
Archives of Razi Institute Journal
Volume

Journal homepage: https://archrazi.areeo.ac.ir

Original Article

Histopathological Diagnosis and Detection of *Avian Pathogenic Escherichia Coli* Virulence Genes in Broiler Chickens in Indonesia

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Article Info:

Received: 6 September 2024
Revised: 13 October 2025
Accepted: 16 October 2024

Keywords:

Colibacillosis, APEC, Gene *hlyF*, Gene *iroN*, Poultry.

ABSTRACT

Colibacillosis is a disease in poultry that often occurs in poultry farms in developing countries, including Indonesia. This disease is generally caused by cage or environmental sanitation problems, as well as poor poultry husbandry patterns. Colibacillosis, caused by *Avian Pathogenic Escherichia coli* (APEC) infection, is one of the significant health problems in the poultry industry, especially in Indonesia, with clinical symptoms such as emaciation decreased appetite, impaired growth, diarrhea, dirty or sticky feathers around the vent, bloated intestines ,and white feces. This study aims to V histopathological identification and detect virulence genes of Avian pathogenic *Escherichia coli* in broiler chickens.The methods used included organ sampling such as heart, liver, jejunum ,and cecum, which were then processed for histopathological preparation using Hematoxylin-Eosin (HE) staining. In addition, molecular diagnosis was performed using *Polymerase Chain Reaction* (PCR) technique to detect virulence genes, *iroN* and *hlyF*. The results showed that avian pathogenic *Escherichia coli* isolates were present in chickens suspected of colibacillosis, with positive blood agar culture showing hemolysin production (β-hemolysis) ,and the gene encoding *hlyF* was found positive but the gene encoding *iroN* was not. Histopathology results of liver, heart, jejunum ,and cecum infected with pathogenic *Escherichia coli* showed damage in the form of hemorrhage, necrosis, rupture of intestinal villi, erythrocyte accumulation, central venous congestion ,and fatty degeneration. Our study demonstrates that *avian pathogenic Escherichia coli* strains can be isolated from broiler chickens suffering from colibacillosis and cause anatomical pathological changes. This study emphasizes the importance of a better understanding of this pathogen to develop effective prevention and control strategies in the poultry farming industry.

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How to cite this article: **Wibisono FJ, Candra AYR, Mardijanto A, Nussa ORPA, Kendek IA, Artha AC, Muhammad Andika Bhayangkara. Histopathological Diagnosis and Detection of *Avian Pathogenic Escherichia Coli* Virulence Genes in Broiler Chickens in Indonesia. *Archives of Razi Institute*. 2025;80(3):693-701.** DOI: 10.32592/ARI.2025.80.3.693

1. Introduction

Colibacillosis is a disease in poultry that has a high economic impact. Economic losses due to colibacillosis in the poultry industry in Indonesia amount to 825 million dollars every month (1). Colibacillosis disease in poultry is caused by avian pathogenic *Escherichia coli* strains. Avian colibacillosis is the main disease affecting the poultry industry throughout the world, including Indonesia (2). *Escherichia coli* bacteria are opportunistic pathogens and can be divided into two large groups: Diarrhogenic *Escherichia coli(*DEC)and Extraintestinal Pathogenic *Escherichia coli (*ExPEC). DEC strains are responsible for gastrointestinal infections, while ExPEC strains cause diseases outside the intestinal tract such as sepsis, urinary tract infections, and meningitis. ExPEC includes pathogenic strains like Avian Pathogenic *Escherichia coli* (APEC) which is responsible for colibacillosis in poultry (2, 3).

It is important to improve understanding of the diagnosis, symptoms, and pathogenesis of colibacillosis, especially in developing countries such as Indonesia. The incidence of colibacillosis in poultry is very high, this can be attributed to predisposing factors such as stress and the misconception that the disease is caused by opportunistic infections, thereby underestimating the virulence of APEC. Generally, pathogenic *Escherichia coli* infections in poultry occur in two forms: systemic and local (1, 4).

