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A comparative study on histopathological features of duodenum, jejunum, and ileum from broiler chicken with avian pathogenic *Escherichia coli* infection

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ABSTRACT

Avian colibacillosis is a high-prevalence chicken farm disease caused by avian pathogenic *Escherichia coli (*APEC). It is necessary to identify and look up to the bacterial activity to stave off a decline in chicken performance. This study aimed to identify and determine the pathogenetic activity of APEC within broiler chickens on the duodenum, jejunum, and ileum histopathology comparison. The samples were collected from a total of 40 broilers, obtained from a highest-colibacillosis historical chicken house, identified with bacterial isolation swab-technique, and analyzed with nonparametric statistical lesions scoring. The identification result showed 82.5% *E. coli* with a 62.5% APEC confirmed. The histopathological feature showed a significant difference ($p<0.05$), where the jejunum appeared to encounter the most significant damage compared to the duodenum and ileum. Thus, it is concluded that APEC field infection varies in characteristics and pathogenicity, which affects intestinal organs based on the histology changes, mainly in the jejunum part of the intestine.

Keywords: Avian colibacillosis, Avian pathogenic *Escherichia coli*, Broiler chicken, Histology, Intestinal injury.

INTRODUCTION

Disease emergence is one of the main challenges in poultry, as chicken is one of the biggest sectors to fulfill protein needs for the human population worldwide. Avian pathogenic *Escherichia coli* (APEC) is one of the main serious challenges in broiler farming disease emergence. Colibacillosis has been shown to affect economic loss due to lower performance, egg production, mortality of the infected chicken, and high management expenses, such as disinfection and cleaning

(Wibisono *et al*., 2022). Colibacillosis is a bacterial disease caused by *E. coli* which is found to be widely prevalent in all chicken groups (Panth, 2019). Colibacillosis occurrence in chicken was reported in high prevalence in all chicken groups (9.52 to 36.73%), especially young ones from 1-2 weeks chicken, with a higher mortality rate if accompanied by other infections such as Newcastle Disease, *M. gallisepticum* infection, or Infectious Bronchitis (Panth, 2019; Dameanti *et al.,* 2020). Colibacillosis is often associated with other diseases as a secondary infection, such as infectious bronchitis, mycoplasmosis, infectious bursal disease, Newcastle disease, and hemorrhagic enteritis (Nordin *et al.*, 2021). Therefore, pathogenic *E. coli* chicken product contamination is zoonotic potential (Byomi *et al*., 2017; Al -Arfaj *et al*., 2016).

Escherichia coli is a natural inhabitant in the chicken intestinal tract. It turns into a pathogenic phase in several conditions such as overcrowding, malnutrition, poor ventilation, and extreme temperature, giving rise to bacteremia (Joshua *et al*., 2022). When transmitted either horizontally by ingestion or vertically by farm instruments. The septicemic (systemic colibacillosis) is mainly found in broilers, characterized by pericarditis, peritonitis, air-sacculitis, and perihepatitis. The acute phase of septicemia can revert to the chronic phase of localized disease type, present in unusual sites such as joints, eyes, brain, and bones (Koutsianos, *et al*., 2020).

The severity of APEC's pathogenicity is detectable based on clinical signs, postmortem gross lesions, bacterial isolation, and histopathological findings. It was reported that three of five farms presented elevating chicken mortality with ataxia, dyspnea, cough, sneezing, white to yellowish diarrhea, and cachexia (Taunde *et al*., 2021). Besides, respiratory distress and organ damage is can be detected such as air-sacculitis or chronic respiratory disease, swollen head syndrome, yolk sac infection (omphalitis), Hjarre's disease, enteritis, and hemorrhagic septicemia (Abalaka *et al*., 2017; Siregar *et al*., 2019). The gross lesions can be specified in histopathological examination of the affected organs. In general, fibrinous exudate along with mononuclear infiltration in the thickening pericardium is usually found. In mild cases, there will be found heterophilic infiltration in the affected organs. When the case is further continued to a heavy infection, the pericardium excessively thickened along with eosinophilic necrotic areas containing heterophils in different stages of degeneration. Besides, severe myopathy due to degeneration in between muscle fibers, leucocytic infiltration dominated with heterophil is also evident (Shah *et al*., 2019). Furthermore, to strengthen the identification, bacterial gene isolation can be detected by using visceral organs (Ibrahim *et al*., 2019). APEC strains have been associated with specific serogroups, namely O1, O2, and O78, while during colibacillosis outbreaks, the serogroup isolated are O8, O15, O18, O35, O88, O109, and O115 (Gambi *et al*., 2022).

