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Investigating the presence of *E. coli* bacteria in broiler chickens and detecting virulence genes (rfbE, eaeA, fliCH7)

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Abstract
The study was conducted for the purpose of detecting the genes (*rfbE*, *eaeA*, *fliCH7*) and confirming their diagnosis using polymerase chain reaction testing and biochemical tests. The experiment was carried out in the laboratories of the Pathology and Poultry Diseases Branch at the College of Veterinary Medicine, Al Qasim Green University, during the period from 8/10/2023 to 9/10/2023. (150) samples were collected from chickens suffering from diarrhea and showing clinical symptoms of the infection. They were weighed at one day old (average weight of the chick: 43±2 g) and then transported to the poultry hall. Chickens in houses divided by wooden barriers. *Escherichia coli.* were first diagnosed by bacteriological methods. Among (150) samples for cases of diarrhea in chickens, (90%) was an isolate of *Escherichia coli*. bacteria. Then, PCR technology was used to detect the virulence genes (*rfbE*, *eaeA*, *fliCH7*) and to confirm the diagnosis, and the result showed (*rfbE*, *eaeA*, *fliCH7*). In this study, this gene was discovered in (135) isolates from (150) samples of observed chickens carrying the *rfb E*, *eae A*, *flich7* gene, which represents (90%).

Keywords: Diarrhea, broiler chicken, *E. coli*, PCR, *rfbE*, *eaeA*, *fliCH7*

Introduction
Infections contracted through food pose a major threat to public health. An estimated 600 million pathogenic illnesses and 420,000 infection-related deaths occur each year as a result of these disorders. Bacteria that cause diarrhea, such *Escherichia coli* Al-Nabulsi *et al*. 2022 [1] and WHO 2015 [2], are the main cause of these diseases. Consequently, a creature that gets into human food chains, like chicken, keeps zoonotic infections alive that can also create poisons and directly endanger human health. 2018 saw Heredia and García [3]. Furthermore, a number of environmental variables (on farms) and human actions associated with animal handling (such as slaughter, processing, and storage) can contaminate animal products. Large numbers of chickens from various sources and with varying histories of healing can be found in live poultry marketplaces. This creates an environment that is ideal for the spread of germs Dantas *et al*., 2008) [5]. Furthermore, the slaughterhouse is located in close proximity to the customer. Bacteria can proliferate quickly and uncontrollably throughout the food chain under the circumstances mentioned above. Sarker *et al*. (2019) [6]. *Escherichia coli* is widely found in the intestines of warm-blooded animals Denamur *et al*. 2010 [7]. While the majority of *E. coli* strains are benign, a small number are categorized as extraintestinal pathogenic *E. coli*. additionally Antibiotic residues in chicken can have a variety of detrimental consequences on health, such as the growth of microbial strains that are resistant to drugs, allergies, and changes to the natural flora in the intestines Rabee *et al*. (2023) [36]. According to Almremdhy (2014) [8] and Al-Bawi and Hussein (2020) [9], *E. coli* supplies AIV by way of a protease-like enzyme, which is required for HA protein cleavability and fission to occur. As a result, this is considered a secondary infection. *E. coli* views this as a secondary infection Almendra (2014) [8]. The cause of colibacillosis, which starts as a respiratory infection and progresses to a systemic infection marked by moderate to severe symptoms, is APEC. Signs of respiratory distress include postmortem breathing, coughing, sneezing, snickering, and strained breathing. Results showed enteritis, fibrinous pericarditis, and fibrinous perihepatitis. In addition, *Escherichia coli* O157:H7 is a dangerous foodborne pathogen that, when it colonizes the intestine and releases toxins that harm host cells, can cause serious illness. Lim and associates (2010) [10]. The virulence-associated genes (rfbE, eaeA, fliCH7) of an *E. coli* strain can be used to assess its virulence (VAGs).

The highly pathogenic *E. coli* strains iutA, hlyF, ISS, iroN, and ompT have been associated with many VAGs. Johnson and others (2008) [11]. Gharaibeh and Shatnawi (2019) [12] have discovered that a significant factor in the colistin resistance of pathogenic bacteria is the presence of mobile colistin resistance (mcr) genes. Over the course of the last nine years, there have been more MCR variants-from MCR-1 in 2015 to MCR-10 in 2020. These variations have been found in pets, agricultural workers, and farm-derived microbes. The likelihood of mcr genes spreading globally is increased by the speed at which they are spreading among bacteria. Lima & associates (2017) [13]. Furthermore, it is critical to understand that the extensive application of antibiotics in food production has altered the microbiome, as noted by Subedi *et al.* (2018) [14]. Understanding *E. coli*'s known function can be gained through qualitative profiling of the bacteria. As stated in Claremont Clermont *et al.*'s publications (2019) [15]. To our knowledge, no prior studies have assessed the prevalence of *E. coli* outside of the virulence genes (rfbE, eaeA, and fliCH7), resistance genes, and phenotypes. Accordingly, the purpose of this study was to ascertain the percentage of broiler chickens infected with *Escherichia coli* from day one to day twenty-eight, as well as the presence of *Escherichia coli* O157:H7. The majority of E. Col strains are commensals, meaning they are found in the digestive tracts of warm-blooded animals and people. Until they acquire VFs from mobile genetic elements like as bacteriophages, pathogenicity islands, and plasmids, however, they rarely cause disease. Furthermore, AMR genes that can be shared by a range of bacterial species, including those that are dangerous to humans and animals, can be accumulated by *E. coli* (Benameur *et al*., 2021) [29].