Local infections may manifest as omphalitis / yolk sac infection, swollen head syndrome, cellulitis, enteritis, venereal colibacillosis, salpingitis,or egg peritonitis, while systemic form of infections in colibacillosis present as colisepticemia (5, 6). This study is aims to document colibacillosis symptoms seen in chickens. Confirmation of diagnosis with necropsy seen in this study can be done by veterinary medical personnel in the field. Direct evidence of the presence of poultry pathogenic *Escherichia coli* bacteria as the causative agent of colibacillosis in poultry remains limited, making further research essential. Colibacillosis is a zoonotic disease with potential negative impact on society. people are more careful about colibacillosis which is not only caused by common *Escherichia coli*, but apparently caused by Avian Pathogenic *Escherichia coli* with virulence genes HlyF and IroN (7, 8).

2. Materials and Methods

2.1. Study Period and Location

The study was conducted from December 2023 toMarch 2024. Purposive sampling was used, selecting chickens based on visible clinical symptoms of colibacillosis. Of 60 observed broiler chickens, 27 showed no colibacillosis clinical signs, while 33 chickens showed clinical symptoms of colibacillosis. Samples originated from live broiler chickens sold in traditional markets in Surabaya city, Indonesia. The traditional markets selected as samples are Wonokromo, Keputran, Pabean, Pucang, Dukuh Kupang, and Benowo. The traditional markets can represent the Surabaya area, Indonesia, with 10 chickens taken from each market.

2.2. Isolation and Identification *Avian Pathogenic Escherichia Coli*

Isolation and identification of Avian Pathogenic *Escherichia coli* using *Mac Conkey agar* (MH081 - HiMedia®, *Triple sugar iron Agar* (M021 - HiMedia®), *Simmon Citrate Agar* (M099 - HiMedia®), *Sulfide Indole Motility* (M181 - HiMedia®), and *methyl red - Voges–Proskauer* (M070 - HiMedia®). *Escherichia coli* were cultured on *Blood Agar* media supplemented with 5% sheep blood. Pathogenic *Escherichia coli* would show the formation of a clear zone around the colony which is considered to be hemolysin production (9).

2.3. Histopathology

After confirming pathogenic *Escherichia coli* bacteria presence, a necropsy was carried out on all samples. Organ samples taken were the heart, liver, jejunum and cecum. The organs were sectioned into lx1xl cm pieces, then the solution was soaked in 10% Buffer Neutral Formalin (BNF) to be made into histopathological preparations using *Hematoxylin Eosin* (HE) staining (10).

2.4. Molecular Diagnose Polymerase Chain Reaction

DNA extraction for Polymerase Chain Reaction was performed using QIAamp® DNA kit (QIAGEN, Germany) to detect gene encoding *iroN* (8) and *hlyF* (7). The forward primer used in iroN was AAGTCAAAGCAGGGGTTGCCCG, while the reverse primer was GACGCCGACATTAAGACGCAG with a target of 667 bp and the forward primer used in *hlyF* is GGCGATTTAGGCATTCCGATACTC, while the reverse primer was ACGGGGTCGCTAGTTAAGGAG with a target of 599 bp under thermal cycler conditions with predenaturation parameters at a temperature of 94°C for seven minutes, denaturation at 94°C for one minute, annealing at 56°C for 30 seconds, extension at 72°C for 30 seconds, cycle repeated 35 times and final extension at 72°C for five minutes. Subsequently, the amplicons were

visualized by electrophoresis using 2% agarose gel (7, 8, 11).

2.5. Statistical Analysis

Data analysis was performed descriptively by identifying avian pathogenic *Escherichia coli* bacteria referring to the Indonesian National Standard(SNI 7388:2009). By comparing the organs in chicken with no colibacillosis clinical signs using, the T test, Histopathological imaging was carried out descriptively by identifying changes in the heart, liver, cecum and jejunum of chicken infected with colibacillosis.

3. Results

3.1. Clinical Examinations and Necropsy

Based on observations, broiler chickens experience symptoms of disease ,including lethargy, emaciation, retarded growth, dirty or sticky feathers, visible feces attached around the vent ,and the consistency of greenish-white feces. The results of the macroscopic examination identified petechiae on the thighs of chickens, as well as, hepatomegaly at necropsy, abnormal heart shape and the presence of fibrin membranes in the heart, the cecum looked normal, and in the jejunum found bleeding (Figure1).