The objectives of the current study was to isolate and characterize *E. coli* strains to determine the pathogenicity compared with the organ histopathological findings at poultry farms in Malang Regency.

MATERIALS AND METHODS

Sample Preparation

Purposive sampling was the technique used for sampling method from the highest colibacillosis historical cage. The chicken was selected according to the modified APEC-specific colibacillosis clinical sign scoring from the previous research study (Table 1). This research has obtained ethical eligibility with the number: 096-KEP-UB-2020 from Animal Care and Use Committee, Universitas Brawijaya.

Intestinal Organ Sample Collection

The anamnesis, signalment, and clinical signs of the infected chickens were recorded and euthanized by decapitation for a necropsy procedure. The anatomy pathological findings were graded using the modified-scoring system based on the severity changes microscopically (Ebrahim-Nik *et al*., 2018) (Table 2).

The intestinal tenue was cut into small pieces and subjected to a luminal swab for bacterial isolation identification. This process was carried out around the Bunsen burner to keep the collection area sterile from other bacterial exposure outside the intestinal lumen. The cotton swab was then inoculated to nutrient broth media to maintain bacterial viability.

Escherichia coli Strain Isolation and Identification

EMBA (Eosin Methylene-Blue) agar was used to obtain pure colonies of *E. coli* bacteria. The media was incubated for 24 hours at 37^{o}C temperature using the 4-quadrant streak method. After incubation, the pure colony was placed on glass objects with several drops of sterile distilled water and fixed on a Bunsen burner fire to dry. Crystal violet was added to the slide to stain the colony. After 2 minutes, the slide was rinsed under running water. Subsequently, aquos iodine was added to fix the staining. After 1 minute, it was rinsed under running water. Subsequently, acetone alcohol was added for a few seconds to remove the remaining staining liquid. Then, safranin was added for 1 minute. Finally, the slide was rinsed under running water. The sample was identified using a microscope at 1000x magnification.

Several biochemistry examinations were carried out to identify the pure *E. coli* isolate. Triple Sugar Iron Agar (TSIA), indole methyl red Voges-Proskauer, and citrate utilization (IMViC), *urease*, and confectionery (glucose, sorbitol, mannitol, sucrose, lactose) media were used to identify the APEC strain. A total of 8-10 drops of Methyl red solution was added to the Methyl-red biochemical test. A total of 8-10 drops of alpha naphthol and KOH 40% were added to the VP medium. A total of 5-10 drops of *Kovac* were added to the Indole test. The *E. coli*-confirmed samples were then subjected to SMCA (Sorbitol-MacConkey Agar) media for APEC confirmation, using 4-quadrant streak. The SMCA media can differentiate the pathogenic and nonpathogenic *E. coli* by the sorbitol fermentation capability of *E. coli.*

Histopathology Slide Preparations

The histopathological preparations making process consists of several stages: fixation, dehydration, purification, paraffin infiltration, embedding, sectioning, pasting, and staining. The organ was cut transversely into a 1 x 1 x 0.5 cm size. The organ was dehydrated with stratified alcohol and purified with xylol. The organ was then placed into paraffin infiltration at 56^{o}C. The organ was then molded into a paraffin block to be cut with a microtome (5 μm thickness). Finally, the tissue was fixed to the glass object.

The fixed tissue was stained using HE staining. The slide was deparaffinized using xylol and subjected to rehydration using graded ethanol. Furthermore, the slide was dyed using Hematoxylin, dipped in acidic alcohol, and rinsed under running water. Preparations were stained using Eosin. The slide was then dehydrated using graded ethanol (70%, 80%, 85%, 90%, 95%, 100%). Finally, the slide was re-rarefied with xylol I, II, and III and preserved by the mounting method.

Histopathology Evaluation

The microscopy organ findings were analyzed using the previous modified scoring system (Table 3) (Abd Elatiff *et al*., 2019; Abdul-Azain *et al.*, 2016). The sample was identified using a microscope at 1000x magnification.

Data Analysis

The APEC isolation identification was conducted using a descriptive method. Meanwhile, the lesion scoring result was analyzed using the score of the duodenum, jejunum, and ileum histology profile. The data which were not normally distributed continued to *Kruskall-Wallis* nonparametric statistical analysis. The data with a significant difference were continued to *the Mann-Whitney* analysis. Finally, the scoring analysis was conducted using *Statistical Product and Service Solutions* (SPSS) program.

