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Microbiological and pathological studies of concurrent infections associated with retarded growth in broilers in Behira governorate

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ABSTRACT

Several broiler flocks in Behira governorate, suffered from growth retardation that caused a lot of economic losses. Thus, this study was done to identify some of the probable causes and their pathological picture. Samples were collected from 50 broiler chicken flocks, suffering from retarded growth during 2018–2021. Chicken anemia virus (CAV) was detected by PCR in five flocks (10%), meanwhile avian reovirus (ARV) was detected by RT-PCR in 29 flocks (58%). Antibodies of ARV and CAV were detected by ELISA in 86% and 12% from the examined flocks, respectively. Five serotypes of *E. coli* (O86a, O112, O128, O114, and O55) were isolated from 19 flocks (38%). *C. perfringens* was isolated from 26 flocks (52%). The observed pathological features were associated with hyper activity of intestinal mucus secreting cells, elongation of cryptal intestinal villi which were partly filled with inflammatory cells and depletion of Payer's patches of ileum. Pancreas showed degeneration and vacuolation of acinar cells. Proventriculus showed dilation of glandular acini, necrosis and sloughing of the glandular epithelium. Bursa showed lymphocytic depletion and atrophy of the bursal follicle, these changes were associated with lymphocytic depletion within the spleen and thymus. Additionally, apoptotic bodies were detected in liver and thymus. This study can indicate the presence of several causes to growth retardation (in vivo) that can do a synergistic effect to aggravate the problem.

INTRODUCTION:

Growth retardation is one of the challenges that threatening poultry industry especially broilers and causing severe economic damages that resulting from poor feed conversion ratio, decreased body weight, excessive culling and carcass condemnations.

Avian reovirus (ARV) has worldwide dis-

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tribution and affects chickens, ducks, ostriches, turkeys, and wild birds. Young chickens show the major damage from ARV infection with up to 10% mortality and 40% morbidity and present many clinical diseases, such as malabsorption syndrome (MAS), viral arthritis/ tenosynovitis, runting–stunting syndrome (RSS), respiratory diseases, myocarditis, hepatitis, neurological signs (Van De Zande and Kuhn 2007, Pantin Jackwood et al. 2008, Devaney et al. 2016, De Carli et al. 2020 and Kim et al. 2022).

Growth retardation and worse feed conversion characterize MAS and RSS. In addition to ARV, growth retardation is caused by chicken anemia virus (CAV) which is the causative agent of chicken infectious anemia (CIA) that is an immunosuppressive disease-causing lymphoid depletion, aplastic anemia, increased sensitivity to secondary viral, bacterial or fungal infections, yellow bone marrow and thymus atrophy and affects mainly in 10–14 days-old chickens (Adair 2000 and Liu et al. 2022).

Escherichia coli (*E. coli*), the gram-negative bacteria, inhabit the intestinal tracts of animals and human as one of the essential components of their microbiota (Santos et al. 2020, Bertelloni et al. 2022 and Méndez Moreno et al. 2022). However, some strains of *E. coli* are pathogenic and can cause severe intestinal or extraintestinal diseases that related to variety of virulence genes (Santos et al. 2020). Necrotic enteritis (NE) is a widespread disease of broiler chickens, is caused by *Clostridium perfringens* (*C. perfringens*).

Klebsiella pneumonia (*K. pneumonia*) was isolated from broilers suffering from poor growth (Elgaos et al. 2019). *K. pneumoniae* virulence factor *magA* (mucoviscosity associated gene A) contributes to the, septicemia, pneumonia, liver and lung abscesses and increases resistance to phagocytosis (Chan et al. 2005, Struve et al., 2005 and Chung et al. 2007).

Staphylococcus aureus (*S. aureus*) is an opportunistic organism of animals and human, that under certain circumstances can cause some disease conditions, such as arthritis, synovitis, tenosynovitis dermatitis, osteomyelitis, femoral head necrosis, omphalitis and bumblefoot (Basset et al. 2011, Abd El Tawab et al. 2017 and Cheung et al. 2021).

Recently, several broiler flocks in Behira governorate suffered from growth retardation that associated with other clinical symptoms such as diarrhea and indigestion. Therefore, this study was done to identify some of the probable causes and their pathological picture in order to proper control this clinical problem.

MATERIALS AND METHODS:

Samples collection:

Samples were collected from 50 chicken flocks (10 samples from each flock), aged 14−47 days and suffered from retarded growth and poor uniformity at different localities in Behira governorate during 2018–2021. Flock capacities ranged from 1500-6500 birds of different breeds (16 Cobb, 8 IR, 16 Ross and 10 Arbor Acres) and all of them were not vaccinated against CAV or ARV. Bone marrow, thymus, liver and spleen were collected when infection with CAV was suspected, meanwhile liver, spleen, proventriculus, pancreas and intestine were collected if ARV was suspected. The tissue samples were ground, homogenized in saline (20% W/V) containing 200 mcg/ ml Streptomycin and 2000 IU/ml Penicillin and centrifuged for 15 minutes at 3000 rpm. Supernatant was collected and kept at -80°C till viral detection. Bacteriological examination was done on heart blood, heart, kidney, lung, liver, spleen, joints that were collected. In addition to intestine and swabs from skin ulcers. For histopathological investigation, proventriculus, intestine, liver, spleen, pancreas, thymus and bursa were placed in 10% neutral buffered formalin, sectioned, stained with hematoxylin and eosin (H& E) (Bancroft and Gamble, 2008). Ten serum samples were collected from each flock and stored at -20°C until to be used for detection of viral antibodies by ELISA.