3.2. Isolation and Identification Results

Based on microbiological examinations conducted on broiler cloacal swab samples, the macroscopic morphology of *Escherichia coli* bacteria on MCA media showed pink colonies that were small, round, separate and irregular (Figure 2a). Further Gram staining is performed to determine the morphology of *Escherichia coli* bacterial cells with a short rod shape (*coccobacillus*) that appeared red (Figure 2b). Physiologically Identification of *Escherichia coli* bacteria used media such as TSIA, SCA, SIM, MR and VP (Figure 3c).

TSIA results showed Acid/Acid (A/A) reactions in both the base (butt) and slope (slant), gas production, and negative H_2S. The Simon Citrate Agar (SCA) test was negative for *Escherichia coli* bacteria, indicated by the absence of green color changes in the media, as *Escherichia coli* did not utilize citrate as a carbon source. The Sulfide Indole Motility (SIM) test showed positive results for Indole, with motility characterized by bacterial spread in the puncture area, and negative for sulfide. The Methyl Red (MR) test results showed a red color change after adding 0.5% Methyl Red reagent, if positive. The Voges-Proskauer (VP) test for *Escherichia coli* produced negative results. Based on the results of the Blood Agar test (Figure 2d), the presence of β-hemolysin, forming a clear zone around the colony was observed.

3.3. Polymerase Chain Reaction (PCR) Results

Based on the PCR test, a positive result was obtained for the gene encoding hlyF at 599 bp, while iroN at 667 bp was not. hly F gene in Avian pathogenic *Escherichia coli* is a virulence coding gene that can determine the ability of APEC to cause disease by hemolyzing, regulating outer membrane vesicles, and inducing autophagy in host cells. These results demonstrate that *Escherichia coli* isolates from colibacillosis-infected are pathogenic to poultry (Figure 3).

3.4. Histopathology Results

Histopathological evaluation was carried out based on the results of macroscopic pathological changes in broiler chickens with colibacillosis. The organs examined included the liver, heart, jejunum, and cecum.The histopathological changes observed in each organ included inflammatory cell infiltration, hemorrhage, and necrosis (Figure 4). The results showed that both chicken without colibacillosis clinical signs and those infected with colibacillosis showed these changes.

Based on the results of histopathological examination of both colibacillosis-infected chickens and chicken without colibacillosis clinical signs in the heart, liver, jejunum, and cecum, there were changes in lesions in the form of inflammatory cell infiltration, hemorrhage and necrosis (Table 1). Hemorrhagic myocarditis results were found in the heart with hemorrhage, edema and heterophils, and inflammatory cell infiltration. The liver showed hemorrhagic hepatitis with hemorrhage, necrosis and inflammatory cell infiltration. The intestines showed hemorrhagic and necrotizing enteritis, characterized by villous necrosis, hemorrhage, edema and neutrophilic inflammatory cells.

Based on Table 2, the average number of inflammatory cell infiltration in the heart of chickens without colibacillosis clinical signs is lower than that of infected chickens with colibacillosis, but the difference was not statistically significant ($P>0.05$).

Similarly, the inflammatory cell infiltration in the liver without colibacillosis clinical signs shows the same value as chickens infected with colibacillosis, which means the difference is notsignificant. However, in the jejunum and cecum, there were significant differences ($P<0.05$), with chickens without clinical signs exhibiting higher inflammatory cell infiltration than those infected with colibacillosis.

