

Investigation of some bacterial and viral pathogens causing nephropathy

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Received in 5/1/2016

Accepted in 24/2/2016

Abstract

Avian pathogenic *Escherichia coli* (APEC), causes infections in extraintestinal organs observed in severe systemic colibacillosis classified as extraintestinal pathogenic *E. coli* (ExPEC). ExPEC showed drastic losses in poultry production. In this study ten layer flocks suffered of severe mortalities and showing kidney and liver lesions have been investigated. Clinicopathological examination revealed peritonitis, pericarditis and severe congestion of internal organs, histological examination showed nephritis, hepatitis with heterophilic infiltration and small abscess in breast muscles. Collection of affected organs for bacteriological and molecular examination from the examined farms was taken place. *E. coli* serotype O125 has been isolated from liver and kidneys. Antibiotic sensitivity test of isolated *E. coli* were multidrug resistant, Detection of virulence-associated genes in ExPEC strain for adhesion (*papC*, *crl*, *tsh*); protectins and Iron acquisition proved positive examined ExPEC strain. Exclusion of nephropathogenic type of Infectious bronchitis virus supported that ExPEC was the prime cause of morbidity. This study concluded that APEC subgroup has to be considered potential infectious agent given certain extraintestinal pathotype.

Key words: APEC, serotyping *E. coli*, histopathology, virulence genes ExPEC.

1. Introduction

Renal failure which is one of most common reasons of economic losses as it causes high percentage of mortalities. There are many bacterial and viral diseases responsible for hepatorenal failure as colibacillosis, Salmonellosis, erysipelas, infectious bronchitis disease, Newcastle Disease, pasteurellosis and botulism (Fossum *et al.*, 2009).

Salmonellosis is the most significant bacterial pathogen which is widely spread (Mir *et al.*, 2015) Salmonellosis is a zoonotic disease which is responsible for many economic losses in layer farms (Manning *et al.*, 2015).

Consumption of contaminated food and eggs are the Major source of Salmonellosis According to the Centers for Disease Control and Prevention. While more than 2,000 *Salmonella* serovars have been identified, 20 *Salmonella* serovars account for 73% of salmonellosis cases in the United States (Cheng *et al.*, 2015)

Salmonella is transmitted either horizontal and vertical, Horizontal transmission includes indirect transmission from the environment, equipment, and vectors (e.g., rodents, flies, humans). In the vertical transmission was occurred through colonization through the ova of laying hens (Theva *et al.*, 2013).

Colibacillosis caused by avian pathogenic *Escherichia coli* (APEC) either local or systemic infection, *Escherichia coli* is bacteria of genus *Escherichia* which is member of the family *Enterobacteriaceae*, which is composed of organisms that can grow aerobically or anaerobically and utilize simple carbon and nitrogen sources, Gram-negative, non-acid-fast, uniform staining, non-spore-forming bacillus, usually 2–3X0.6 µm. Organisms grown in culture are more variable in size and shape. (Barnes *et al.*, 2008). Classification of *E. coli* serotypes according to Kauffmann scheme resulted in (180 O, 60 H, and 80 K antigens (Barnes *et*

al., 2008).

E. coli either veterinary or medical is subdivided into three categories intestinal pathogenic, extraintestinal pathogenic (ExPEC), and commensal *E. coli* (Kaper *et al.*, 2004). ExPEC, which include uropathogenic (UPEC), new born meningitis-causing (NMEC), and avian pathogenic (APEC) shows variability in virulence factors (Ewers *et al.*, 2007).

Bacterial infections due to APEC, a subgroup of ExPEC are responsible for severe economic losses for the poultry farms, which is important industries in many countries. APEC causes systemic infections in birds, which known as avian colibacillosis (Barnes *et al.*, 2003).

Acute colibacillosis in layers is being seen with increasing frequency (Vandekerchove *et al.*, 2004). Death usually occurred suddenly without predictive signs, although depression was observed in some affected hens in approximately half of the flocks. Weekly mortality was significantly higher in affected flocks. Perihepatitis, pericarditis and peritonitis were present in most birds at necropsy (Dhillon and Jack, 1996). Congestion of hepatic sinusoids was commonly seen in acute septicaemia of *E. coli* (Arp, 1982; Chevielle and Arp, 1978) with aggregates of heterophils (Zanella *et al.*, 2000). Mild to moderate renal lesions was shown in acute colibacillosis (Siller, 1981).