Viral detection by PCR and RT-PCR:

Nucleic acid was extracted from samples using QIAamp MinElute virus spin kit (Qiagen, Germany) according to the manufacturer's instructions. PCR was applied to detect-

ed DNA of CAV using 12.5 µl of Emerald Amp Max PCR Master Mix (Takara, Japan), 1 µl of each primer, 5.5 µl of water, and 5 µl of DNA template (table 1). RT-PCR was done to detect RNA of ARV using 25 µl of 1-Step PCR ReddyMix buffer (Thermo Fisher Scientific, USA), 1 µl of Verso Enzyme Mix, 2.5 µL of RT Enhancer, 13 µl of Nuclease-free water, 5 µl of RNA template and 2 µl of each primer (table 1). The reaction was done in Applied biosystem 2720 thermal cycler (Germany) (table 2). Electrophoresis of PCR products was done in 1.5% agarose gel along with 100 bp DNA ladder (Fermentas, Thermo, Germany).

2.3. Bacterial isolation and identification:

For isolation of *C. perfringens,* intestinal samples were inoculated anaerobically into cooled cooked meat medium (CMM) (Oxoid) and incubated at 37°C for 24 hours in a Gaspak anaerobic jar. A loopful of inoculated medium was streaked onto neomycin sulphate (200ug/ml) sheep blood agar plates then re-incubated anaerobically for 24 h at 37°C (Amer et al. 2016). *C. perfringens* suspected colonies were tested on egg yolk agar medium for lecithinase activity to determine pathogenic type. Typical colonies (lecithinase producer and that with double zone of haemolysis on blood agar medium) were picked up, sub-cultured and purified for further biochemical identification tests.

E. coli isolation was performed on MacConkey and Eosin Methylene Blue (EMB) and confirmed by biochemical tests (IMVIC) according to Quinn et al. (2002). Serotyping of the identified isolates was done according to Kok et al. (1996). *K. pneumoniae* was isolated on MacConkey agar and identified by biochemical tests (lactose fermentation test, Indole production test, Voges-Proskauer test, Citrate utilization test) according to Arya et al. (2020).

S. aureus was isolated on Baird parker agar and mannitol salt agar then identified by biochemical identification: according to Quinn et al. (2002)**.** Detection of *Salmonella* was done according to **(ISO 6579, 2002).**

2.4. Detection of bacterial toxin and virulence factors coding genes by PCR:

DNA was extracted from the identified bacterial colonies using QIAamp DNA Mini kit (Qiagen, Germany) according to the manufacturer's instructions with slight modification. Briefly, 200 µl of colony suspensions were incubated at 56^{O}C for 10 min with 200 µl of lysis buffer and 10 µl of proteinase K, 200 µl of 100% ethanol were then added to the lysate, nucleic acid was then washed and eluted with 100 µl of elution buffer.

Alpha toxin, *magA, iss* and *eaeA* coding genes were identified using 12.5 µl of Emerald Amp Max PCR Master Mix (Takara, Japan), 1 µl of each primer (table 1), 4.5 µl of water, and 6 µl of DNA template. The reaction was performed in an Applied biosystem 2720 thermal cycler with the thermal profiles indicated in table 2 and the electrophoresis was done by the same way as previously mentioned during viral detection.

2.5. Detection of ARV and CAV Antibodies by ELISA:

Serum samples were tested by ARV and CIAV antibodies ELISA test kit (Synbiotics Corporation, San Diego, CA, USA) according to the manufacturer's instructions.

Table 1. Primers used in the study.

Agents	Target genes	Primer sequences (5'-3')	Amplified products (bp)	References
CAV	VP1	CTAAGATCTGCAACTGCGGA CCTTGGAAG CGGATAGTCAT	418	Hussein et al. 2002
ARV	S2	CCC ATG GCA ACG ATT TC TTC GGC CAG GTC TCA AC	399	Bruhn et al. 2005
C. perfringens	*Alpha toxin*	GTTGATAGCGCAGGACATGTTAAG CATGTAGTCATCTGTTCCAGCATC	402	Yoo et al. 1997
E. coli	*iss*	ATGTTATTTTCTGCCGCTCTG CTATTGTGAGCAATATACCC	266	Yaguchi et al. 2007
	eaeA	ATG CTT AGT GCT GGT TTA GG GCC TTC ATC ATT TCG CTT TC	248	Bisi-Johnson et al. 2011
K. pneumoniae	*magA*	GGTGCTCTTTACATCATTGC GCAATGGCCATTTGCGTTAG	1282	Yeh et al. 2007

Table 2. Thermal profiles used in PCR and RT-PCR.