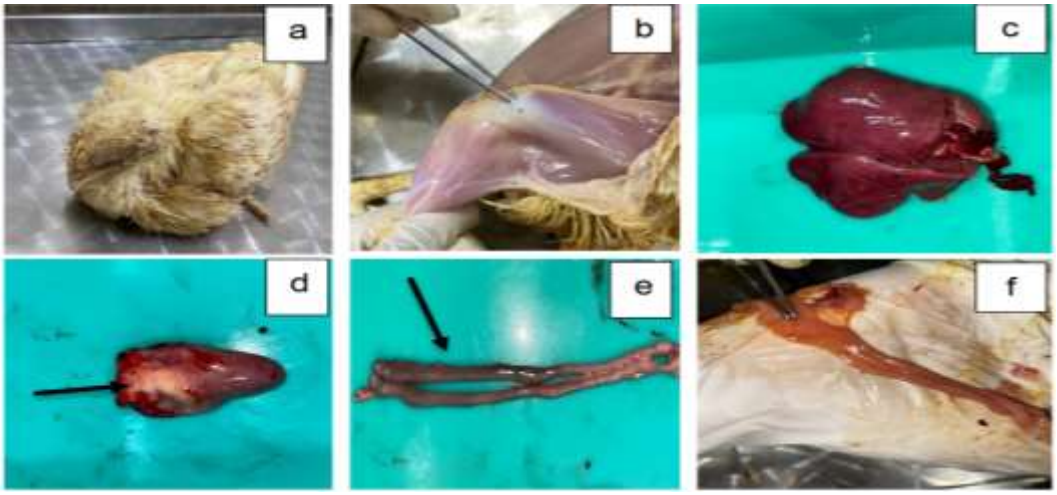

Figure 1. Necropsy (a); presence of petechiae (b); hepathomegaly (c); heart abnormalities (d); hemorrhage in the cecum (e); and hemorrhage in the jejunum (f).

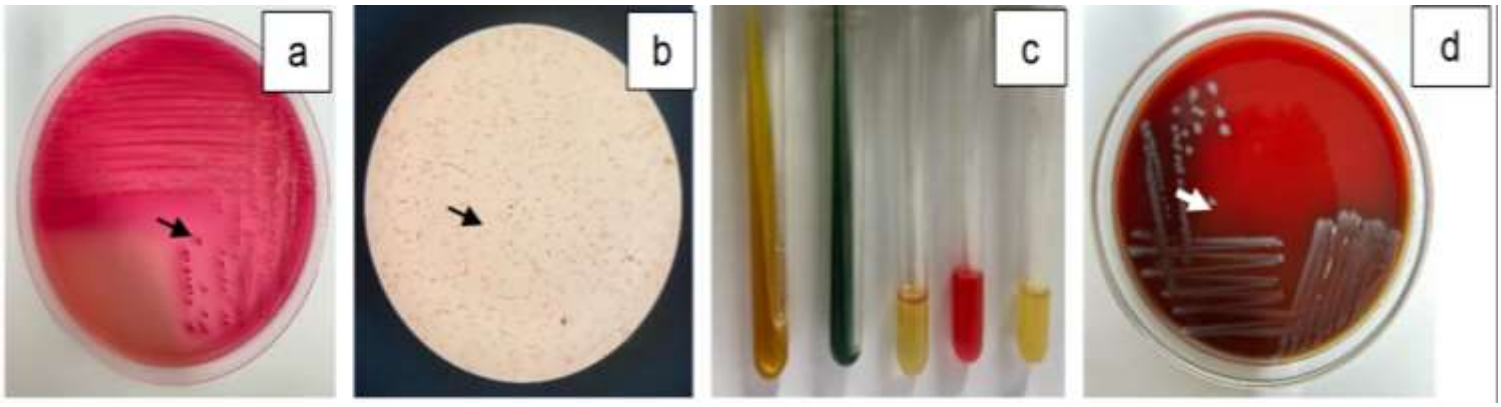

Figure 2. *Escherichia coli* colonies on *Mac Conkey Agar* (a); Gram staining on *Escherichia coli* using a microscope at 1000x. (b); Biochemical test results for *Escherichia coli* (c); Hemolysin production test on Blood Agar media (β-hemolysis) (d).