RESULTS

Clinical Symptoms and Necropsy

Table 1. Clinical symptoms modified scoring of APEC infection

Score	Respiratory symptoms	Systemic Symptoms
0	No symptoms/normal.	No symptoms/normal.
1	Mild breathing disorders.	Hanging feathers, chicken reaction normal.
2	Heavier respiratory distress (increased movement of the chest when breathing).	Hanging feathers, the chicken's reaction slow.
3	In severe respiratory distress, the beak opens to help breathe.	Solitude, feathers hang down, eyes closed, chickens do not react to the presence of stimuli.
4	Very severe respiratory distress (deep breathing with light frequency).	Solitude, feathers hang down, eyes closed, consciousness decreases, chickens do not react to the presence of stimuli.

Source: Antão *et al*. (2008).

Table 2. Macroscopic changes modified scoring in APEC infections

Score	Description of organ lesions
0	No change
1 (Minimum)	Airsac/pericardium/ turbid hepar; the presence of fluid in pericardium; there is little fibrin exudate in the surface is hepar; the intestines were unchanged.
2 (Lightweight)	Airsac thickens, fibrin extends to the pericardium cavity, swelling of the liver with enveloped exudate, and bleeding at some points of the intestine.
3 (Medium)	There is thick fibrinopurulent exudate in the Airsac, pericardium, and hepar; the intestine has widespread bleeding
4 (Weight)	Severe and extensive fibrinous airsacculitis, pericarditis/ In Perihepatitis, the intestines undergo necrosis.

Source: Ebrahimi-Nik *et al*. (2018).

Table 3. Microscopic lesions modified scoring of intestinal organs of APEC infection

Organ	Score	Description of organ lesions
Duodenum	0	No change/normal.
	1	Minimal damage (edema and shortening of villi)
	2	Minor damage (villi are lightly torn/dulled, and goblet cells proliferate)
	3	Moderate damage (infiltration of inflammatory cells)
	4	Severe damage (necrosis)
Jejunum	0	No change/normal.
	1	Minimal damage (edema and shortening of villi)
	2	Minor damage (villi are lightly torn/dulled, and goblet cells proliferate)
	3	Moderate damage (infiltration of inflammatory cells)
	4	Severe damage (necrosis)
Ileum	0	No change/normal.
	1	Minimal damage (edema and shortening of villi)
	2	Minor damage (villi are lightly torn/dulled, and goblet cells proliferate)
	3	Moderate damage (infiltration of inflammatory cells)
	4	Severe damage (necrosis)

Sources: Abd Elatiff *et al*. (2019); Abdul-Azain *et al*. (2016).

Table 4. Systemic and respiratory clinical symptoms percentage of APEC-infected chickens

Chicken samples	Percentage (Σ positive chickens/ total sample)				
	Clinical Symptom Scoring				
	0	1	2	3	4
Cage 1	0/10	0/10	5/10	4/10	1/10
Cage 2	0/10	0/10	7/10	3/10	0/10
Cage 3	0/10	0/10	5/10	3/10	2/10
Cage 4	0/10	0/10	7/10	3/10	0/10
Sum	0	0	24	13	3
Percentage	0%	0%	60%	32.5%	7.5%

Table 5. Biochemical test results

Biochemical Test	Interpretation
	S: Acid B: Acid
Urease	-
Indol	√
Methyl Red	+
Voges-Proskauer	-
Citric	-
Motility	√
Glucose	+
Lactose	+
Sucrose	√
Sorbitol	√
Mannitol	-

Description: +: Positive reaction
–: Negative reaction
√: there was a sample showing both positive and negative reactions

Table 6. Mean and standard deviation of duodenum, jejunum, and ileum severity

Small Intestine	Small Bowel Injury Score Mean ± SD
Duodenum	0.45 ± 0.08
Jejunum	1.25 ± 0.10
Ileum	0.98 ± 0.04

Table 7. Saphiro-Wilk normality of duodenum, jejunum, and ileum severity

Small Intestine	Saphiro-Wilk
	P
Duodenum	.000
Jejunum	.000
Ileum	.020

The results showed $p<0.05$; which means it is not normally distributed.

Sample determination analysis showed that there was no clinical sign for the scoring 0 and 1 (0%). There were 24 chickens (60%) scored 2, 13 (32.5%) scored 3, and 3 chickens (7.5%) scored 4 of the clinical symptoms (Table 4)**.** Besides, other several clinical symptoms were found such as dwarfism, compared to chickens in one cage population, hair loss in some areas of the skin, diarrhea, swelling in the abdominal area, and osteoarthritis (Figure 1). Besides, the chickens showed a body condition scoring 2 out of 5.