Target gene	Reverse transcription	Primary denaturation	Amplification (40 cycles)			Final extension
			Secondary denaturation	Annealing	Extension	
VP1	-	95˚C-3 min	95°C-30 sec.	50°C-30 sec.	72°C-30 sec.	72˚C-10 min.
S2	50˚C-30 min.	95˚C-15 min.	94°C-30 sec.	54◦C-30 sec.	72◦C-60 sec.	72˚C-10 min.
Alpha toxin	-	94˚C-5 min.	94˚C-60 sec.	55˚C-60 sec	72˚C-60 sec	72˚C-10 min.
Iss	-	94˚C-5 min.	94˚C-60 sec.	54˚C-30 sec.	72˚C-120 sec.	72˚C-7 min.
EaeA	-	95˚C-5 min.	95˚C-30 sec.	60˚C-30 sec.	72˚C-30 sec.	72˚C-7 min.
MagA	-	95˚C-5 min.	95˚C-60 sec.	50˚C-60 sec	72˚C-120 sec.	72˚C-7 min.

RESULTS:

Clinical examination:

Growth retardation was reported in all of the 50 examined broiler flocks. 10%-70% of the broilers in the affected flocks showed growth retardation that was 15%-65% reduction in body weight than the expected weight (according to each breed) (table 3). In addition, affected flocks showed short feather, decreased feed consumption, watery diarrhea with undigested food, soiled vents and dehydration (Fig.1, A). Other broilers showed pale legs and beaks (in 13 flocks). Lameness and bilateral arthritis in hock joints (that contained straw yellow-colored exudates) were observed in five flocks (Fig.1, B). Three flocks showed skin ulcer and suppurative dermatitis (Fig.1, C). The common necropsy findings were enlarged mottled proventriculus with thick-walled ulcerated mucosa (Fig.1, D), hypertrophy and/or atrophied spleen in different cases (Fig.1, E), atrophied pancreas, pale thin intestinal wall that were filled with a large amount of fluid and poorly digested food (Fig.1, E) and covered with pseudo-membrane in 27 flocks (Fig.1, F).

Table 3. Growth retardation in relation to the identified agents:

The identified agent	No. of farms	Broilers with retarded growth (%)	Reduction in body weight (%)	Mortality (%)
ARV+CAV+*E. coli*+ *C. perfringens* + *K. pneumoniae*	1/50	70	65	17.5
ARV+*K. pneumoniae*+ *S. aureus*	4/50	20-35	28-35	13.5
CAV+*S. aureus*	2/50	17-20	18-25	12.5
CAV+*C. perfringens* +*K. pneumoniae*	2/50	18-25	37-39	10.5
E. coli	17/50	10-16	15-18	8-10
ARV+*E. coli*	1/50	60	61	15
ARV+*C. perfringens*	23/50	52-60	58-60	2-10