Antibiotic is widely used in control and treatment of *E. coli* in poultry but after long time of usage antibiotic resistance has been developed (Blanco *et al.*, 1997; Salehi and Bonab, 2007). Antibiotic resistance become a widely distributed problem in human and veterinary field (Habrun *et al.*, 2010).

Staphylococcal infections is widely distributed in poultry as they are responsible for economic losses through inducing weight loss, drop in egg production, and carcasses condemnation at slaughter time. The morphology of these microorganisms; appeared gram-positive, coccoid in shape, found in clusters of grapes when grown on solid media. (Andreasen, 2008).

Coagulase-negative staphylococci are of high significance especially in nosocomial infection (Pfaller and Herwaldt, 1988). Antibiotic drug

resistance is widely distributed due to massive usage of antibiotics in treatment of human and animal (Akbar and Anal 2013).

Infectious bronchitis virus (IBV) is the most common causes for renal lesion in chickens. Glemulonephritis, tubularnecrosis and subsequent hyperuracemia were the highly predominant lesions caused by IBV (Condrón and Marshal, 1985).

The objectives of this study were to examine the chicken flocks clinically and histopathologically that showing mortalities then isolation of bacterial causative organism, and study their molecular characterization. Molecular detection for a suspected concomitant virus infection causing morbidity.

2. Materials and Methods

2.1. Samples:

Freshly dead and live red and white chickens foreign breeds layers (25 weeks old) suffering of 20% mortalities. Organs (liver, kidneys, intestine, lung and ovary) were aseptically collected for bacteriological isolation, showing severe systemic congestion and renal problems from local farms in Giza Province.

2.2 Histopathology:

Liver, kidneys, proventriculus, intestine and skeletal muscles were harvested from euthanized birds and fixed in buffered formalin saline solution. The tissues were later transferred through several changes of xylol and ethanol, embedded in low-melting-point paraffin wax. Sections of 4µm in thickness were cut and mounted on microscope slides that were stained with hematoxylin and eosin then covered with cover slips (Bancroft and Gamble, 2008).

2.3 Bacterial Isolation:

Different samples were collected from farms as internal organs (liver, heart, lung, kidney and intestine) which were submitted to reference laboratory for veterinary quality control on poultry production. All samples were examined bacteriologically for presence of *Salmonella*, *E. coli* and *Staphylococcus*. Under aseptic conditions loop full from (liver, lung, heart) were directly cultivated onto MacConkey agar

then E.M.B. The plates were incubated at 37°C for 24-48 hours and then examined for the characteristic *E. coli* colonies. Pure colonies were subjected to biochemical test to identify and differentiate between members of *Enterobacteriaceae* according to **Quinn et al. (2002)**. Isolation and Identification of *Salmonella*, *E. coli* and *Staphylococcus* was done according to standard methods (**ISO 6579: 2002; Lee and Arp 1998 and ISO 6888-1: 2003**) respectively.

2.4 Serological typing:

Serological typing of *E. coli* was carried out according to **Lee et al., (2009)** by using Known antisera for each organism (Sifin).

2.5 Antibiotic sensitivity test:

The antibiogram of Bacterial Isolates were done by disc-diffusion test for *E. Coli* against 11 antimicrobial (oxoid) (**table 1, 2**): Ampicillin, Chloramphenicol, Ciprofloxacin, Colistin sulphate, Doxycycline, Erythromycin, Gentamycin, Levofloxacin, Norfloxacin, Tetracycline, Trimethoprim –sulfamethoxazole (SXT), while *Staph* tested against 10 antibiotics (Oxoid): Ampicillin, Chloramphenicol, Ciprofloxacin, Doxycycline, Gentamycin, Levofloxacin, Norfloxacin, Penicillin, Tetracycline, Trimethoprim –sulfamethoxazole (SXT), according to the Clinical and Laboratory Standards Institute/ Formerly National Committee for Clinical Laboratory Standard (**CLSI/NCCLS, 2009**).