There were several macroscopical changes found in the chickens. A total of 13 chickens scored 1 with clinical symptoms stage 2, 6 chickens scored 2 with clinical symptoms stage 3, 4 chickens scored 1 with clinical symptoms 3, and 2 chickens scored 3 with clinical symptoms 4 (Figure 2).

APEC Identification

The identification result showed that 33 from 40 samples identified an *E. coli* typical growth within EMBA media. *E. coli* is characterized by the green metallic sheen-colored formed colonies (Figure 3). The Gram staining results showed Gram-negative bacteria in a short rod or coco basil shape (Figure 4).

Biochemistry Examination

The result was then continued to APEC strain identification. The SMCA were used (Figure 5) and other biochemistry examination showed that there were 29 samples are *E. coli* confirmed (Table 5). The colonies were using the plate-streak technique and incubated at 37^{o}C for 24 hours. Pathogenic *E. coli* doesn't ferment sorbitol and remained colorless. Based on the EMBA isolation, Gram staining, and biochemistry examination, it was shown that 29 samples (72.5%) indicated *E. coli,* whereas the 25 samples (62.5%) were APEC strain confirmed.

Histopathology Result (Duodenum, Jejunum, Ileum)

According to the scoring method, there were microscopy findings varied from the score of 0-5. There were no abnormal changes in the samples that scored 0 (Figure 6). There were laminae propria edema and shortened villas in the samples that scored 1 (Figure 7). There were villi ruptures and epithelial erosion, along with goblet cell hyperplasia in the samples scored 2 (Figure 8). There were thickened vili in lamina propria due to inflammatory cell infiltration and epithelial desquamation in the samples scored 3 (Figure 9) and a higher inflammatory cell infiltration along with necrosis in the samples scored 4 (Figure 10).

Non-Parametric Scoring Identification

The Kruskal-Wallis and Mann-Whiteney test was conducted to obtain the average score in the overall samples. The average damage ratio to each part of the small intestine organ showed an average standard deviation of the duodenal injury score as the lowest (0.45 ± 0.08), ileum (0.98 ± 0.04), and jejunum as the highest (1.25 ± 0.10) (Table 6). The Saphiro-Wilk test showed that the data were not distributed normally ($p<0.05$) (Table 7).

The probability value was smaller than 0.05 ($p<0.05$) (Table 8). It concluded that there was a significant difference in the average score between the duodenum, jejunum, and ileum. This result was also supported by the mean and standard deviation test, where the average injuries of the duodenum, jejunum, and ileum were different.

The Mann-Whitney Post Hoc test showed that there was a significant difference ($p<0.05$) among the intestinal injuries (Table 9). The comparison between the duodenum and jejunum ($p=0.000$) and jejunum and ileum ($p=0.019$) showed a significant difference, respectively. Thus, the average scoring of duodenal, jejunum, and ileum injuries infected with APEC was significantly different. Based on the Kruskal Wallis test data, the average value and standard deviation, and the Mann-Whitney Post Hoc test, it concluded that the most severe injury lies in the jejunum.

DISCUSSION

Escherichia coli infection varies in manifestations in chicken. The infection is spread by the fecal-oral route. Once the bacteria entered the gastrointestinal tract of the chicken, it attracts heterophils and macrophages. The heterophils are the fastest to *E. coli* within 6 hours post-infection response, contributing to the clearance mechanism by degranulation and antibacterial compounds (Mol *et al*., 2019). Besides, the macrophage is a key component to contribute to tissue self-repair inflammation (Li *et al*., 2022). Thus, when there is either a localized or systemic infection, the organ will be changed functionally, resulting in poor performance of the chicken. The osteoarthritis obtained is one of the results of localized infection within the chicken bone, resulting in an inflammatory reaction in its structure. Furthermore, the systemic infection will lead to endothelial vasodilatation inflammatory-mediated, increasing vascular permeability (Azhimah *et al*., 2018), resulting in blood plasma leakage to the eye area, and inducing an excess eye discharge.

APEC identification can be conducted using media isolation. EMBA is a typical growth of *E. coli. E. coli* bacteria are suspectible when the green metallic sheen-colored colonies-like appear in the media (Khasanah *et al.,* 2021). When the result leads to a suspect, a further specific examination is needed. SMCA is a selective and differential biochemical medium to distinguish pathogenic serotype *E. coli* from commensal *E. coli* through the fermentation ability of sorbitol (Izevbuwa and Okhuebor, 2020). SMCA is designed specifically to isolate and distinguish the *E. coli* pathogens that tend to slow or the non -sorbitol fermenting *E. coli* (Eluchie *et al*., 2019). A recent research reported a set of *E. coli* APEC strains isolates containig 2 or more virulence markers. To be specific, several genes (*hlyF, ompT, iroN, iss,* and *iutA*) are located on the virulence-plasmid. These virulence genes could potentially jump to other species and cause human infection which act as zoonotic pathogens (Kim *et al.,* 2020).