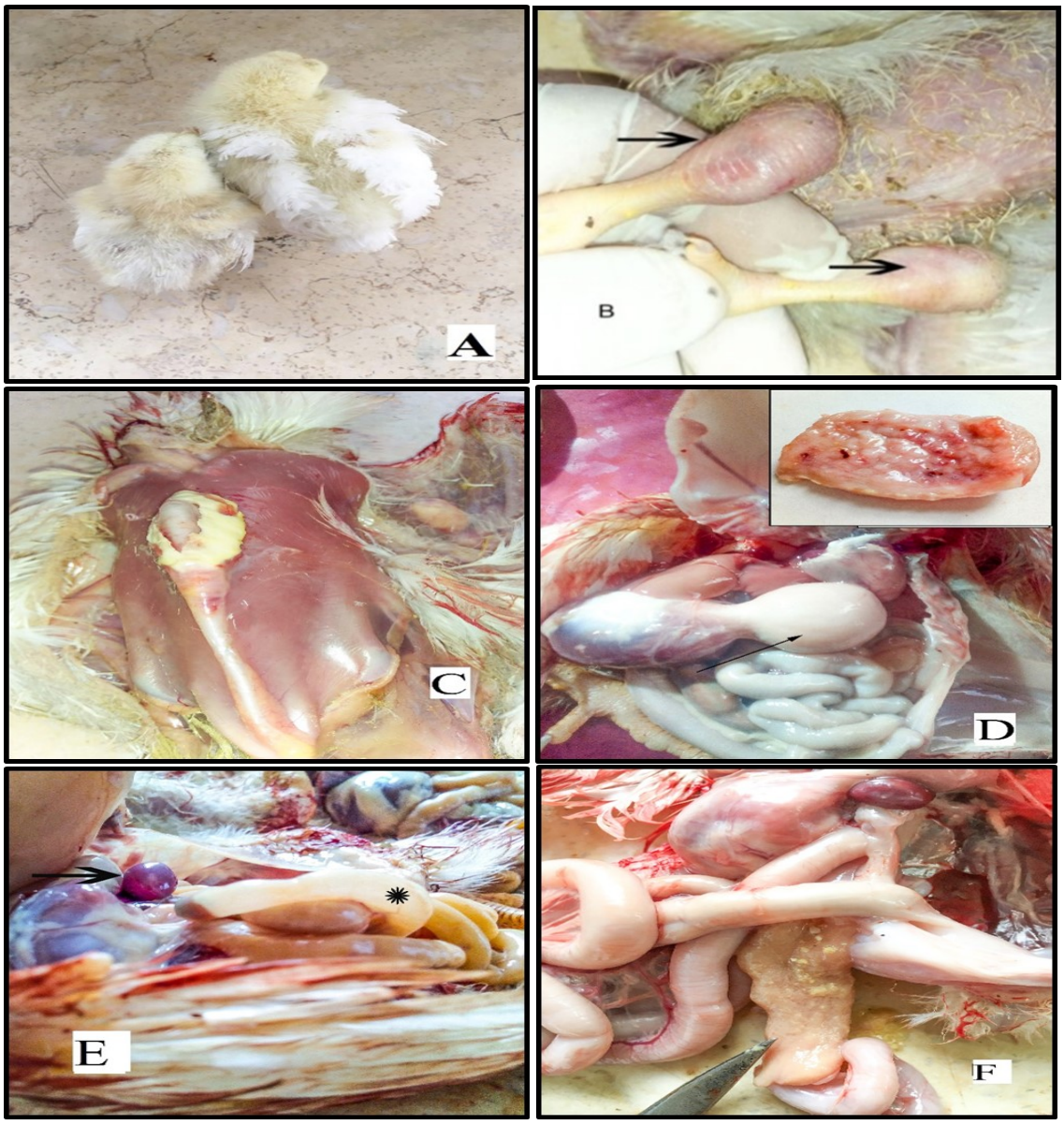

Fig. 1: Broiler chickens (A) Broiler (23 days) showing retarded growth, ruffled and curled feather. (B) showing bilateral arthritis (arrow). (C) showing suppurative dermatitis (D) showing enlarged proventriculus (arrow), ulceration in proventriculus mucosa. (E) showing thinning and ballooning of intestine (asterisk), enlarged spleen (arrow). (F) showing pseudo membrane of intestinal mucosa with undigested food.

Molecular detection of CAV and ARV:

CAV was detected in five flocks (10%), meanwhile ARV was detected in 29 flocks (58%) from which one flock had mixed infection (table 3).

Bacteriological investigation:

C. perfringens was isolated from 26 flocks (52%) with 60 % isolation rate (156 isolates) within positive flocks. Toxigenic isolates represented 53.2% (83 isolates) from the total isolates and were confirmed by molecular detection of alpha toxin gene by PCR (Fig.2.A).
E. coli was isolated from 19 flocks (38%). Serotyping of the 19 *E. coli* isolates revealed 5 serotypes. Seven isolates were serotyped as O86a, five as O112, three as O128, two as O114, and two as O55. PCR result revealed identification of *iss* gene in 60% from the identified isolates of *E. coli*. Meanwhile, 40% from the identified *E. coli* found to be positive for *eaeA* gene (Fig.2.B and C) from which 67% were also positive for iss gene. *K. pneumoniae* and *S. aureus* were isolated and identified in seven (14%) and six (12 %) flocks, respectively. Additionally, *magA* gene was identified by PCR in 29% from the identified *K. pneumoniae* isolates (Fig.2.D). None of the flocks under examination had any *Salmonella* detected.