Table (1). The break point of *Enterobacteriaceae* according to (CLSI/NCCLS, 2009).

Antimicrobial Discs	code	Disc Potency Mg/disc	Interpretation		
			Zone diameter (mm)		
			Sensitive ≤	Intermediate	Resistant ≥
Ampicillin	A ¹⁰	10 µ G	17	14-16	13
Chloramphenicol	C ³⁰	30µg	18	13-17	12
Ciprofloxacin	CF ⁵	5 µg	21	16-20	15
Colistin (MethanSulphate)	CL ¹⁰	10 µ G	11	9-10	8
Doxycycline hydrochloride.	DO ³⁰	g µ 30	14	11-13	10
Erythromycin.	E15	G µ 15	18	14-17	13
Gentamicin	G ¹⁰	10µg	15	13-14	12
Levofloxacin	LEV 5	5µg	17	14-16	13
Norfloxacin	NX ¹⁰	gµ10 µg	17	13-16	12
Trimethoprim-sulfamethoxazole	SXT	23.75µg	16	11-15	10
Tetracycline	T ³⁰	30 µg	15	13-15	11

Table (2). The break point of *Staphylococcus* (CLSI/NCCLS, 2009).

Antimicrobial Discs	code	Disc Potency Mg/disc	Interpretation		
			Zone diameter (mm)		
			Sensitive ≤	Intermediate	Resistant ≥
Ampicillin	A ¹⁰	10 µg	29	-	28
Chloramphenicol.	C ³⁰	30 µg	18	13-17	12
Ciprofloxacin.	CF ⁵	5 µg	21	16-20	15
Doxycycline.	DO ³⁰	30µg	16	13-15	12
Gentamicin.	G ¹⁰	10 µg	15	13-14	12
Levofloxacin	LEV 5	5 µg	19	16-18	15
Norfloxacin.	NX ¹⁰	10 µg	17	13-16	12
Penicillin	P ¹⁰	10 I.U.	29	-	28
Tetracycline.	T ³⁰	30	19	15-18	14
Trimethoprim-sulfamethoxazole	SXT	23.75 µg	16	11-15	10

2.6. DNA extraction:

DNA extraction from samples was performed using the QIAamp DNA Mini kit (Qiagen, Germany, GmbH) with modifications from the manufacturer's recommendations. Briefly, 200 µl of the sample suspension was incubated with 10 µl of proteinase K and 200 µl of lysis buffer at 56°C for 10 min. After incubation, 200 µl of 100% ethanol was added to the lysate. The sample was then washed and centrifuged following the manufacturer's recommendations. Nucleic acid was eluted with 100 µl of elution buffer provided in the kit (Ewers *et al.*, 2004).

2.7. Oligonucleotide Primer:

Primers used were supplied from Metabion (Germany) are listed in (table 3).

2.8. PCR amplification

For *pap* Cuniplex PCR, primers were utilized in a 25- µl reaction containing 12.5 µl of EmeraldAmp Max PCR Master Mix (Takara, Japan), 1 µl of each primer of 20 pmol concentration, 4.5 µl of water, and 6 µl of DNA template. The reaction was performed in an Applied biosystem 2720 thermal cycler. For *ibeA*, *iss* and *tsh* multiplex PCR, primers were utilized in a 50 µl reaction containing 25 µl of EmeraldAmp Max PCR Master Mix (Takara, Japan), 1 µl of each primer of 20 pmol concentration, 9 µl of water, and 10 µl of DNA template.

For *iron* and *crl* duplex PCR, primers were

utilized in a 50- µl reaction containing 25 µl of EmeraldAmp Max PCR Master Mix (Takara, Japan), 1 µl of each primer of 20 pmol concentration, 11 µl of water, and 10 µl of DNA template.

2.9. Analysis of the PCR Products.

The products of PCR were separated by electrophoresis on 1% agarose gel (Applichem, Germany, GmbH) in 1x TBE buffer at room temperature using gradients of 5V/cm. For gel analysis, 20 µl of the uniplex PCR products and 40 µl of the multiplex PCR products were loaded in each gel slot. Gelpilot 100 bp plus DNA Ladder (Qiagen, Germany, GmbH) was used to determine the fragment sizes. The gel was photographed by a gel documentation system (Alpha Innotech, Biometra) and the data was analyzed through computer software.