The poor clinical presentation presented due to the bacterial capability to colonize the gastrointestinal tract of the chicken. APEC strains of *E. coli* inhabit the intestinal tract and have a genes-carrying plasmid which becomes its distinct characteristic that is required through horizontal gene transfer (Fancher *et al*., 2020). The nonparametric examination showed that the jejunum was more prone to injury due to APEC pathogenicity which was confirmed by the intestinal histopathology. The bacteria penetrate the epithelial layer, causing epithelial erosion and breaking the intestinal wall barrier. The later infection then results in submucosa damage. The villi rupture calls out the inflammatory cells to the infection sites and promotes the goblet cells to proliferate. This mechanism occurs to counter the infection and prevent the bacteria enter the deeper intestinal structure. Once *E. coli* infecting chicken, it enters bloodstream expands to systemic infection. Inflammation occur as an inflammatory response, characterized by an increase of cytokine IL-1, IL-6, and TNF-alpha. The vascular premeability is affected and increases the fluid and protein accumulation in tissues, result in a gelatinous exudate (Dameanti *et al.,* 2022). The severity of infection is noted by the cells around the intestinal parts. The macrophages and inflmmatory cells are characterized by dark purple cell within the tissue histopathology, as it colored by the methylene blue and eosin during the staining process. All the cells come out to tackle bacteria and microorganism by phagocytosis process (Hayer *et al.,* 2021).

The presence of *E. coli* pathogens will affect the digestive system process and nutrient absorption. The food passes to the small intestine, which completes the protein digestion through the digestion enzymes and intestinal juices to finally enter the bloodstream (Clavijo and Florez, 2018). Gastrointestinal tract of chicken contains the highest amount of bacterial diversity and abundance. The imbalance (dysbiosis) quantitively or qualitatively of intestinal microflora leads to the put growth and virulence gene expressions of pathogens becoming an intestinal disease (Fancher, 2020; Abdelhamid *et al*., 2020). APEC

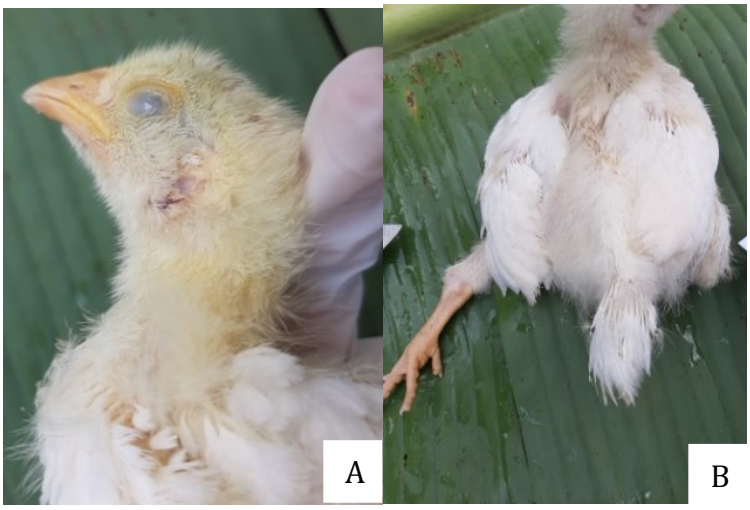

Figure 1. (A) Systemic symptoms (eye closed, decreased response), (B) Osteoarthritis.

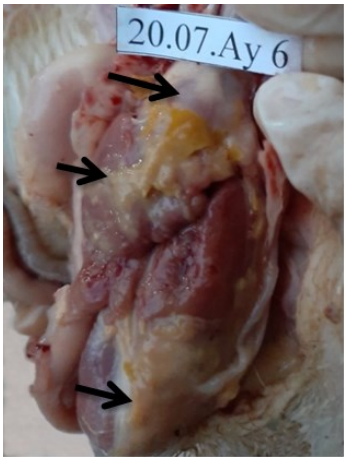

Figure 2. Pericarditis, perihepatitis with fibrinous exudate, and airsaculitis on the necropsy results (starting at the uppermost arrow sequentially downwards).