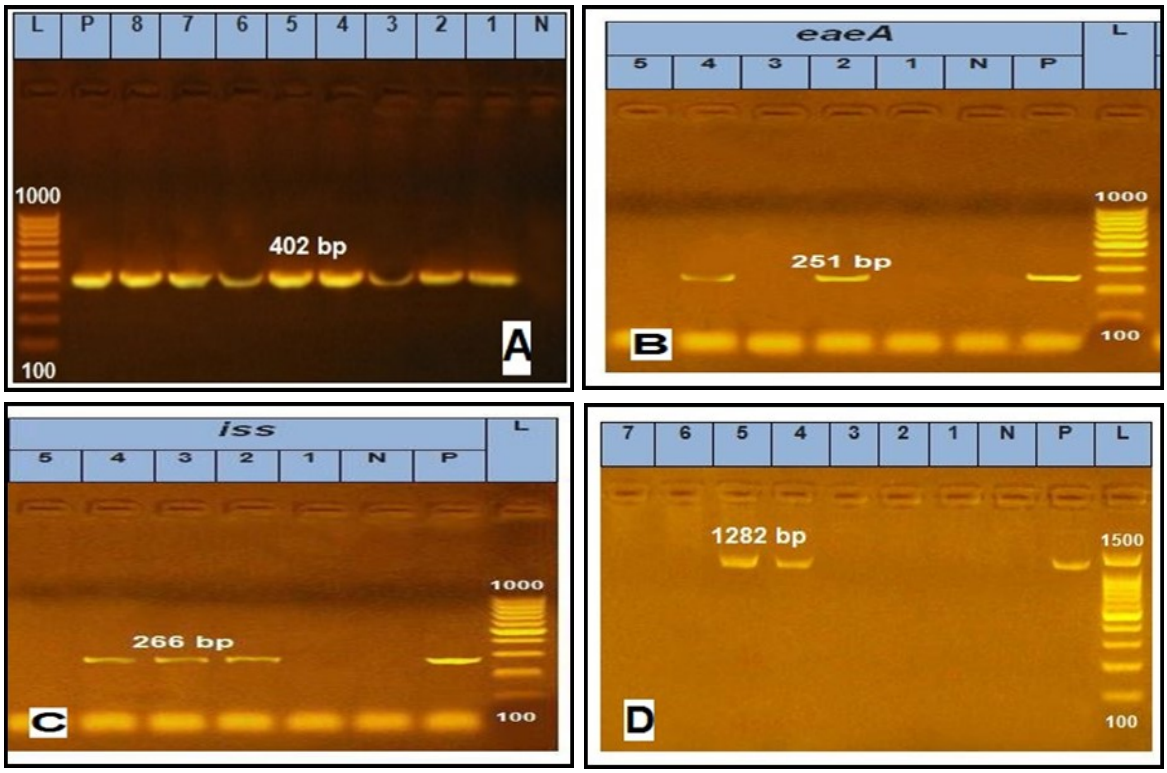

Fig. 2: Electrophoretic patterns of PCR:
(A): Alpha toxin PCR assay: Lane (L) :100 bp DNA ladder, lane (N): control negative and lanes (1-8): showed positive isolated *C. perfringens* strains (402 bp), Lane (P): control positive.
(B): Electrophoretic pattern of eaeA gene of *E. coli*: Lane (L) :100 bp DNA ladder, Lane (P): control positive, lane (N): control negative, lane (1): poly 2, O55, Lane (2): poly 1, O114, Lane (3) poly1, O86a, Lane (4): poly1, O128, Lane (5): poly7, O112. and Lane (2& 4) positive eaeA gene.
(**C): Electrophoretic pattern of iss gene of *E. coli*: Lane** (L) :100 bp DNA ladder, Lane (P): control positive, lane (N): control negative, lane (2,3,4 positive iss gene(266bp).
(D): Electrophoretic pattern *K.pneumonae* magA gene: Lane (L) : DNA ladder, Lane (P) control positive, lane (N): control negative, Lane(4&5) positive magA gene

Histopathological findings

Most of the histopathological changes were found in the jejunum and ileum and characterized by fusion of intestinal villi (Fig.3 A). In many cases, there were necrosis and sloughing of the villus epithelium and these changes were associated with round cell infiltration and hyper activity of mucus secreating cells (Fig.3 B), many cases showed variable degree of cryptal distention where many of them were filled with eosinophilic necrotic material (Fig.3 C), these changes were associated with lymphocytic depletion within the Peyer's patches of ileum (Fig.3 D), the histopathological examination of pancreas of most cases revealed acinar vacuolation (Fig.3 E). Examination of the proventriculus revealed necrosis of the glandular epithelium associated with round cells infiltration (Fig.3 F).

Histopathological examination of bursa of fabricius showed variable degrees of follicular atrophy (Fig.4 A), some cases showed dystrophic calcification and marked lymphocytic depletion (Fig.4 B). Examination of the thymus revealed lymphoid depletion within the cortex giving a starry sky appearance (Fig.4 C), these were associated with intranuclear inclusion bodies within many macrophages (Fig.4 D) and apoptotic changes (Fig.4 E). Also, apoptotic changes were seen in the liver of many cases (Fig.4 F) and these were associated with hepatocytic necrosis, interstitial edema and round cell infiltration.

