to antibiotics, are found in poultry and can spread to people through direct contact or the food chain. Consequently, monitoring and managing *E. coli* in poultry is critical for both animal health and public health, notably in preventing infections and minimizing the spread of antibiotic resistance. Only a small number of *E. coli* serotypes are thought to be nonmotile due to the arrangement of peritrichous flagella (Percival and Williams, 2014). It is prevalent in domesticated cattle species and is regarded as one of the most prevalent bacterial infections worldwide (Nolan *et al.,* 2020). In terms of toxicity and virulence, *E. coli* is a very diverse bacterial species. It is extensively dispersed across the ecosystem, indicating a significant risk to human health (Ghanem and Haddadin 2018). According to Jang *et al.,* (2017), *E. coli* can grow and survive outside of various hosts because it can reproduce in water and soil in tropical and subtropical regions. Hatcheries are a common source of spreading infections (Papouskova *et al.,* 2023).

MATERIALS AND METHODS

Sample collection

A total of 150 samples were gathered as part of the study population during the September–December 2025 period in the Dinajpur district, namely in the vicinity of the Hajee Mohammad Danesh Science and Technology University (HSTU). Now the initial round of sample collection only 100 fecal and 50 intestinal samples were collected from the surrounding areas of Hajee Mohammad Danesh Science and

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Technology University, Dinajpur. The Phosphate buffer Solution (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

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

Isolation and biochemical identification

Samples were taken from broiler farms in Dinajpur and brought to the Department of Microbiology at HSTU, Dinajpur, in an aseptic ice box filled with 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. A loopful of enriched suspension was streaked in MacConkey agar and incubated at 37◦C for 24 h for isolation of *E. coli*; because *E. coli* is a lactose-fermenting bacterium, colonies on MacConkey agar appear pink to rose-red. The fermentation of lactose produces acidic by products that lower the pH of the medium, leading the pH indicator (neutral red) to turn pink. *E. coli* on MacConkey agar were submitted to Gram staining, which exhibited 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 detected and then sub-cultured on selective media Eosin-Methylene Blue (EMB agar) at 37°C for 24 hours.

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

PCR-based confirmation using gel electrophoresis and genomic DNA isolation was carried out. 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. 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 2. (MR Test-Positive) (TSI-Positive) (DNA Extraction)

Molecular characterization of virulence genes

To aid in 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 microcentrifuge 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. The extracted genomic DNA-containing supernatant was carefully collected and put into a new microcentrifuge tube to be stored at -20°C until further PCR analysis. The genomic DNA was isolated from *E. coli* isolates using a modified boiling cell approach (Pui *et al.*, 2011). Cultured *E. coli* 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 discarded. 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 brand-new Eppendorf tube and kept at 20°C until PCR amplification.

The concentration and purity of the DNA recovered from the *E. coli* culture were quantified spectrophotometrically (Quawell, UV-Vis Spectrophotometer Q5000) at 260 and 280 nm, with acceptable ratios ranging from1.6-2. One virulence gene (invA) was amplified by PCR using a Bio-Rad T100TM Thermal Cycler (Bio-Rad, USA). Five microliters of Invitrogen Master Mix, one microliter each of forward and reverse primers, 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 per-formed to amplify the virulence genes of *E. coli.* Detailed information on the primers (Macrogen, Inc., Seoul, South Korea), including their melting temperatures (Tm) and amplicon sizes, was performed as protocol. Pre-denaturation at 95°C for 3.5 minutes, 95°C for 3 seconds, 56°C for 30 seconds, 72°C for 60 seconds with 35 cycles, 72°C for 10 minutes, and 4°C with infinite were the cycling conditions. 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 100 bp DNA ladder was loaded with five microliters of PCR product combined with loading dye. The gel electrophoresis was done at 90 V for 60 min, and DNA bands were observed under a UV transilluminator (Platinum Q9, U Vitec Cambridge).

The primer used in PCR for E. coli was Ee116Srna F(5'GACCTCGGTTTAGTTCAC)AGA-3' and Ee116Srna R(5'CACACGCTGACGCTGACCA)3'. To examine PCR results using agarose gel electrophoresis, gel was generated by dissolving 0.75 grams of agarose powder in a mixture of 5 mL of TAE buffer and 45 mL of distilled water, which generates a 1.5% gel solution; this solution is heated in a microwave until the agarose was completely

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dissolved. In Micro wave at 30 second interval the solution was mixed by shaking with hands for four times at comparable time interval. Then let to cool somewhat before adding a DNA-intercalating dye, and is lastly poured into a casting tray with a comb to make wells. Once the gel has set, it is placed into an electrophoresis chamber filled with TAE buffer that submerges the gel, and the PCR products, combined with a loading dye, are carefully pipetted into the wells alongside a DNA ladder for size comparison. The negatively charged DNA fragments moved through the gel matrix in the direction of the positive anode when the lid was fastened and an electric current was applied; smaller fragments traveled farther and quicker than bigger ones. Once the dye front has moved far enough, the power is switched off, the gel is examined under a UV lamp, and the band pattern that results is examined to verify the size and existence of the amplified DNA targets from the first PCR reaction.