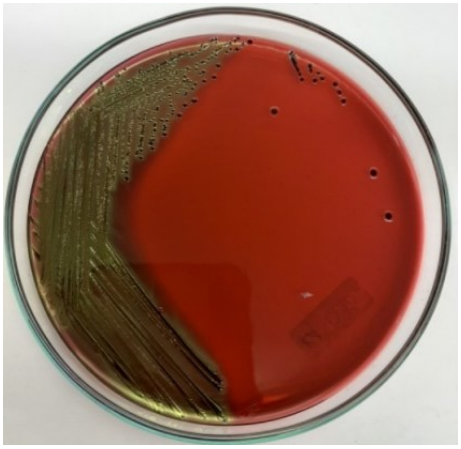

Figure 3. green metallic sheen-colored formed colonies on EMBA media.

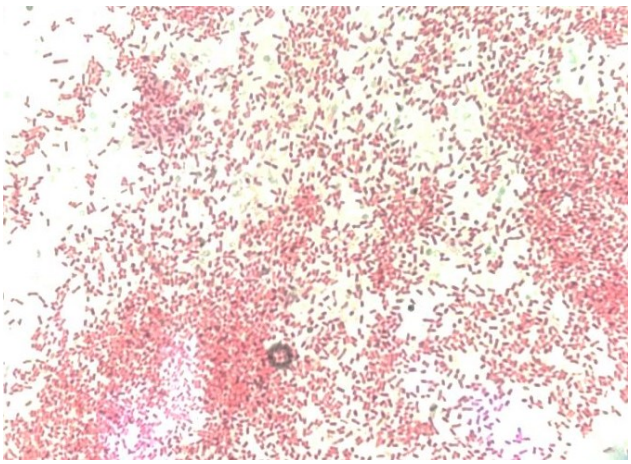

Figure 4. Short bacillus *E. coli* bacteria on Gram Staining results.

Figure 5. Colorless colony on SMCA media.

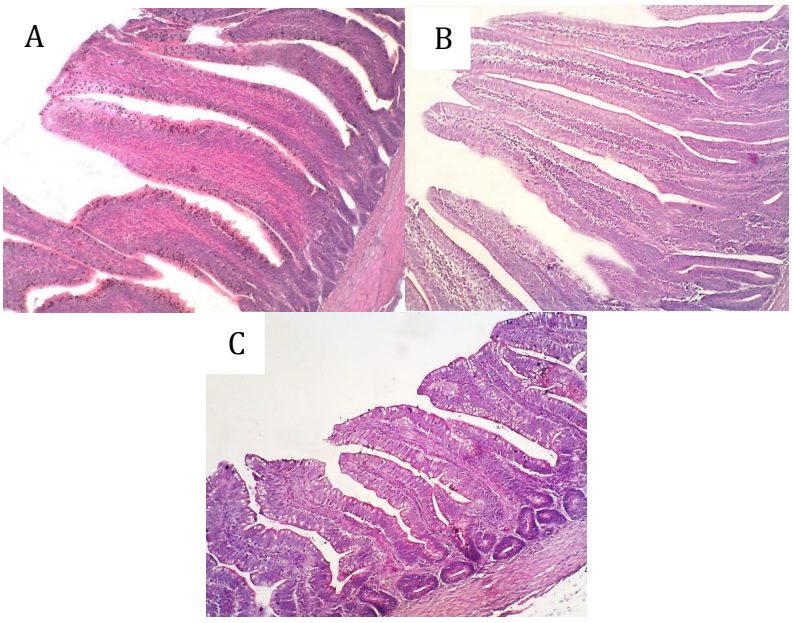

Figure 6. Scoring 0: (A) Duodenum; (B) Jejunum; (C) Ileum. HE Staining 1000x.

Table 8. Kruskal-Wallis test comparison of average scoring duodenum, jejunum, and ileum values.

Variable	N	Df	P
Scoring Cedera Small Intestine	25	2	0,00*

*The test results showed a significant difference ($p<0.05$) in the average injury scoring results between the duodenum, jejunum, and ileum.

Table 9. Mann-Whitney Post Hoc Test result.

	Duodenum	Jejunum	Ileum
Duodenum	-	0.000*	0.000*
Jejunum	0.000*	-	0.019*
Ileum	0.000*	0.019*	-

*Shows a significant difference ($p<0.05$).