Materials and Methods

250 chicks were utilized as experimental animals in this investigation. After being weighed at one day old (with an average weight of 43 g±2), each chick was taken to the poultry hall. The chicks are housed in houses that are divided by 1 x 1.5 m wooden walls. Sawdust, about 7 cm in thickness, was used as bedding in the chicken coop. Free access to food and drinking water is provided, along with a continuous lighting system.

PCR master mix preparation: A ready-to-use combination of premium Taq DNA Polymerase, deoxynucleotides, and reaction buffer at a 2X concentration is called GoTaq® G2 Green Master Mix. It includes every reagent required for DNA amplification. An inert green dye and a stabilizer are included in the GoTaq® G2 Green Master Mix, enabling direct loading of the finished products onto a gel for analysis carried out in accordance with business guidelines as shown in the following table (3-11).

Components	Concentration	Volume (50 µl)
2X PCR *Taq* Master Mix	1X	25 µl
Forward primer	10 µM/µl	4 µl
Reverse primer	10 µM/µl	4 µl
ddH_2O	-	13 µl
DNA	40 ng	4 µl

PCR Thermocycler Conditions

PCR thermocycler conditions were done by using convential PCR thermo cycler system is same for each gene following table (3-12).

Phase	Tm (°C)	Time	Cycles
Initial denaturation	94 °C	5 min	1X
Denaturation	94 °C	30 sec.	35X
Annealing	56 °C	30 sec.	
Extension	72 °C	1 min	
Final extension	72 °C	5 min	1X

Results and Discussion

Isolation of *Escherichia coli*

In order to promote bacterial growth, ileum samples were inoculated on a variety of bacteriological media, including Blood agar, Nutrient agar, MacConkey agar, and EMB agar. The samples were then incubated at (37 °C) for 18 to 24 hours. The findings were shown in Figure (1).

According to (Cheesbrough, 2006; Abdallah *et al*. 2016 and Rabee *et al*. 2023) [16, 17, 36], bacterial techniques such colony morphology, Gram's staining, and cultural and biochemical properties were initially utilized to diagnose bacterial colonies. In (150) ileum samples from chickens who had diarrhea, (90%) strains of *Escherichia coli* were discovered. The findings were displayed in Figure (2).