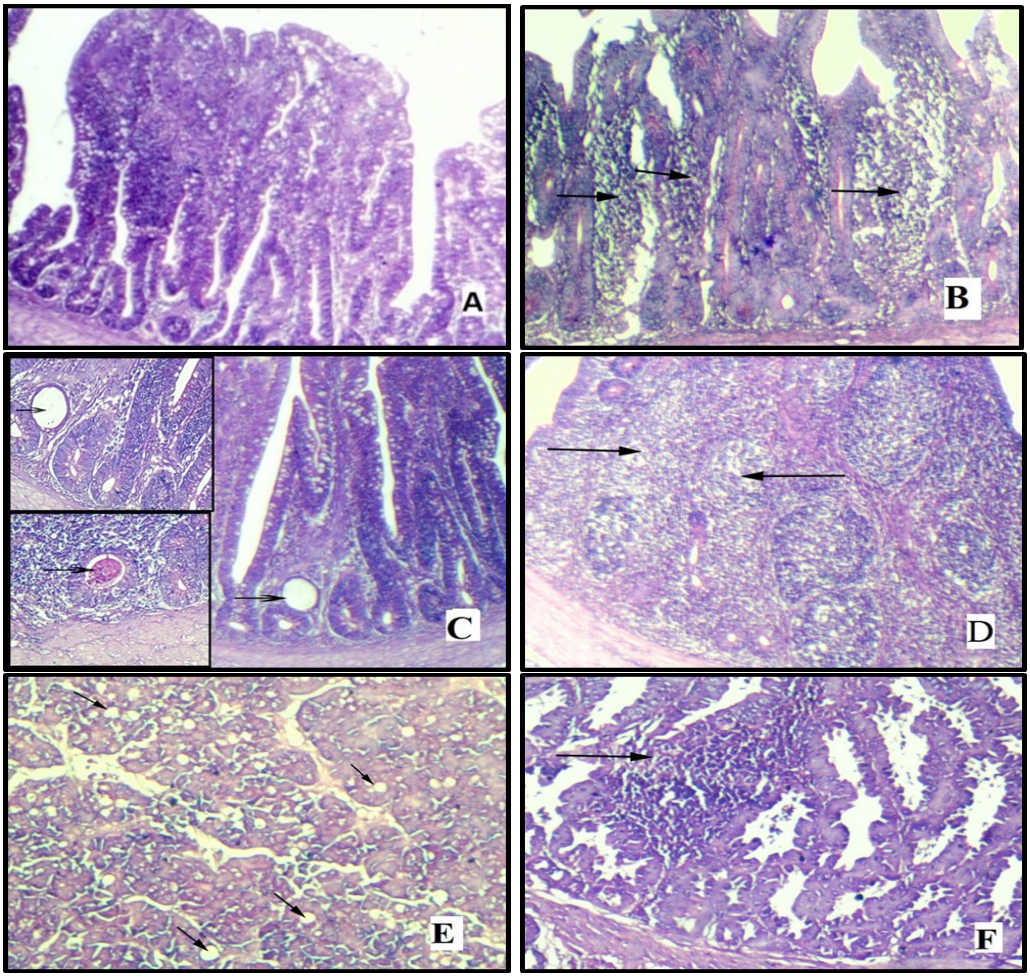

Fig. 3: Intestine showing **(A)** Fusion of intestinal villi **(B)** Necrosis and sloughing of the villus epithelium associated with round cell infiltration (arrow) **(C)** Cryptal distention on which many of them are filled with eosinophilic necrotic material (arrow) **(D)** Lymphocytic depletion within the Peyer's patches of ileum (arrow). **(E)** Acinar vacuolation of pancreas (arrow). **(F)** Necrosis of the glandular epithelium of proventriculus associated with round cells infiltration (arrow). (A, B, D) H&EX100, (C) H&EX100-200, (E, F) H&EX 200 .

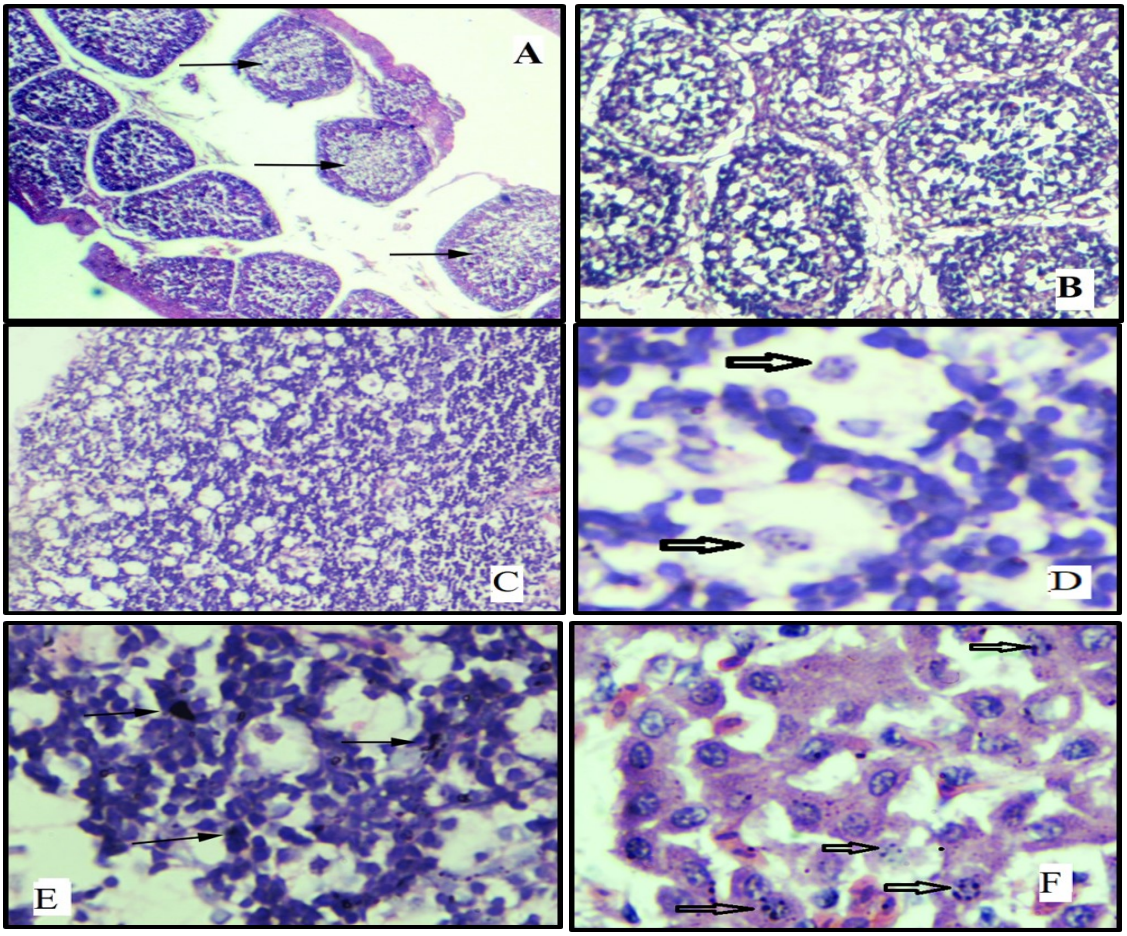

Fig. 4: Bursa of Fabricius: **(A, B)** showing variable degrees of follicular atrophy, lymphocytic depletion and interstitial edema (arrow). Thymus: **(C)** showing lymphoid depletion within the cortex giving a starry sky appearance **(D)** showing intranuclear inclusion bodies within many macrophages (arrow) **(E)** showing apoptotic changes in cortex (arrow) **(F)** showing liver showed marked apoptotic changes (arrow). (A, C) H&EX100, (B) H&EX200, (D, E, F) H&E X 1000.