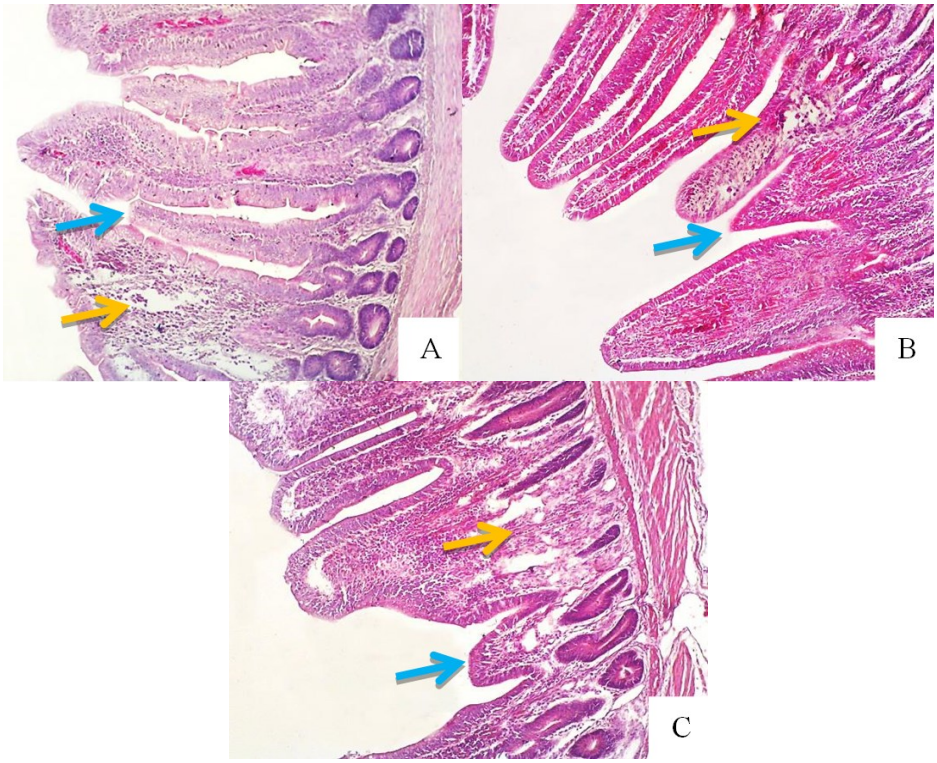

Figure 7. Scoring 1: (A) Duodenum; (B) Jejunum; (C) Ileum. HE Staining 1000x. The yellow arrow indicates the presence of edema in the lamina propria; the blue arrow indicates the shortening of the villi.

also has an acetate assimilation system in replication within macrophages to become an intracellular pathogen. This mechanism then enhances the bacterial ability to exploit its metabolic pathway uptaking hots nutrient sources for survival and proliferation within host intracellular compartments (Zhuge *et al*., 2019).

A different scoring and significance in different parts of the intestine are potentially due to the function of intestinal compartments. Bile salts in the duodenum are secreted by hepatocytes that are formed by the conjugation of bile acids with taurine or glycine (Zaefarian *et al*., 2019). The presence of bile salts is considered to help to protect the overgrowth condition of microorganisms. It was reported that total bacteria, total fungi, *E. coli,* and *Salmonella* were decreased in a broiler chicken-fed diet supplement-

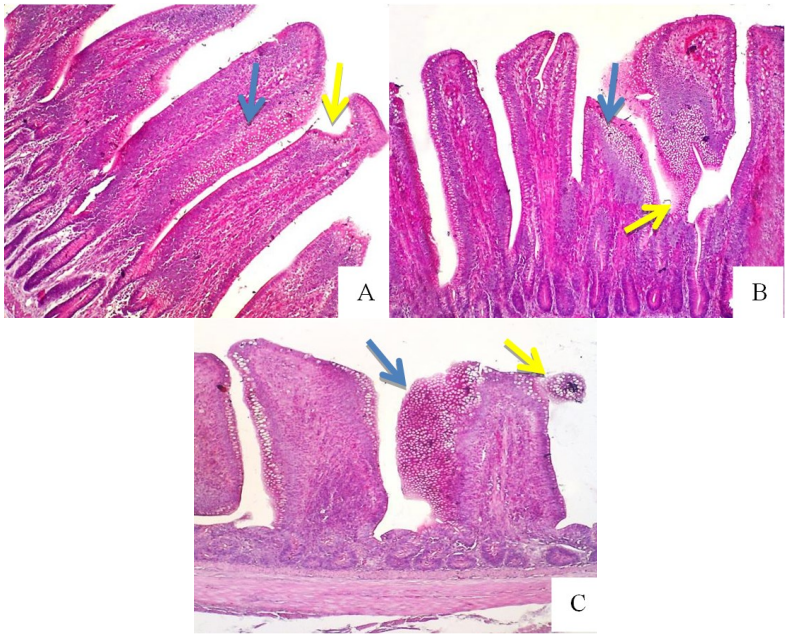

Figure 8. Scoring 2: (A) Duodenum; (B) Jejunum; (C) Ileum. HE Staining 1000x. Yellow arrows indicate torn villi and epithelial erosion; blue arrows indicate goblet cell hyperplasia.

