ISSN: 0975-8585

Research Journal of Pharmaceutical, Biological and Chemical Sciences

Experimental Infection of Dogs with Attaching- Effacing E.coli Identified by PCR: Clinical, bacteriological and Pathological Studies.

Khaled M Abdelhakim[1] and Reda M. S. Korany[2]*.

[1]Police Officer, Police Academy, PhD., Faculty of Veterinary Medicine, Cairo University, Giza, Egypt.
[2]Lecturer, Pathology Department, Faculty of Veterinary Medicine, Cairo University, Giza, Egypt

ABSTRACT

This study was conducted to investigate the clinical and pathological effect of attaching-effacing E.coli O26:K60 on experimentally infected dogs. This strain was isolated from 1.5 years old male German shepherd dog after sudden death. Ten mongrel dogs of 6 months old were used in this study. These dogs were divided into two groups. The first group kept as a control negative group, while the second group was orally given 1×109 CFU /ml of isolated E.coli strain (infected group). Two dogs of the infected group showed a slight rise in rectal temperature after 24 hours, followed by depression and then died at the 7th day of the experiment. Other three dogs were euthanized after 14 days of the experiment. E.coli O26:K60 was isolated from rectal swabs, intestine, liver, kidneys and lungs of naturally and experimentally infected animals and by PCR the strain showed amplification with a molecular length of 384 bp corresponding to the eaeA gene. Microscopically, intestine of naturally and experimentally infected dogs revealed enteritis. Histopathologic examination of liver of infected dogs showed congested blood vessels, vacuolar degeneration and coagulative necrosis of hepatocytes. Kidneys of dogs revealed coagulative necrosis of lining epithelium of renal tubule in both cortex and medulla. Lungs showed interstitial pneumonia in both naturally and experimentally infected dogs. In conclusion, E. coli O26:K60 that express eaeA gene responsible for attaching- effacing (A/E) lesion and isolated from dog can cause severe lesions in most body organs and subsequent death of experimentally and naturally infected dogs.

Keywords: Dogs, eaeA gene, E.coli O26:K60, Histopathologic, PCR.

***Corresponding author**

ISSN: 0975-8585

INTRODUCTION

Escherichia coli are facultative anaerobes presents in the intestine of humans and animals (1). *Escherichia coli* are major component of the normal flora of animals intestine. *Escherichia coli* have different serotypes and can be isolated from the feces of both healthy and diseased animals. Although most of these bacteria are non-pathogenic, some of them have acquired genes that encoded the virulence (2). According to virulence factors present with diarrhea, five groups of diarrheagenic E. coli were established: enteropathogenic (EPEC), enterotoxigenic (ETEC), enteroinvasive (EIEC), enterohemorrhagic (EHEC) and enteroaggregative (EAEC) (3). The lesions of EPEC infections in the intestine are named attaching and effacing (A/E) lesions. This lesion is characterized by intimate bacterial adherence to the intestinal epithelium (4). Intimin, an outer membrane protein, encoded by eaeA, is a bacterial adhesion molecule that mediates the intimate bacterium host cell interaction characteristic of A/E lesions (5). EPEC classified into two types: typical EPECs, which cause A/E lesions and have the eae gene and the EAF plasmid but do not have the stx gene; and atypical EPECs, which cause A/E lesions and have the eae gene but do not have the stx gene and the EAF plasmid. Thus, studies on virulence factors, forms and clinical signs are essential to understand the pathogenesis of *Escherichia coli* (1). Association of typical EPEC with enteritis in dogs and the identification of pet dogs as reservoirs of *Escherichia coli* are still arguable (6). *Escherichia coli* is a clonal species with clones identified by their combination of O and H (and sometimes K) antigens. O antigens such as O157, O111 and O26 are of certain importance in human disease. A clonal population structure presents for these pathogenic E. coli strains have indicated that E. coli O26 strains posses variability with unique virulence profiles. O26 has appeared as an O antigen present in pathogenic strains (7). Attaching-effacing *Escherichia coli* O26:K60 and O157:H7 serogroups share common colonization factors and prior colonization by E. coli O26 may show reduced colonization by E. coli O157 (8). Understanding the genetic structure and molecular mechanisms of the bacterial virulence of E. coli has highlighted the mechanisms of pathogenesis associated with these different groups of bacteria. PCR is a powerful molecular biology technique that was conducted to facilitate the identification of virulence genes of different microorganisms; however, its direct use in fecal samples is limited by the presence of inhibiting factors, so most studies using this technique used isolated colonies and/or extraction and partial DNA purification to detect the virulence factors (5).