2.10. RNA extraction and molecular diagnosis for Infectious bronchitis virus IBV

Viral RNA was extracted using the QIAamp Viral RNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. RNA was eluted in 70 µL nuclease-free water, aliquoted at 10 µL and stored at -20°C until use. A commonly used primer pair for amplification of genotypically diverse IBV strains is oligo S15' mod (forward): 5'-TGA-AAA-CTG-AAC-AAA-AGA-3' and CK2 (reverse): 5'-CNG-TRT-TRT-AYT-GRC-A-3' (Gelb *et al.*, 2005).

Table (3). Primers sequences, target genes, amplicon sizes and cycling conditions.

Target gene	Primers sequences	Amplified segment (bp)	Primary denaturation	Amplification (35 cycles)			Final extension	Reference
				Secondary denaturation	Annealing	Extension		
<i>papC</i>	5'TGA TAT CAC GCA GTC AGT AGC3'	501	94°C 5 min.	94°C 30 sec.	59°C 45 sec.	72°C 45 sec.	72°C 10 min.	Wen-jie <i>et al.</i> , 2008
	5'CCG GCC ATA TTC ACA TAA3'							
<i>tsh</i>	5'GGT GGT GCA CTG GAG TGG3'	620	94°C 5 min.	94°C 30 sec.	54°C 45 sec.	72°C 45 sec.	72°C 10 min.	Delicato <i>et al.</i> , 2003
	5'AGT CCA GCG TGA TAG TGG3'							
<i>iss</i>	5'ATG TTA TTT TCT GCC GCT CTG3'	266						Yaguchi <i>et al.</i> , 2007
	5'CTA TTG TGA GCA ATA TAC CC3'							
<i>ibeA</i>	5'TGG AAC CCG CTC GTA ATA TAC3'	342						Ewers <i>et al.</i> , 2007
	5'CTG CCT GTT CAA GCA TTG CA3'							
<i>iroN</i>	5'ATC CTC TGG TCG CTA ACT G3'	847	94°C 5 min.	94°C 30 sec.	50°C 45 sec.	72°C 45 sec.	72°C 10 min.	
	5'CTG CAC TGG AAG AAC TGT TCT3'							
<i>Crl</i>	5'TTT CGA TTG TCT GGC TGT ATG3'	250						Ghanbargpour and Salehi, 2010
	5'CTT CAG ATT CAG CGT CGT C3'							

3. Results

3.1. Clinical and postmortem examination:

Severe systemic congestion, perihepatitis and peritonitis, congested liver and kidney were observed. Small focal swelling on breast muscles. All birds showed lesions of acute septice-mia at postmortem examination.

3.2. Bacterial isolation:

Bacteriological isolation from internal organs resulted in isolation of *E. coli* from liver and kidneys and *staphylococcus* from intestine. But, was negative for *Salmonella* isolation.

3.3. Serological identification:

E. coli serogroup was O125 K70.

3.4. Antibiotic sensitivity test:

Isolates of *E. coli* was multidrug resistant. It was sensitive to chloramphenicol and Trimetho-prim-sulfamethoxazole while was resistant to ampicillin, colistin sulphate, ciprofloxacin, doxycycline, erythromycin, gentamycin, levofloxacin, norfloxacin and tetracycline. For *Staph. Spp.* sensitive to gentamycin and intermediate sensitive to ciprofloxacin, levofloxacin

and norfloxacin, while resistant to ampicillin, chloramphenicol, doxycycline, penicillin and tetracycline.

3.5. Histopathology:

Internal organs showed peritonitis, pericarditis and congestion of internal organs. Liver showed congested hepatic sinusoids vacuolar degeneration with heterophilic infiltration also disruption of hepatic plates (fig. 1 A, B). Proventriculus showed lymphocytic infiltration in the mucosal layer with congested blood vessels and edema in submucosal layer (fig.1 C). Small intestine showed hyperplasia of epithelium of intestinal villi with infiltration of lymphocytic cells (fig. 1 D). Kidney showed glomerulonephritis with renal cast and focal necrosis of renal tubules (fig. 1E, F). Skeletal muscles showed focal abscess of aggregated heterophils (fig. 1 g, h).