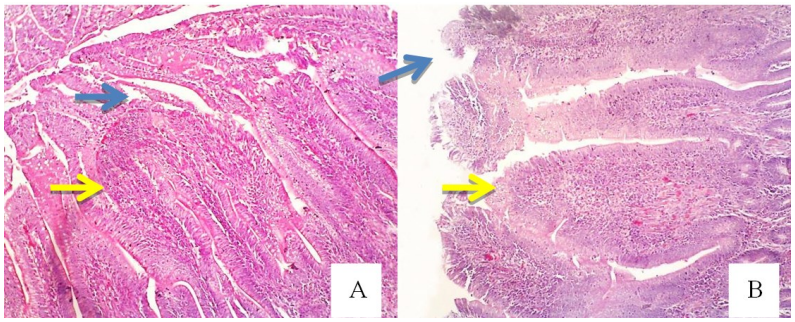

Figure 9. Scoring 3: (A) Jejunum; (B) Ileum. HE Staining 1000x. The yellow arrow indicates the thickening of the villi due to infiltration of inflammatory cells in the lamina propria, the blue arrow indicates the desquamation of the epithelium

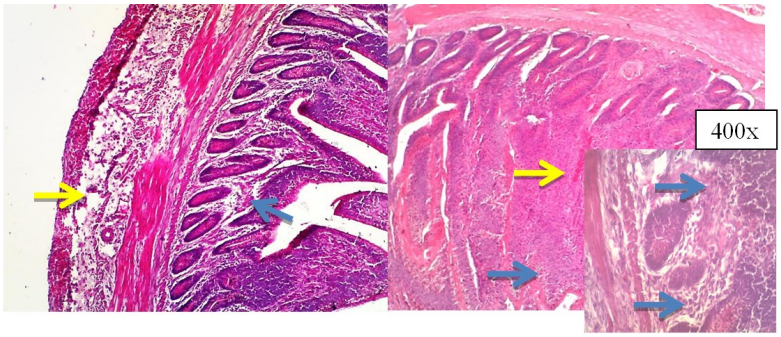

Figure 10. Scoring 4: Jejunum; HE Staining 1000x..The yellow arrow indicates the area that has undergone necrosis, and the blue arrow indicates the infiltration of inflammatory cells.

ed with bile salts (Mohamed *et al*., 2020). Jejunum is an intestinal part with a longer structure compared to the duodenum and ileum. Sittiya *et al*. (2019), reported that length of chicken intestinal parts of jejunum is 3.04 cm/100gBW, ileum is 2.96 cm/100gBW length, and duodenum is 1.24 cm/100gBW. A long structure potentially affects the APEC infection, resulting in a higher damage score that was obtained. Kayal *et al*. (2022), also reported that jejunal mucosa was diverse in the microbiota as it highly supports the microbial community membership. Thus, the jejunum is susceptible to APEC colonization. Meanwhile, the ileum is a structure that functions to absorb the remaining nutrients missed by the proximal small intestine. The lower nutrient absorption is in connection with the shorter villi and thicker mucus layer compared to the duodenum and jejunum. Besides, a high frequency of goblet cells expands the number of GAPs (Goblet cell-associated antigen passages), which deliver luminal antigens to DCs (Dendritic cells) within the lamina propria upon goblet cell secretion during homeostasis (Brown and Esterhazy, 2021). Similarly, increased frequency and density of Payer's patches (PPs) within the ileum may increase microfold cell (M-cell) mediated antigen uptake, which all play roles in the regulation and stimulation of gut immune defense (Shini and Bryden, 2022).

CONCLUSION

APEC is a pathogenic bacterial disease, manifests vary from respiratory abnormality to osteoarthritis, and organ changes. From a total of 33 samples, 29 were confirmed based on the biochemical examination, and 25 were included as APEC strains. The most affected part of the small intestine is the jejunum, compared to duodenum and ileum. The underlying reason lies in the complexity of the intestinal structure, environmental exposure, digestive physiology, nutrition sufficiency, and the other external stressors that influence chicken immunity.

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