Aim of the work: The present study was carried out to investigate the clinical and pathological effect of attaching- effacing (A/E) *E.coli* O26:K60 on naturally and experimentally infected dogs.

MATERIALS AND METHODS

I- Bacterial strain: *E.coli* strain was isolated from 1.5 years old male German shepherd dog at K-9 department clinic at police academy, Cairo, Egypt after sudden death (naturally infected dog). Samples from rectal swab, intestine, liver, kidneys and lungs of this dog were enriched on sheep blood agar and MacConkey agar plates for colony isolation. The agar plates were incubated in 5% CO_2 at 37°C and were observed for bacterial growth after 24, 48, and 72 h of incubation. Lactose- positive colonies isolated from tissue specimen and rectal swab were selected for identification using standard methods, based on their characteristic reactions in triple-sugar-iron agar (the ability to produce indole and to hydrolyse o-nitrophenyl galactoside and the inability to split urea). In addition, selected hemolytic colonies from blood agar plates were subcultured on MacConkey's agar to assess lactose fermentation. Identification also confirmed by PCR assay.

Serotyping: Isolated strain was serotyped for its 'O' and 'K' antigens by Ministry of Health Laboratories, Cairo, Egypt using O and K specific antisera, according to standard methods.

II-Animals: Ten healthy mongrel female dogs, about six months old were used in this study. Dogs were acclimatized for seven days before the onset of the study, they were fed dry food. The drinking water was offered ad libtum.

III-Experimental design: Dogs were randomly divided into two groups. The first group (contain five dogs) served as a control. The second group (contain five dogs) was given a single dose of isolated *E.coli* strain inoculum. The inoculum was administered orally at a dose of 10 ml bacterial suspension of 1×10^9 CFU/ml.

The rectal temperature and clinical signs were recorded. All dogs of the first and the second groups were euthanized by intravenous injection of overdose of barbiturates at the end of the experiment (after 14 days).

All procedures of using experimental animals in this study met the regulations of Ethics of Research Committee at Faculty of Veterinary Medicine, Cairo University.

IV- Isolation and identification of recovered E-coli isolate: Rectal swabs were collected daily from dogs till the end of the experiment for bacteriological examination. After 14 days samples from intestine, liver, kidneys and lungs were collected for bacteriological examination also. Each sample was treated as before for isolation, identification and serotyping of *E.coli* strain.

V- PCR assay: The presence of the eaeA gene, which encodes intimin, was verified by PCR analysis.

DNA extraction: DNA samples for PCR analyses were obtained from suspicious cultures. One colony was selected, implanted on BHI broth (5 mL) and incubated at 37ºC for 12 h. The culture was centrifuged and the sediment resuspended in 200 µL phosphate buffered saline 0.05M, pH 7.2 (PBS), 10mg/ml lysozyme was added and the sample was incubated for 1 h at 30ºC, followed by addition of 200 µg/mL proteinase K and incubation at 55ºC for 1 h. Then, 300 µL 10% sodium dodecyl sulphate (SDS) were added and incubation was performed at 65ºC for 10 minutes; 600 µL chloroform and 400 µL protein precipitation solution (potassium acetate 5M and acetic acid 11%) were also added. The tube was then centrifuged at 10,000 rpm for 10 min and the supernatant was transferred to a clean tube then 1 mL absolute ethanol was added for later centrifugation for 5 min. The sediment was added to 1 mL ethanol 70% and centrifuged for 2 min. After the sediment was dried, DNA was eluted in 50 µL Tris-EDTA and incubated at 65ºC for 5 min. The extracted DNA was quantified in a Nanodrop spectrophotometer.