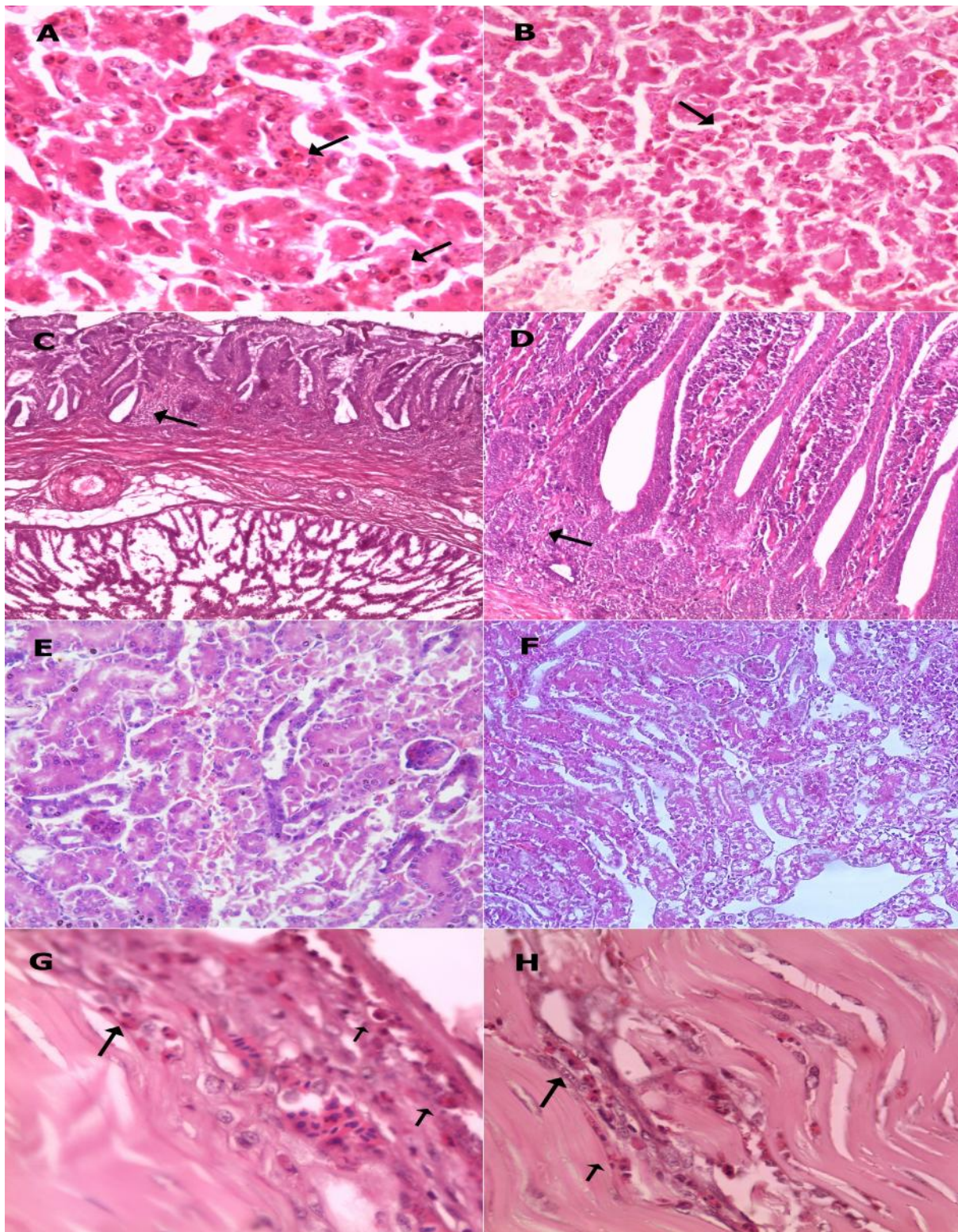


Figure 1. Histopathology of chickens showing colibacillosis. A) liver showed heterophilic infiltration (arrows) associated with vacuolar degeneration H&E x400. B) Liver showed focal necrosis with heterophilic infiltration (arrow) H&E x200. C) proventriculus showed focal lymphocytic infiltration in mucosal layer (arrow) H&E x100. D) Deudenum showed enteritis with lymphocytic infiltration H&E x200. (E, F) Kidney showed necrosis of renal tubules and renal cast in other sections. H&E x200. (G, H) skeletal muscles showed focal abscess with heterophilic cells aggregation H&E x400.