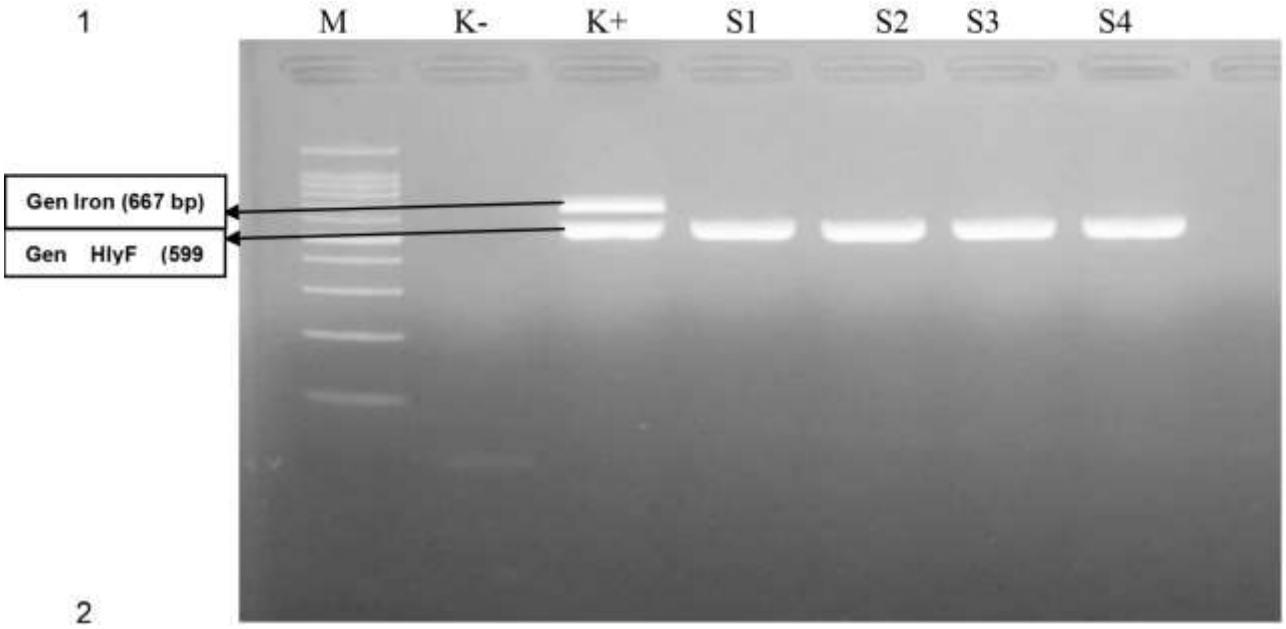

Figure 3. PCR results of the hlyF gene for *Escherichia coli* isolates positive for pathogens. Sample codes S1, S2, S3, and S4; M: marker; K-: negative control; K+: positive control.