PCR: For PCR, 10 µL DNA template were mixed with 2.5 U Taq DNA polymerase, 50 pmol of each primer, 200 µM deoxynucleoside triphosphate, 1.5 mM MgCl and PCR buffer 1X in a final volume of 25 µL. Following an initial denaturation at 94°C for three minutes, the material was subjected to 35 thermal cycles of 94ºC (denaturation) for one minute, 56ºC (annealing) for one min, and 72ºC for 40 seconds. The reactions were carried out in a thermocycler. A 5µL volume from each reaction was subjected to electrophoresis on 0.8% agarose gel, stained with ethidium bromide/SybrGold and later visualized in a transilluminator (Ultra Violet Products). The amplification of an 384 bp fragment, corresponding to eae gene, was expected. For the amplification of eae gene, the following primers were used:

eaeA 1: 5’ GACCCGGCACAAGCATAAGC 3’
eaeA 2: 5’ CCACCTGCAGCAACAAGAGG 3’

VI-Postmortem and histopathological examination: At the time of euthanizing (after 14 days), dogs were subjected to postmortem examination to detect any abnormal gross changes. Tissue specimens from intestine, liver, kidneys and lungs of dogs were collected, fixed in 10 % neutral buffered formalin, processed and embedded in Paraffin wax, sectioned at 4 µm and stained with Hematoxylin and Eosin (9) and examined under an Olympus microscope (Olympus, Japan). Also the same samples were collected from naturally and experimentally infected dead dogs and handled as before.

RESULTS

Concerning to group 1 (Control group), no abnormal clinical signs or pathological changes were detected.

In naturally and experimentally infected animals (group 2, infected group), the recorded results were as follow:

Clinical signs: Naturally infected dog died suddenly without any apparent abnormal signs. Two dogs of the infected group showed a slight rise in rectal temperature after 24 hours from the onset of the experiment, followed by depression, these two dogs died at the 7th day of the experiment. Other animals in the same group did not show apparent clinical signs.

ISSN: 0975-8585

Bacteriological findings: E. coli strain was recovered from faeces, intestine, liver, kidneys and lungs of naturally infected dog after death, and also isolated from rectal swabs of infected group 24 hours post-inoculation and then fecal shedding continued till the end of the experiment. The organism was isolated from intestine, liver, kidneys and lungs of experimentally infected dead dogs at day 7th of the experiment (case I and II), and from the same organs of the other experimentally infected animals at the end of the experiment (day 14th). Organism was isolated, identified and serotyped as E,coli O26:K60.

PCR findings: *E.coli* strain was tested by PCR and showed amplification with a molecular length of 384 bp (Fig. a), corresponding to the eaeA gene which encoding the outer membrane protein intimin.

Fig a: PCR finding:

Agarose gel stained with ethidium bromide with polymerase chain reaction (PCR) products of E. coli isolates, showing amplification with a molecular length of 384 bp.