3.6. Virulent genes associated in isolates APEC:

Genes of adhesions as *papC* uniplex PCR, primers were utilized in a 25- µl reaction containing gave positive reaction compared with standard positive sample. For *ibeA*, *iss* and *tsh*

multiplex PCR, primers were positive amplification of *iss* and *tsh* and negative amplification of *ibeA*. For *iroN* and *crl* duplex PCR, both two genes are positive amplification compared with positive standard.

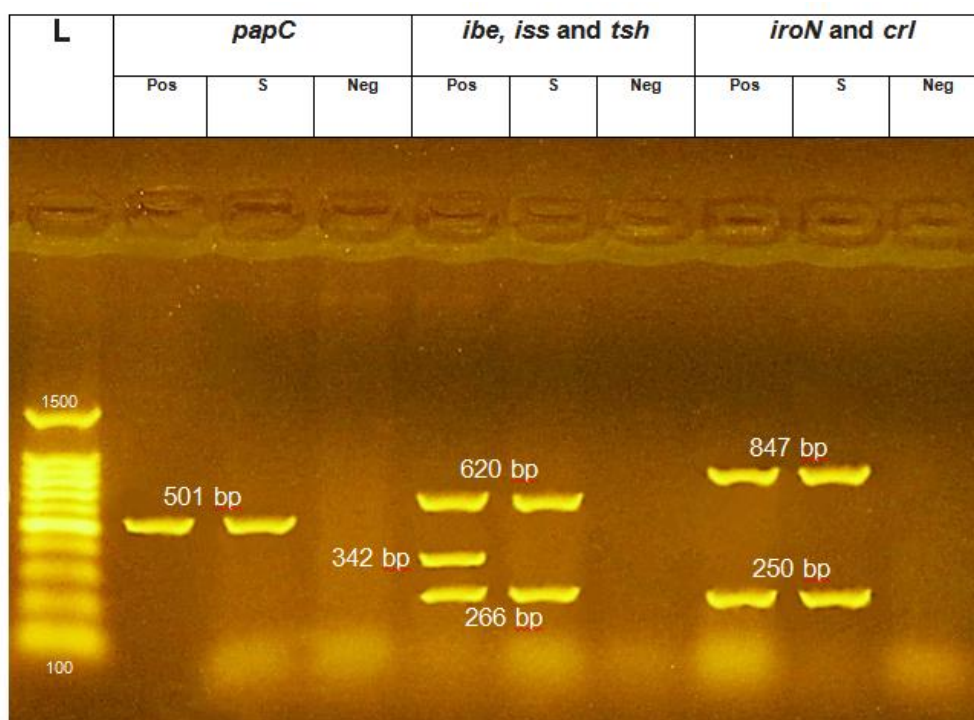


Figure 2. Agarose gel of amplicons generated in in virulence genes of ExPEC PCR protocol. Lane 1, ladder; lane 2–10, APEC strains used virulence assay including *papC* band in lane 2 correspond positive control and in lane 3 is sample with positive band at 501 bp. Lane 5-7 showed positive bands of *iss* and *tsh* and negative *ibe*, lanes 8-10 showed positive amplification of *iroN* and *crl*.

3.7. Molecular diagnosis for Infectious bronchitis virus IBV:

Negative amplification of IBV was figured out.

4. Discussion

Colibacillosis is a major cause of morbidity, mortality, and condemnation of carcasses in the poultry industry (Allan *et al.*, 1993; Goren, 1990), especially colisepticemia (Barnes and Gross, 1997; Ewers *et al.*, 2003).

Extra intestinal pathogenic *Escherichia coli* (ExPEC) infections are widely distributed all over the world causing public health hazard, on the other hand they are resulting in severe economic losses in poultry industry. Several studies investigated the relation between avian and

human (ExPEC) as poultry products considered as reservoir for (ExPEC) in human. (Blaak *et al.*, 2015).