Table 4. Histopathological score lesions within different organs:

Organ	Lesion	1	2	3	4	5	6	7
Intestine	Fusion of intestinal villi	+++	++	-	++	++	+++	+++
	Necrosis and sloughing of the villus epithelium	+++	++	-	++	++	+++	+++
	Cryptal distention with eosinophilic necrotic material	+++	+++	-	+	++	+++	+++
	Depletion of Peyer's patches of Ileum	+++	+++	-	+	+	+++	+++
Pancreas	Acinar vacuolation	+++	+++	-	+	++	+++	+++
Proventriculus	Dilation of glandular acini	+++	+++	+	+	++	+++	+++
	Necrosis of glandular epithelium with lymphocytic infiltration	+++	+++	+	+	++	+++	+++
Bursa of Fabricius	Atrophy of the bursal follicles	+++	+++	++	++	-	+++	+++
	Lymphoid depletion	+++	+++	+++	+++	-	+++	+++
Thymus	Lymphoid depletion	+++	++	+++	+++	-	+++	+++
	Intranuclear inclusion bodies and apoptotic bodies	+++	-	+++	+++	-	-	-
Liver	Necrosis with lymphocytic infiltration	+++	++	+	++	++	++	++
	Apoptosis	+++	-	+++	+++	-	-	-

Appreviations: +++ = severe, ++ = moderate, + = mild and - = no lesions.
1 (+ve ARV, CAV, *E. coli, C. perfringens* and *K. pneumoniae*), 2 (+ve ARV, *K. pneumoniae* and *S. aureus*), 3 (+ve CAV and *S. aureus*), 4 (+ve CAV, *C. perfringens* and *K. pneumoniae*), 5 (+ve *E. coli*), 6 (+ve ARV and *E. coli*) and 7 (+ve ARV and *C. perfringens*)

Seroprevalence of CAV and ARV:

Antibodies of ARV were detected in 86% from the examined flocks (10-100% within flocks). Meanwhile Antibodies of CAV were detected in 12% from the examined flocks (10-90% within flocks). Antibodies of both ARV (30%) and CAV (70%) were detected in only one flock.

DISCUSSION:

Growth retardation was reported at several broiler flocks in Behira governorate causing high economic losses. Accordingly, this study was done to identify some of the concurrent possible causes and their histopathological pictures in some broiler's flocks. Malabsorption syndrome (MAS), viral arthritis/tenosynovitis and runting–stunting syndrome (RSS) that are caused by ARV, are characterized by growth retardation (De Carli et al. 2020; Kim et al. 2020; De Oliveira et al. 2021). At arthritis/ tenosynovitis disease, poor growth and feed conversion can occur due to inability of lame bird to reach feed. The affected joint is swollen and inflamed and the gastrocnemius tendon ruptures in severe cases (Egaña Labrin et al. 2017 and De-Carli et al. 2020). Besides, growth retardation is caused by CAV (Liu et al. 2022).

During this study ARV was identified in 58% from the examined broiler flocks. In addition, Antibodies of ARV were detected in 86% from these flocks. These results indicate the wide spread of ARV infection among broiler flocks. Lower molecular and serological detection rates were determined to CAV.

C. perfringens causes necrotic enteritis (NE) that has two forms; the acute clinical form that causes death of chicken and the subclinical form that induces retarded growth due to disruption of villus-crypt micro-architecture and reduction of nutrient digestion and absorption which negatively affects feed conversion and growth performance (Van Immerseel et al. 2004, Chan et al. 2015, Bansal et al. 2020 and Thi et al. 2021). *C. perfringens* was isolated from 60 % of the collected samples that is close to the isolation rate recorded by Thi et al. (2021), (63.39%) and slightly lower than that reported by Samy et al. (2017), (66.67%) but higher than that reported by Amer et al. (2016), (18.3%). Alpha toxin producing *C. perfringens* represented 53.2% from the total isolates (Fig.2. A). This toxin has been implicated in the destruction of mucosal tissue of small intestine (Rood 1998). Subclinical form of *C. perfringens* infection is caused by type A and to a lesser extent type C that producing the alpha toxin (Van Immerseel et al. 2004).

E. coli was the second predominant bacterial isolate that was identified in 19 flocks (38%) . Serotyping of *E. coli* isolates revealed four EPEC (O86a, O128, O114, and O55) serotypes and one enteroenvasive *E. Coli* (O112) (Orskov and Orskov 1992). This diversity in the prevalence of serotypes among avian pathogenic strains was also reported by El Sawah et al. (2018). Several studies associated growth retardation with pathogenic *E. coli* infections (Mahmoud and Edens 2005, Ask et al. 2006 and Ekim et al. 2020). *E. coli* strains that causing diseases in the intestinal tract (diarrheagenic *E. coli*) are divided into seven pathotypes from which enteropathogenic *E. coli* (EPEC) are frequently involved in enteric disorders of animals and human and able to produce an adherence factor (*intimin*) that is encoded by the *eaeA* gene which is present in the locus of enterocyte effacement (LEE) at the large chromosomal pathogenicity island (PAI). Diarrhea that is induced by these pathotypes is a result of loss of intestinal microvilli due to attaching and effacing (A/E) lesions that occur after bacterial adherence on enterocytes (Santos et al. 2020 and Bertelloni et al. 2022). In addition, eaeA gene that encodes adherence factor (*intimin*), was identified in 40% from the identified *E. coli* isolates (Fig.2.B). This factor is produced by strains of EPEC, facilitating their adherence on the enterocytes and subsequently causing attaching and effacing (A/E) lesions that is resulted in loss of intestinal microvilli, which can explain the occurrence of diarrhea and growth retardation (Santos et al. 2020 and Bertelloni et al. 2022). The virulence factor (*iss* gene) of septicemic *E. coli* strains, encodes protein that is associated with the bactericidal resistance to the serum and production of

systemic and localized infections (López et al. 2017, Sarowska et al. 2022, Wilczyński et al. 2022). This gene was determined in 60% from the identified isolates (Fig.2.C) and 67% from the positive *eaeA* isolates which can explain the systemic infection.