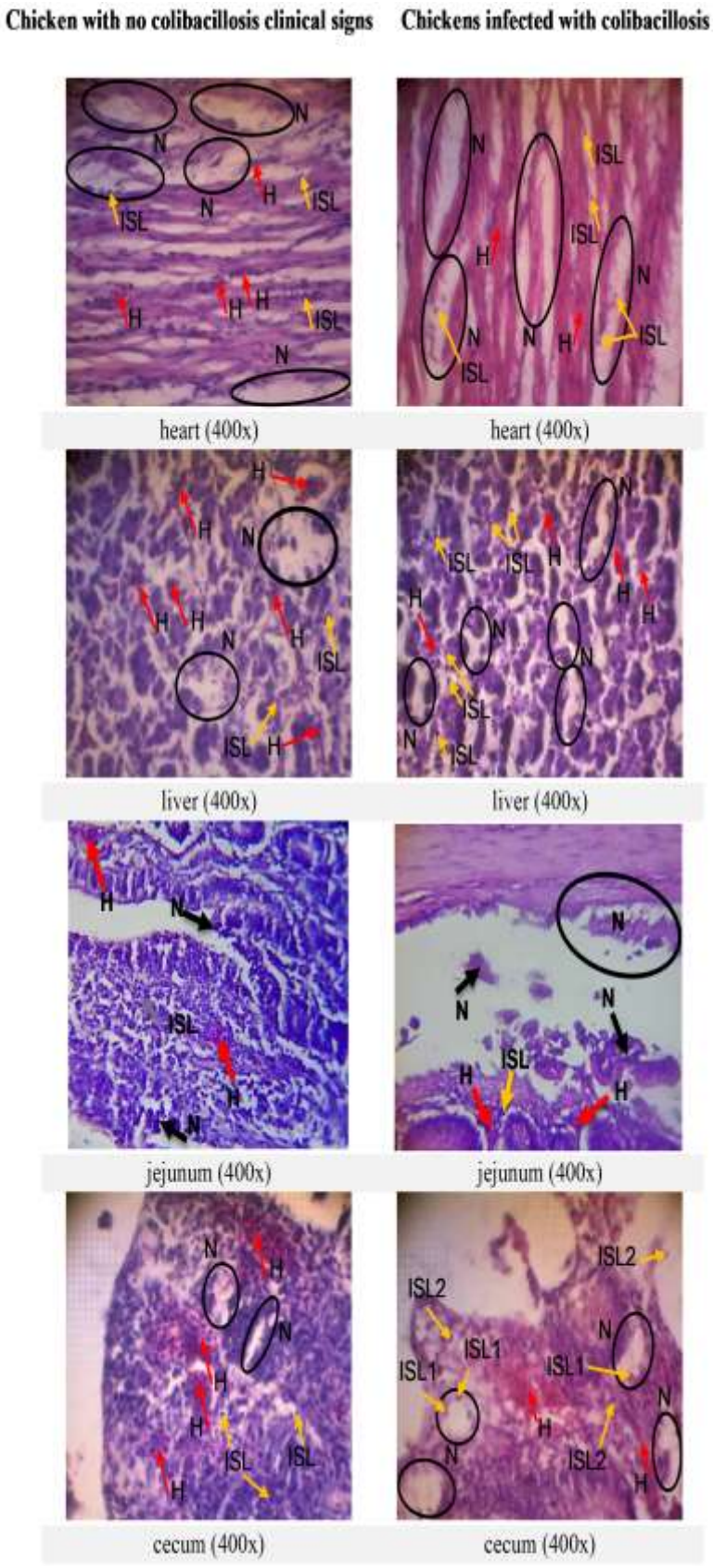

Figure 4. Results of histopathological examination (magnification: 400x).

4. Discussion

Examination results have identified petechiae in several chicken organs, swelling of the liver, abnormal heart bases, and the presence of fibrin membranes in the heart (pericarditis), hemorrhage was also observed in the jejunum and cecum. *Escherichia coli* is associated with colibacillosis caused by the APEC strain (12). Colibacillosis in poultry is a significant challenge in poultry production, leading to economic lossesand increased mortality in poultry (13). The incidence of colibacillosis in live poultry farms and markets is often linked to poor sanitation, hygiene and environment all conditions (14, 15). *Escherichia coli* can spread through the bloodstream (bacteremia), reaching target organs such as the heart, where it can colonize and cause inflammation, leading to fibrin formation that may spread to other organs such as the liver. Emphysema is found in the liver. Emphysema is an abnormal dilation of the air spaces accompanied by damage to the alveoli that can reduce maximum expiratory airflow due to decreased elastic recoil of the lungs (16). Infected chickens' jejunum with colibacillosis can experience intestinal distension, obstruction and bleeding within the digestive tract. These symptoms may be caused by enterotoxins in *Escherichia coli* that attach to the intestine, increasing blood vessel capacity (17), as illustrated in Figure 1.

Based on the Blood Agar test results shown in Figure 2c, positive results in testing the ability of bacteria to hydrolyze blood and protein are indicated by the formation of a clear zone (transparent zone) around the colony. The formation of the hemolysis zone's results shown in Figure 2c is linked to the release of active glycolipid compounds on substrates that are hydrophilic by bacterial strains. The test results on the *hlyF* virulence gene showed 100% positive rate (4/4) in *Escherichia coli* isolates from chicken cloacal samples. This result is higher than previous studies, which reported 83.3% incidence in Bangladesh (18) and 80% in Korea (19). In Indonesia, the virulence gene was identifies in native chickens at a rate of 100% (11) and in ducks there was a virulence gene of 60% (7). The *hlyF* gene , found in APEC, encodes a toxin that causes cells to undergo lysis and damage, motility, inducing host cell vacuolization, colonization, biofilm formation, agglutination, outer membrane vesicle formation, further contributing to bacterial virulence including cytolytic swelling toxin (CDT) ,and cytolysin factor A (ClyA) (20).

Table 1. Results of observations of histopathological changes in chickens infected with colibacillosis and healthy chickens.