Avian colibacillosis is a complex syndrome characterized by multiple organ lesions. Clinical and histopathological findings designated features of acute colibacillosis in layers which was supported by Vandekerchove *et al.*, (2004). The rate of weekly mortalities, systemic congestion and the obviously observed polyserositis in necropsy support the infection of bacterial infection (Dhillon and Jack, 1996). Similar pathological findings were reported by Hassanain, (1977) and Mukhopadhyaya and Mishra (1992), who isolated *E. coli* from liver, kidneys and yolk materials.

Histopathological picture observed in liver and kidney were reported by **Chevielle and Arp, (1978); Arp, (1982); Siller, (1981) and Zanella et al., (2000).**

E. coli was the most predominant isolate causing the morbidity followed by less significant isolation of *Staphylococcus* which can be found normally on chicken skin, feathers, respiratory and intestinal tracts (**Casey et al., 2007, Suleiman et al., 2013**).

This study has variable antimicrobial drug resistance for *E coli* which is similar to the findings of (**Zhao et al., 2005, Salehi and Bonab, 2007, Anyanwu et al., 2010, Nasrin et al., 2012 and Zhang et al., 2013**). Antibiotic resistance for *E coli* developed as a result of misuse of antibacterial drugs resulting in appearance of resistant strains (**Lerbec et al., 2014**).

However, absence of invasion genes (*ibeA*) indicated no relationship between strains of 125 APEC and NMEC strains. Genes associated with *ColV* plasmids (*tsh*, *iss*) were in general more prevalent in APEC than in UPEC strains, indicating that APEC could be a source of *ColV*-located genes or complete plasmids for other ExPEC strains (**Ewers et al., 2007**).

Investigation of potential infectious bronchitis virus nephropathogenic type by real time PCR revealed negative conventional PCR supported the prime cause of infection with avian pathogenic *E. coli* (APEC). However, other viruses infectious bursal disease virus IBDV, and low pathogenic avian influenza could cause renal lesions but age of layer flocks and absence of vasculitis or endothelial destruction in respiratory or digestive tract gave a clue for virus choice.

In conclusion, there was a relation between the colisepticemic pathological lesions of diseased flocks and isolated APEC. There was high prevalence of virulence genes among ExPEC, and multidrug resistance was common in the APEC. The majority of APEC causing colisepticemic cases, remains one of the major endemic diseases afflicting the poultry industry worldwide.

The study emphasize on the importance of characterization of *E. coli* from different re-

gions.

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الكشف عن الأمراض البكتيرية والفيروسية المسببة للقصور الكلوي د.منى على عبد الحليم ، د.محمود كمالى ، د. محمد احمد محمد على سليمان

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المخلص العربى

تسبب بكتريا الايشيريشيا كولى الضارة للطيور *Avian pathogenic E. coli* عدوى خارج الامعاء و تظهر فى الاصابة كولى المنتشرة فى عديد من الاعضاء الحشوية وتسمى بكتريا الايشيريشيا كولخارج الامعاء Extraintestinal *pathogenic E. coli*. وتتسبب فى خسائر شديدة لقطعان البياض فى الدواجن. فى الدراسة تم الكشف عن المسبب المرضى المسبب فى زيادة النفوق فى قطعان البياض واحتقان حشوى عام والتهاب بريتوني والتهاب كلوى وكبدى وخراريج سطحية على عضلات الصدر بالفحص الهيستوباثولوجى. بالعزل البكتيري يتم عزل بكتريا الايشيريشيا كولى من الكبد والكلى سلالة serotype O125. وتم عزل الميكوب العنقود من بعض الحالات. استبعاد الاصابة بفيروس الالتهاب الشعبى النوع الكلوى يدعم ويؤكد الاصابة بكتريا الايشيريشيا كولى الضارة للطيور كمسبب رئيسى للأصابة والنفوق . فحص جينات الضراوة الخاصة بالسلالات الضارة خارج الامعاء وجد ايجابية الفحص الجزئ للجينات *(papC, srl, tsh)*; protectins and Iron acquisition تثبت وجود علاقة قوية لشدة ضراوة المعزولات من قطعان البياض للدجاج .