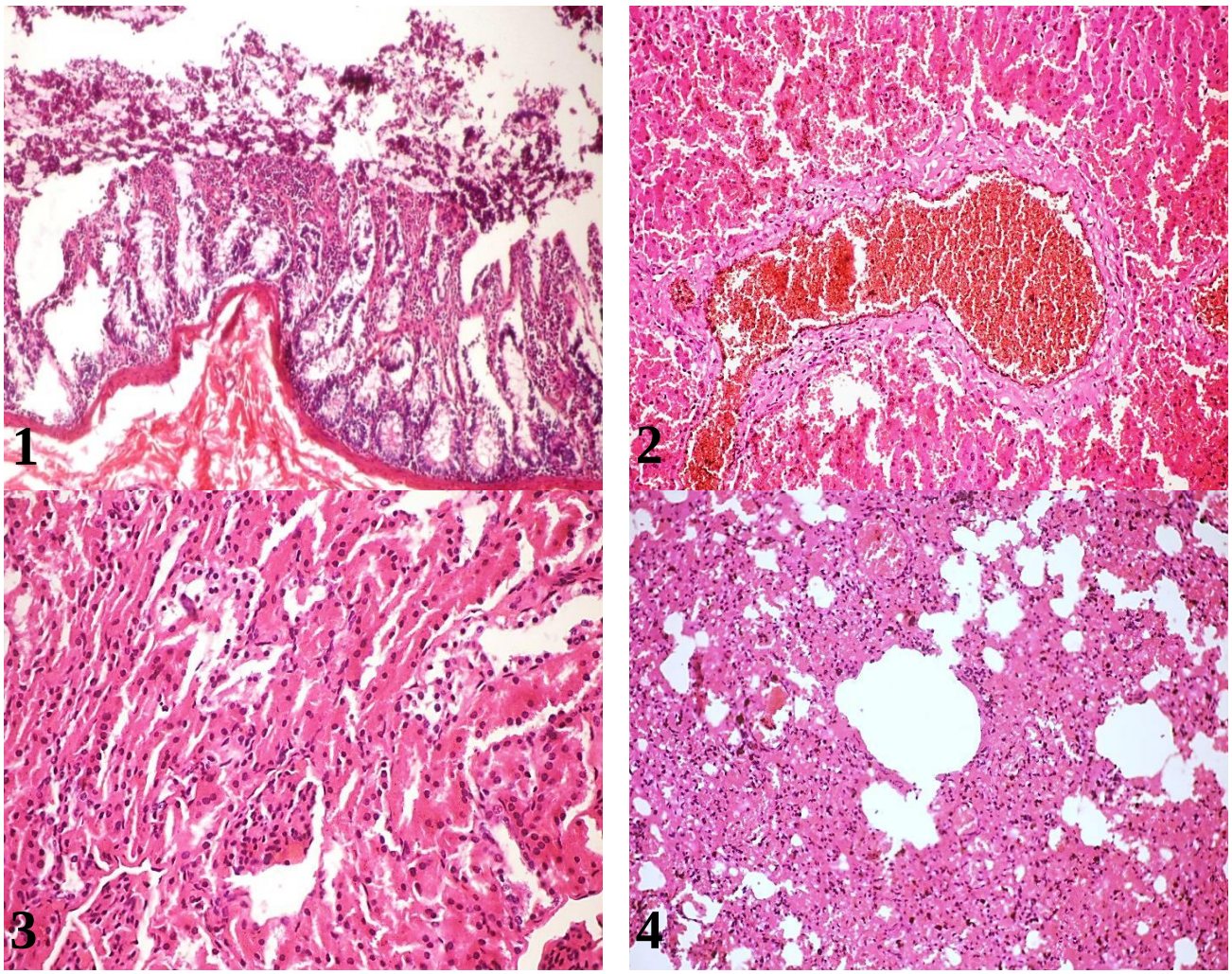
Fig b: Gross findings:

Small intestine, naturally infected dog showing multifocal roundedareas of congestion

Postmortem and histopathological findings: Concerning naturally infected dog, gross examination of intestine showed multiple rounded areas of congestion in some areas of mucosa (Fig. b) while other areas showed severe diffuse congestion. Macroscopic picture of liver, kidneys and lungs revealed presence of severe congestion. Intestine of naturally infected dog developed necrotizing enteritis by microscopic examination, in which there was coagulative necrosis of the enterocytes lining intestinal villi with complete fusion of these villi

ISSN: 0975-8585

at areas of necrosis. Lining epithelium of intestinal glands also showed coagulative necrosis with atrophy or complete disappearance of some intestinal glands. Lamina propria and submucosa of intestine infiltrated with mononuclear inflammatory cells (lymphocytes, macrophages and plasma cells) with presence of inflammatory edema dispersed submucosal connective tissue. Also there was necrotic tissue and inflammatory exudate covered the intestinal mucosa (Fig. c1). Also there was congestion of blood vessels at tips of intestinal villi and congestion of proprial and submucosal blood vessels. Histopathological examination of liver of naturally infected animal showed severe congestion of central veins and blood sinusoid with presence of hemorrhage in-between hepatocytes. Portal area showed fibroplasia with severe congestion of portal blood vessels (Fig. c2), there was periportal hepatocellular coagulative necrosis. Kidneys of infected dog revealed coagulative necrosis of lining epithelium of renal tubule in both cortex and medulla (Fig. c3) with congestion of interstitial blood vessels. Lungs of naturally infected animal revealed interstitial pneumonia in which there was thickening of alveolar wall that infiltrated with mononuclear cells and severely congested interstitial blood vessels with presence of hemorrhage, some alveoli were compressed and some formed giant alveoli. There was also hyperplasia of lining epithelium of bronchi and bronchiole and peribronchial and peribronchiolar fibrosis with congestion of peribronchial blood vessels (Figs. c4&c5).