No.	Sample	Healthy chicken	Chickens infected with colibacillosis
1.	Heart	**(N).** visible rupture of tissue experiencing necrosis **(H).** Haemorragic **(ISL).** There was also heterophils, inflammatory cell infiltration in the heart muscle fibers	**(N).** Rupture of tissue experiencing necrosis **(H).** Haemorragic **(ISL).** There was also heterophils, inflammatory cell infiltration in the heart muscle fibers
2.	Liver	**(H).** Haemorragic **(N).** Necrosis (**ISL).** Inflammatory cell infiltration	**(H).** Haemorragic **(N).** Necrosis (**ISL).** Inflammatory cell infiltration
3.	Jejunum	**(H).** Haemorrhage in the lamina propria **(N).** necrosis of the epithelial layer **(ISL).** Inflammatory cell infiltration of the lamina propria	**(H).** Hemorrhage in the submucosa **(N).** There is very visible rupture/necrosis of the villi in the muscularis **(ISL).** Inflammatory cell infiltration of the submucosa
4.	Cecum	(H). Haemorrhage in the crypts of Liberkuhn (N). Necrosis of the Lieberkuhn crypts (ISL). Inflammatory cell infiltration in the crypts of Lieberkuhn	**(H).** Haemorrhage in the tunica mucosa **(N).** Necrosis of the Lieberkuhn crypts **(ISL1).** Inflammatory cell infiltration in the crypts of Lieberkuhn. **(ISL2).** Inflammatory cell infiltration of the tunica mucosa, The mucous membrane that lines the digestive tract and is the innermost layer of the digestive tract.

Table 2. Average (±Standard Deviation) number of histopathological lesions in healthy broiler chickens and those infected with colibacillosis which experienced inflammatory cell infiltration, hemorrhage and necrosis.

		Healthy chicken	**Chickens infected with colibacillosis**
Heart	Inflammation	$1,60 \pm 0,54^{a}$	$2,60 \pm 0,54^{a}$
	Hemorrhage	$4,20 \pm 1,09^{a}$	$6,20 \pm 3,56^{b}$
	Necrosis	$4,40 \pm 1,67^{b}$	$5,20 \pm 1,09^{b}$
Liver	Inflammation	$2,60 \pm 0,54^{c}$	$2,60 \pm 0,54^{c}$
	Hemorrhage	$4,20 \pm 1,09^{c}$	$6,60 \pm 3,20^{d}$
	Necrosis	$4,40 \pm 1,67^{d}$	$6,00 \pm 2,00^{d}$
Jejunum	Inflammation	$0,20 \pm 0,00^{d}$	$2,60 \pm 0,89^{e}$
	Hemorrhage	$0,80 \pm 0,44^{e}$	$2,20 \pm 0,83^{e}$
	Necrosis	$1,20 \pm 0,45^{e}$	$2,60 \pm 0,54^{e}$
Cecum	Inflammation	$3,00 \pm 0,00^{e}$	$2,60 \pm 0,54^{f}$
	Hemorrhage	$4,20 \pm 1,09^{e}$	$6,20 \pm 3,56^{f}$
	Necrosis	$4,40 \pm 1,67^{f}$	$6,80 \pm 1,78^{f}$

^{a,b,c,d,e,f} superscripts of the same letter in the same column indicate insignificant differences ($P>0,05$), while superscripts of different letters in the same column indicate significant differences ($P<0,05$).

PCR testing results showed negative results for the *iroN* virulence gene. While reports have shown that the iroN gene is present in 100% of APEC strains in Bangladesh (18), 92% in China (21), 97% in Qatar, 100% in Korea (19) our results suggest otherwise. Another study on APEC chickens in Indonesia ,in irongen showed 100% (11). The iroN gene encodes siderophores, such as aerobactin, salmochelin,and yersiniabactin, which are secondary metabolites involved in iron absorption, thereby increasing bacterial growth and development. The ability of the iroN gene to enter the blood serum is very important because *Escherichia coli* causes sepsis and infections in various organs that are *iron*- deficient (20). Although PCR has detected the iron gene, it is important to note that this gene is caused by *Escherichia coli* isolated from feces, so it is more accurately clssified as *Avian Fecal Escherichia coli* (AFEC) (8).