Klebsiella pneumoniae was isolated and identified in seven (14%) flocks which is close to the isolation rate **(**14.4%) that obtained from broilers with weakness, gasping and poor growth (Elgaos et al. 2019) and higher than that (6.6%) obtained from broilers with respiratory disorders, diarrhea and growth retardation (Marwa et al. 2018)**.** *K. pneumoniae* virulence factor *magA* (that has an important role in septicemia, pneumonia, liver and lung abscesses, and increasing resistance to phagocytosis) was found in 29% from the identified *K. pneumonae* (fig.2 D), (Chan et al. 2005, Struve et al. 2005 and Chung et al. 2007).

S. aureus was identified in six flocks (12%) that mostly suffered from lameness and arthritis (Youssef and Hamed 2012 and Abd El Tawab et al. 2017). Lameness considers the biggest factor contributing to poor chicken welfare and significant financial losses in the production of meat-type poultry (Gustaw et al. 2022) and from which ARV or CAV was identified (table 3). Growth retardation at these flocks can be returned to the inability of the infected bird to reach food and water due to arthritis and the co-infection with ARV or CAV.

Wide range of reduction in body weight (15-65%) was reported with the highest weight reduction was found in one flock in which ARV, CAV, *E. coli, C. prfringens* and *K. pneumoniae* were identified. Meanwhile, the lowest weight reduction was reported in flocks with only *E. coli* infection. These findings can reflect the synergistic effect of the identified microorganisms in growth retardation.

Pathologically, the most affected intestinal part is jejunum that showed necrosis and sloughing of the villus epithelium with round cell infiltration and hyper activity of goblet cells. These results are similar to that reported by Qamar et al. (2013) and De Oliveira et al. (2021)**.** Some area showed clubbing and fusion of intestinal villi as reported by De Oliveira et al. (2021). Also, there were variable degree of distention of the crypts of Lieberkühn, the epithelial cells lining the crypts become more degenerated and detached from the cryptal wall resulting in its flattening and distention. The crypts got filled with cellular debris and degenerated cells that reduced the absorptive capacity causing diarrhea, these results agreed with Qamar et al. (2013), Ter Veen et al. (2017) and De Oliveira et al. (2021). While, Otto et al. (2006) mentioned that the severity of lesions depends upon the extended loss of enterocytes, which are an absorptive cell lining the intestinal villi and the target for infectious agents (viruses). Because of degeneration and loss of enterocytes, digestion and absorption of electrolytes and nutrients is interrupted resulting in maldigestion, malabsorption and osmotic retention of water which is considered as one of the lesions responsible for the poor weight gain and retarded growth. Pancreas showed vacuolation of acinar cells similar to the results of Nili et al. (2007) and Qamar et al. (2013) who reported that due to inflammatory reaction in the exocrine ducts, these ducts are obstructed resulting in atrophy of pancreas, and as the pancreas is the major source of digestive enzymes, an obstruction of the pancreatic ducts interferes with the production of digestive enzymes that results in the disturbance of the digestive process accompanied by undigested feed material in the intestine. The proventriculus revealed necrosis of the glandular epithelium associated with round cells infiltration. These results agreed with Nili et al. (2007) and Noiva et al. (2015), while, Shapiro and Nir (1995) and Qamar et al. (2013) did found any specific lesions in proventriculus. The function of the proventriculus is to secrete hydrochloric acid, pepsin, and other digestive enzymes; therefore, any impairment can result in poor digestion and absorption of feed. The liver as one of the digestive organs also showed hepatocytic necrosis, interstitial edema and round cell infiltration as reported by Ashwin et al. (2013)**.** In addition, bursa showed atrophy of the bursal follicles, increased inter follicular space and lymphoid depletion agreed with Nili et al. (2007) and Qamar et al. (2013).

Spleen and thymic cortex showed diffuse lymphoid depletion, giving a (starrysky appearance) and many macrophages contains inclusion bodies. In addition, there were apoptotic bodies in thymus agreed with Mohie et al. (2012) and Eid et al. (2016), and liver agreed with Hegazy et al. (2010).

CONCLUSION:

Finally, we concluded a combination of viruses (ARV, CIAV) especially ARV, aerobic bacteria mostly *E.coli* and *C. perfringens* were the most common co-infection type associated with retarded growth and low uniformity in broiler chicken flocks in this investigation. Our results are indicating the importance of continuous epidemiological surveillance for detection of possible causes of retarded growth in broilers to plane strategy to overcome it. Vaccination of broiler breeder is the cornerstone of the control of ARV and CIAV via overcome vertical spread of the viral diseases, in addition to prophylactic measures to control bacterial infections along with improved biosecurity measures and good poultry house management to limit retarded growth problem in broiler chicken farms.

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