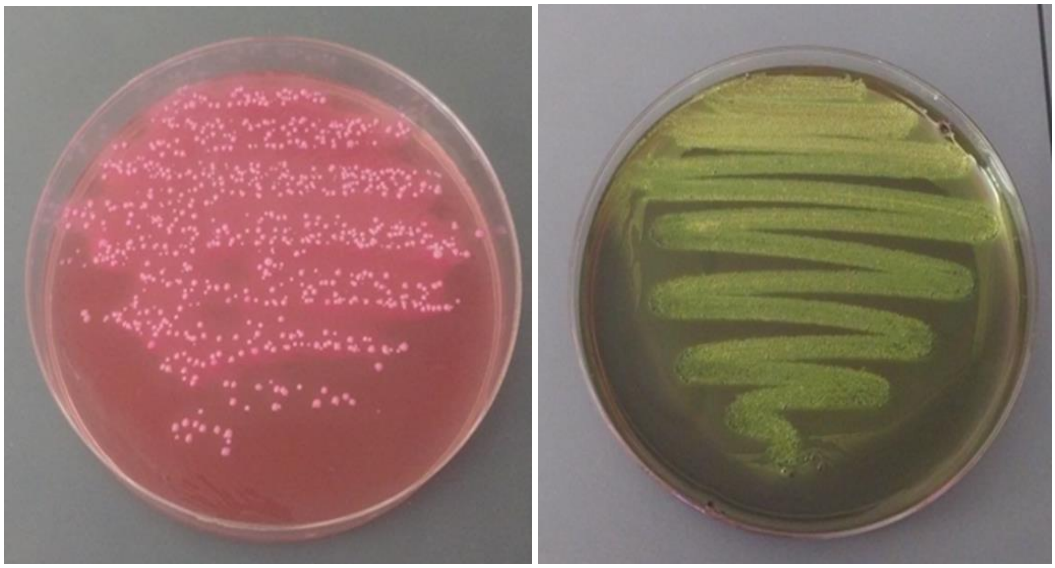

Fig 1: Bacterial Isolates of green diarrhea cases in chicken

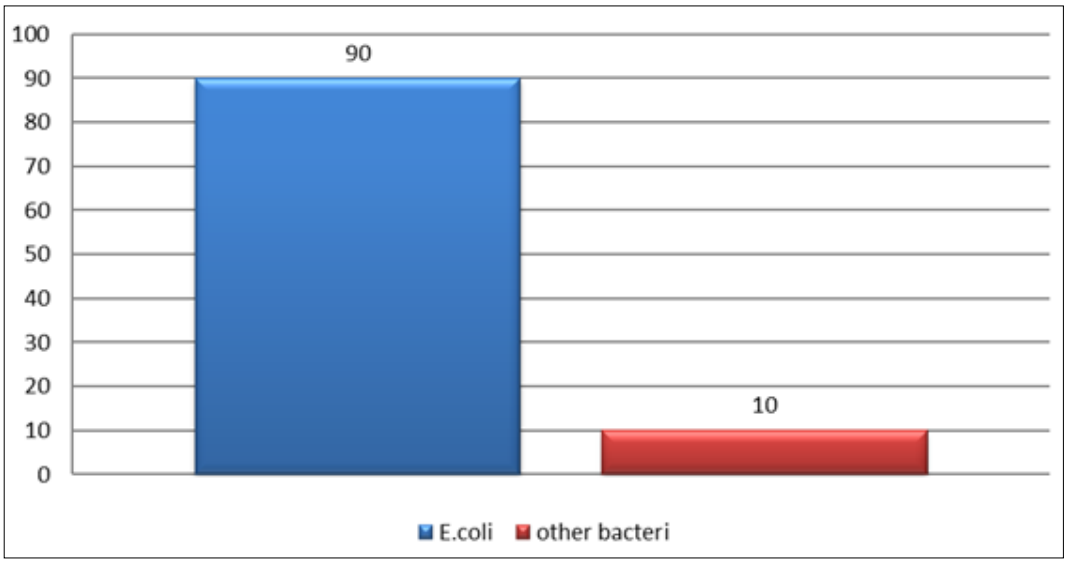

Fig 2: Percentage of Bacterial Isolates of diarrhea cases in chicken

In these The findings are consistent with those of Abbassi *et al*. (2017) [18], who found that the prevalences of 67% and 100% were found in rectal swabs of native and caecal samples of broiler chicken, respectively. In contrast, Yulistiani *et al*. (2019) [19] discovered that the prevalence of *E. coli* in healthy broiler chickens was found in the caecum, ileum, and duodenum samples to be 88%, 38%, and 25%, respectively. Adelaide *et al*. (2008) [20] reported that E. Col. was the source of 99% of instances of diarrhea, with other bacteria accounting for 1% of cases. However, Roshdy *et al*. (2021) [21] discovered that E. Col. was the cause of diarrhea in 40% of broiler chicks. Different investigations' findings about prevalence may be the result of samples' mixed infection with other microorganisms, which has been shown to affect the prevalence of some bacteria like *E. coli*. The prevalence of *E. coli* varies among related species and may be connected to environmental factors, geographic location, and other host condition (Gambushe *et al*., 2022) and Jangid *et al*., 2024) [22, 23].

Detection and Characterization of *eaeA, fliCH7 and rfbE* Gene

To determine if *E.coli* isolates was *E.coli* O157H7, we characterized the isolates on the basis of the presence of virulence genes (*eaeA, fliCH7 and rfbE)* by using PCR technique. Figure (3) shows Agarose gel electrophoresis image that showed the PCR product analysis of *eaeA, fliCH7 and rfbE gene* in *Escherichia coli* isolated from ileum samples. According Indraswari *et al*., 2021 [37].