ISSN: 0975-8585

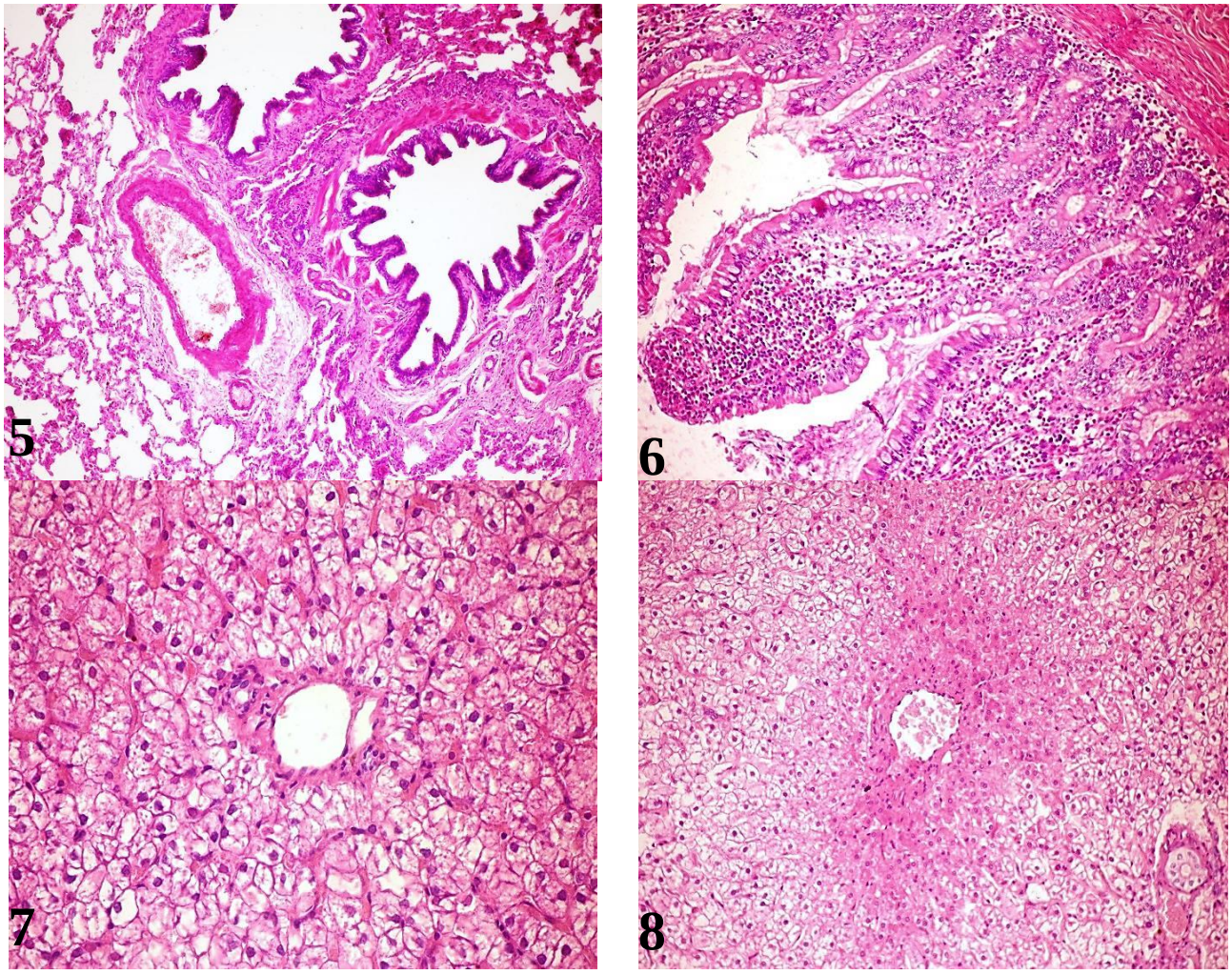


Fig c: Histopathological findings:

1: Micrograph of small intestine, naturally infected dog. Note coagulative necrosis of the enterocytes with fusion of intestinal villi. Lining epithelium of intestinal glands also showing coagulative necrosis with atrophy of some intestinal glands, there are also inflammatory cells infiltrating submucosal connective tissue. Also there is a necrotic tissue and inflammatory exudate covered intestinal mucosa (H&E X 100)
2: Micrograph of liver, naturally infected dog. Notice severe congestion of portal blood vessels and blood sinusoides with presence of hemorrhage in-between hepatocytes. Portal area showing fibroplasia. (H&E X 200)
3: Micrograph of kidneys, naturally infected dog revealing coagulative necrosis of lining epithelium of renal tubule in cortex (H&E X 200)
4: Micrograph of lungs, naturally infected dog showing interstitial pneumonia in which there is thickening of alveolar wall that infiltrated with mononuclear cells. Some alveoli are compressed and some form giant alveoli (H&E X 200)
5: Micrograph of lungs, naturally infected dog revealing hyperplasia of lining epithelium of bronchiole and peribrochiolar fibrosis. Note congestion of peribronchial blood vessels (H&E X 200)
6: Micrograph of small intestine, experimentally infected dog revealing enteritis in which lamina propria and submucosa of intestine infiltrated with mononuclear inflammatory cells with presence of inflammatory edema dispersed submucosal connective tissue. Also there is hyperplasia of goblet cells (H&E X 200)
7: Micrograph of liver, experimentally infected dog showing vacuolar degeneration of hepatocytes with activation of Kupffer's cells (H&E X 400)
8: Micrograph of liver, experimentally infected dog revealing central vein congestion with centrilobular hepatocellular coagulative necrosis. Note vacuolar degeneration of hepatocytes (H&E X 200)

Concerning experimentally infected dogs, gross examination of liver and kidneys revealed enlargement and paleness of both, while lungs revealed presence of congestion and lung hepatization. Intestine of these dogs developed enteritis by microscopic examination, in which lamina propria and submucosa of intestine infiltrated with mononuclear inflammatory cells (lymphocytes, macrophages and plasma cells) with presence of inflammatory edema dispersed submucosal connective tissue. Also there was hyperplasia of goblet cells (Fig. c6). Histopathological examination of liver of experimentally infected dogs showed vacuolar degeneration of hepatocytes with activation of Kupffer's cells in all examined cases, central veins were congested with centrilobular hepatocellular coagulative necrosis (Figs. c7& c8). Portal area showed fibroplasia with congestion of portal blood vessels. Kidneys of experimentally infected dogs revealed cell swelling and coagulative necrosis of lining epithelium of renal tubule in both cortex and medulla, with congestion of interstitial blood vessels. Lungs of experimentally infected dogs revealed interstitial pneumonia in which there was thickening of alveolar wall by infiltrated mononuclear cells and there was also congestion of interstitial blood vessels, hyperplasia of lining epithelium of bronchi and bronchiole and peribronchial and peribronchiolar fibrosis.

DISCUSSION

Escherichia coli is one of intestinal normal inhabitant of most mammalian species. Most E. coli are non-pathogenic, but some of them are an important cause of disease in man and animal (10). Canine species have been incriminated as a reservoir of the virulent *E.coli* strains (11). These bacteria have several serotypes and can be isolated from the feces of both healthy and diseased animal. Although most of them are not pathogenic, some have acquired genes that can convey virulence (5). This study was conducted to detect the presence of the eaeA gene responsible for attaching- effacing (A/E) lesions of *E.coli* by PCR and also to investigate the clinical and pathological effect of this highly pathogenic strain on naturally and experimentally infected dogs. In this study naturally infected dog died suddenly without apparent clinical signs. Two dogs of the infected group showed a slight rise in rectal temperature after 24 hours of inoculation followed by depression, these two dogs died at day 7^{th} of the experiment and this result was compatible with results that recorded by (12).