Based on the results of histopathological testing of chickens infected with colibacillosis and those without clinical signs revealed similar changes in the heart, liver, jejunum, and cecum.Findings included inflammatory cell infiltration, hemorrhage and necrosis. Myocarditis haemorrhagica was observed in the heart with hemorrhage, edema, and inflammatory cell infiltration. Although the incidence of inflammation and edema is small, this pattern may reflect the heart's role as one of the predilection organs of *Escherichia coli* bacteria. The liver exhibited hepatitis with hemorrhage, necrosis and inflammatory cell infiltration. The presence of histopathological lesions in the form of hemorrhage and edema is generally caused by toxins produced by bacteria, absorbed into the bloodstream, damaging endothelial cells. Necrosis typically results from infection and/or toxin activity during colibacillosis (16).

The intestine exhibitshemorrhagic and necrotizing enteritis characterized by necrosis of the villi, hemorrhage, edema, and neutrophilic inflammatory cells. In cases of colibacillosis, the inflammation is characterized by the presence of neutrophilic inflammatory cell infiltration observed under microscopic examination. Inflammatory cell infiltration represents the body's defense mechanism. The dominance of neutrophil inflammatory cells occurs because neutrophils are essential in the body's defense system against microorganism invasion, especially bacterial invasion. The presence of histopathological lesions in the intestine is due to pathogenic *Escherichia coli* strains adhering to, colonizing and proliferating on the intestinal mucosa, releasing toxins. Pathogenic bacteria that colonize the intestine along with the toxins produced can cause inflammation, damage the epithelium, haemorrhage, necrosis, edema, damage the intestinal barrier and weaken the body's immune function (22).

Inflammatory cell infiltration is also seen in the digestive tract, liver, and lungs which are the initial organs that come into direct contact with *Escherichia coli* infectious agents. This triggers defense responses involving lymphocytes, heterophils, and macrophages that infiltrate into the tissues of these organs (16), The histopathological picture of acute intestinal infection caused by *Escherichia coli* toxin is characterized by the presence of heterophil in the intestinal mucus ,which causes congestion in the intestinal wall,along with increased macrophages and plasma cells (23). Necrosis is an irreversible damage to tissue caused by various factors, such as infection of old cells or chemicals. Necrosis begins with nuclear morphological changes, such as loss of chromatin appearance, wrinkling, increased density, and darkening of the nucleus, known as pyknosis (24). Necrosis begins with an inflammatory reaction in the liver in the form of hepatocyte swelling and tissue death (25).

Colibacillosis is a common and widely occurring disease in poultry that can cause significant economic losses, especially in developing countries including Indonesia. Avian pathogenic *Escherichia coli* as the causative agent of colibacillosis has virulence genes such as *hly* F, IroN, *iss*, *fim* H, etc. These virulence genes play role in APEC infection, including in host cell invasion, persistence in the bloodstream, absorption of metals from body fluids for bacterial growth and host cell damage. The zoonotic potential of APEC, as a cause of colibacillosis in poultry, shouldnot be underestimated, this is because APEC has the ability to cause urinary tract infections and meningitis in humans. Several studies have identified the possibility of zoonotic transmission of APEC from poultry to humans through contaminated food. The Discussion of colibacillosis is important for the poultry industry and human health, this is one way to provide education and knowledge to the public as an effort to control colibacillosis. Prevention strategies include educating farmers, treating chickens with mild symptoms, taking vitamins, maintaining good environmental hygiene, or immediately isolating and treating infected chickens exhibiting symptoms.

Acknowledgment

The authors would like to thank Wijaya Kusuma University Surabaya, Indonesia, through the Institute for Research and Community Service, which has provided Enimas internal grants in accordance with the Decree of the Chancellor of Wijaya Kusuma University Surabaya, number 37 of 2024, concerning recipients of research budgets for the 2024 funding year.

Authors' Contribution

Study concept and design: FJW.
Acquisition of data: AYRC, IAK, ACA.
Drafting of the manuscript: FJW, AM.
Statistical analysis: ORPAN, MAB.
Administrative, technical, and material support: FJW, AYRC, AM, IAK, ORPAN.

Ethics

Animal ethical approval was obtained from the Research Ethics Commission of the Faculty of Veterinary Medicine, Wijaya Kusuma Surabaya University, Surabaya, Indonesia, Ethical clearance number 148-KKE.

Conflict of Interest

The authors declare that they have no conflict of interest.

Data Availability

The data supporting the findings of this study are available in the research records conducted by researchers.

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