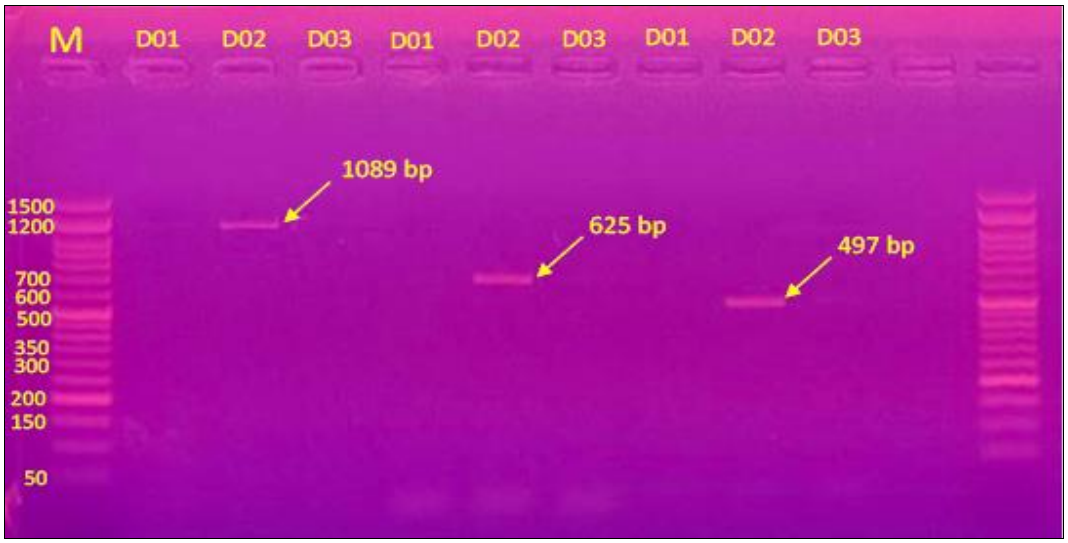

Fig 3: Electrophoresis on agarose gel (1.5%) potential difference (90) for 45 minute, which shows the results of the PCR assay for the investigation of the gene *eaeA* =1089bp, gene *fliCH7* = 625bp and gene *rfbE* = 497bp, for E.coli bacteria according to Marker ladder (50-1500bp) and Lane (1-3) data.

The results of this study were consistent with those of Li *et al*. (2023) [24] developed a dual-gene RPA-TeaPNA-LFA methodology that specifically recognized target amplicons, including the eaeA gene, to eliminate false-positives in LFA readout. The eaeA gene amplification technique was employed in several investigations to detect *E. coli* O157 bacteria. In order to detect the intimin gene (eaeA) from *E. coli* O157:H7, (Zeinoddini *et al*., 2022) [25] optimized a colorimetric LAMP assay, offering a quick and easily noticeable technique. (Suleiman *et al*. 2020) [39] Showed the value of molecular characterisation for infection confirmation by using real-time PCR to find the eaeA gene

in *E. coli* O157:H7 isolates from calves. (Rani *et al*., 2021) [27] assessed the role of gene selection in the detection of *E. coli* O157:H7 and discovered that the pathogen could be successfully identified by the rfbE gene, which contains the eaeA gene. The eaeA gene was detected in 9 out of 14 isolates of *E. coli* O157 strains studied by (Osek and Gallien 2018) [28], suggesting a connection to the gamma intimin variation.

Gram-negative bacteria used the O-antigen, an immunodominant component, to mask their identity as a disease and elude the host's immune system. Previous research suggests that viruses may be more susceptible to the host's killing mechanism if they lack O-antigen. (2015) Hanying *et al*. The eaeA gene plays a critical role in the synthesis of proteins involved in the creation of glucose-free and LPS by bacteria. The eaeA gene encodes intimin, an outer membrane protein necessary for the development of AE lesions, according to Boucher *et al*. (2009) [31]. According to Yamazaki *et al*. (1997) [32], the eaeA gene does not directly contribute to the synthesis of glucose or lipopolysaccharides (LPS) in *E. coli* bacteria.

Figure 3 shows that the PCR primers used in these experiments amplified a 625 bp fragment of the chicken *E. coli* isolates' fliCh7 gene. Koev *et al*. 2020 [33] have noted similar outcomes (2020). But according to (Ayodele *et al*., 2020) [34], specific primers provided by the manufacturer (Macrogen) are made to amplify the fliCh7 gene, which codes for the Flagellar type 7 (H7) product. These primers are then electrophoresed onto a 1.5% agarose gel, UV-examined, and dyed with ethidium bromide. This gene is thought to be diagnostic for H7.

On the other hand, Figure 3 illustrates how the rfbE gene of the chicken *E. coli* isolates was amplified to a length of 497 bp using the PCR primer results. Valiallahi *et al*. (2022) [35] also noted comparable outcomes. The first strain of *Escherichia coli* that produces the O157 Shiga toxin is O157, and it encodes perosamine synthase. Based on an extensive alignment of bacterial rfbE gene sequences, a group of researchers created a unique set of genus-specific primers (rfbE) for *E. coli* O157 H7 that enabled the detection of the majority of species and strains of the bacterium. The rfbE gene is employed in PCR assays to separate serotype O157 from non-O157 STEC in clinical specimens. They also discovered that the gene was more specific for the detection of *E. coli* O157 H7 (Indraswari *et al*., 2021) [37]. The prevalence and molecular detection characterisation of *E. coli* serotype O157:H7 recovered from raw meat and meat products collected from Saudi Arabia were found by Hessian *et al*. (2015) [38], which was in agreement with the results of this study.

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