Statistical analysis

The collected data were processed and evaluated utilizing Microsoft Excel software.

RESULTS AND DISCUSSION

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

The findings of a microbiological study on enteric bacterial pathogens (*E. coli*) from two sample sources intestinal tissue and feces are shown in this table. First, 150 samples were cultured on MacConkey Agar, a generic medium for Gram-negative bacteria, and subsequently on Eosin Methylene Blue (EMB) Agar, a selective medium. Through biochemical testing, all samples that developed on EMB Agar were conclusively identified as target pathogens.

Table 2. Table of Fecal and Intestinal Samples

Category	Total Samples	Positive Samples on Culture in MacConkey Agar	Positive Samples on Culture in Eosin Methylene Agar	Confirmed by Biochemical Test	Prevalence, %
Fecal	100	73	73	73	73
Intestine	50	31	31	31	62
Total	150	104	104	104	69.33

The final Prevalence% is derived from these verified positives, demonstrating a very high infection rate: 73% in fecal samples (73 out of 100) and 62% in intestinal samples (31 out of 50), with an overall prevalence of 69.33% (104 out of 150). The exact numbers across the last three columns imply perfect agreement between selective

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culture and biochemical confirmation in this dataset, pointing to a substantial burden of enteric bacterial illness in the examined population.

Picture 3. *E. coli* Positive in EMB Agar

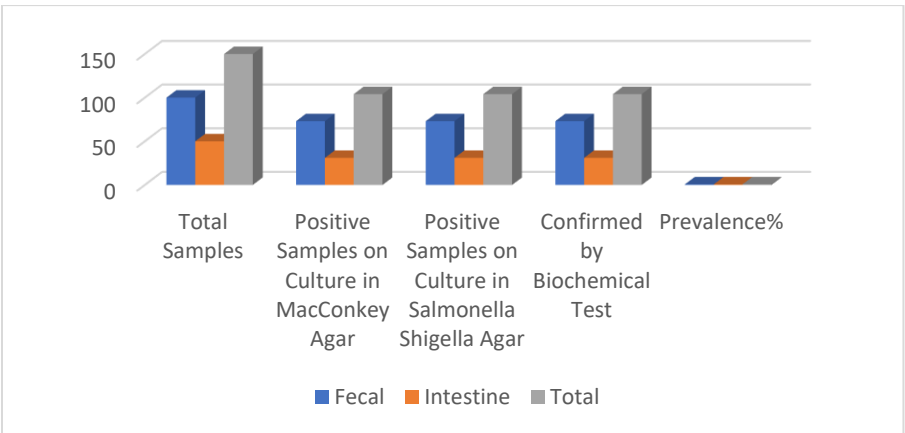

Figure 1. Bar showing Fecal and intestinal positive samples

This clustered bar chart visually compares the results for fecal and intestinal poultry samples across four major criteria. Each measure (e.g., Positive on MacConkey Agar) is represented by a pair of bars blue for fecal and orange for intestinal. The chart clearly demonstrates that for every parameter, the count for fecal samples (Total=100) is higher than for intestinal samples (Total=50), which is predicted due to the bigger sample size. The most important comparison, however, is found in the Prevalence% bars on the far right, where the orange bar (intestinal, 20%) is taller than the blue bar

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(fecal, 15%). This suggests that, despite the lower total number of tests, *E. coli* was confirmed at a greater proportional rate in intestine samples.

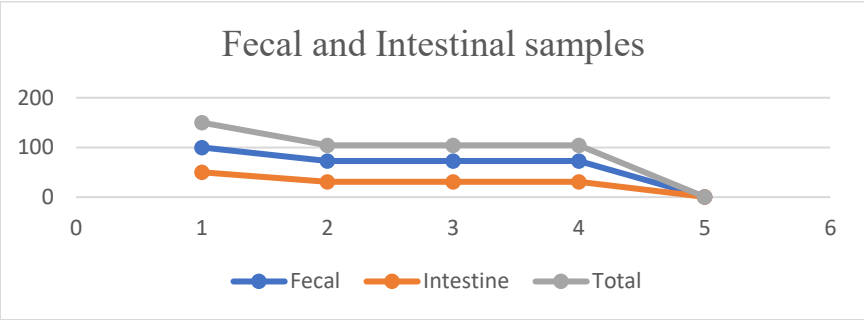

Figure 2. Chart showing positive Fecal and Intestinal sample

Fecal, Intestine, and Total are the three sample categories listed. This is a simple label structure, likely used to organize or present data from a microbiological or epidemiological study comparing two types of biological specimens' fecal samples (such as stool) and intestinal tissue samples (possibly collected post-mortem or surgically) with a Total row that sums or summarizes results from both categories. Three sample categories are listed in it: total, fecal and intestinal. This is a simple label structure, likely used to organize or present data from a microbiological or epidemiological study comparing two types of biological specimens' fecal samples (such as stool) and intestinal tissue samples (possibly collected post-mortem or surgically) with a Total row that sums or summarizes results from both categories.

Molecular confirmation by PCR

Few Sample seems to be better for PCR as *E. coli* as on EMB Agar. A crucial distinguishing feature of *E. Coli* colonies is their dark-centered appearance and green metallic sheen. This is owing to its capacity to ferment lactose, which causes the colors in the medium to precipitate and form dark, almost black colonies with an iridescent shine. The incidence of *Escherichia coli (E. coli*) in poultry fecal matter is constantly high, often ranging from roughly 50% to over 80%, depending on factors like farm management, geographic region, and specific testing procedures.

Conversely, Blaak *et al.,* (2015) found extended-spectrum beta-lactamase (ESBL)-producing *E. coli* in 81, 60, and 57% of samples taken from surface water, dust, and rinse water near chicken flocks, respectively. The overall prevalence of *E. coli*

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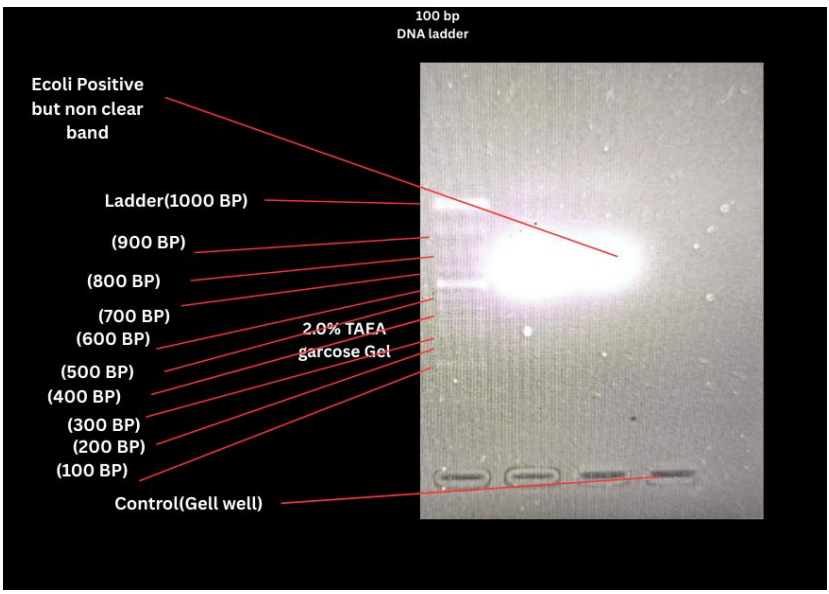
reported in this study was 77.7%, which coincides with other recent studies conducted in Bangladesh (Davies *et al.,* 2024; Al-Salauddin *et al*., 2015) and Turkey (Telli *et al*., 2022). In contrast, our results surpass the lower prevalence rates reported in countries like Sri Lanka, the United States, the United Kingdom and even Bangladesh at 66.8%, 67.9%, 36.4%, 63.5% and 56.5%, respectively (Ranasinghe *et al.,* 2022). These findings unmistakably support one another.



Picture 4. PCR for identification of *E. coli*

CONCLUSION

This study found a notable discrepancy between biochemical and molecular confirmation, even though *E. coli* was successfully isolated from 69.33% of chicken samples. Biochemically confirmed isolates frequently failed to produce strong, unambiguous positive results in PCR assays, often showing only faint bands. To resolve this discrepancy and achieve more reliable PCR confirmation, future work will require larger sample sets drawn from varied regions.

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