Other animals in the same group did not show apparent clinical signs till the end of the experiment. E. coli strain was recovered from faeces, intestine, liver, kidneys and lungs of both naturally and experimentally infected dogs after death and this result was compatible with that recorded by (13), and also isolated from rectal swabs of experimentally infected group 24 hours post-inoculation and then fecal shedding continued till the end of the experiment and this result was compatible with that recorded (12). Organism was isolated, identified and serotyped as *E.coli* O26:K60. Increased association has been observed between certain zoonotic non-O157 strains (specially serogroups O26, O103, and O111) and outbreaks or sporadic cases of *E.coli* infection (10). *E.coli* strain was tested by PCR and showed amplification with a molecular length of 384 bp, corresponding to the eaeA gene which encoding the outer membrane protein intimin and this result was similar to that reported (5). The definition of EPEC by serotyping is inaccurate and must be substituted by methods that detect the virulence properties of EPEC (14). Molecular techniques have been designed to detect the virulence genes and are the most sensitive methods for detection, Isolation frequency of eaeA genes from different E. coli strains have been reported in epidemiological studies from different locations all over the world (5). Concerning naturally infected dog, gross examination of intestine showed multiple rounded areas of congestion in some areas of mucosa while other areas showed severe diffuse congestion. Multifocal areas of congestion are due to presence of A/E lesions which cause multifocal bacterial adherence to epithelial cells, microvilli are effaced from apical membrane and that is mediated by intimin and associated with inflammatory response (4).Concerning naturally and experimentally infected dogs, Intestine developed enteritis by microscopic examination, in which lamina propria and submucosa of intestine infiltrated with mononuclear inflammatory cells (lymphocytes, macrophages and plasma cells) with presence of inflammatory edema dispersed submucosal connective tissue and this result was compatible with that recorded by (12). Intimin, an outer membrane protein, encoded by eaeA gene and considered as a bacterial adhesion molecule that mediates the intimate bacterium host cell interaction characteristic of A/E lesions (5). Intimin facilitates the adhesion of the bacteria to the intestinal villi producing A/E lesions (10). Histopathological examination of liver showed congestion of central veins and blood sinusoids with hemorrhage between hepatocytes, there was hepatocellular coagulative necrosis. Portal area showed fibroplasia with severe congestion of portal blood vessels and these results were similar to that recorded by (12). Kidneys of infected dogs revealed coagulative necrosis of lining epithelium of renal tubule in both cortex and medulla with congestion of interstitial blood

vessels and this result was agreed with (15), these changes may be due to alpha and beta – hemolysis which cause lysis of cells of kidney parenchyma (15). Lungs revealed interstitial pneumonia in which there was thickening of alveolar wall by infiltrated mononuclear cells and severely congested interstitial blood vessels, there was also interstitial hemorrhage and these results was similar to that observed by (13). There was also hyperplasia of lining epithelium of bronchi and bronchiole and peribronchial and peribrochiolar fibrosis. The congestion, hemorrhage and edema in different internal organs occurred as a result of septicemia which occurred as a result of lipopolysaccharides, a major constituent of gram negative bacteria (13).

From this study we concluded that, E. coli O26:K60 that express eaeA gene that responsible for attaching- effacing (A/E) lesion and isolated from dog can cause severe lesions in most body organs and subsequent death in naturally and experimentally infected dogs. The efficiency of PCR in the identification of the studied strain indicated that, this technique can be recommended for the diagnosis of EPEC.

REFERENCES

[1] Gouveia E, Silva I, Nakazato G, Onselem VJ, Corrêa RA *et al.*, 2013. Action of phosphorylated mannanoligosaccharides on immune and hematological responses and fecal consistency of dogs experimentally infected with enteropathogenic *Escherichia coli* strains. BJM, 44 (2): 499-504.

[2] Kantere M, Athanasiou L, Chatzopoulos DC, Spyrou V, Valiakos G *et al.*,2014. Enteric Pathogens of Dogs and Cats with Public Health Implications. Am J Anim Vet Sci, 9 (2): 84-94.

[3] Gouveia E, Silva I, Nakazato G, Araujo FR and Chang MR, 2011. Experimental infection with enteropathogenic *Escherichia coli* identified by PCR using enteric-coated capsules in boxer pups. Acta Cir Bras, 26 (2): 144-148

[4] Bentancor A, Vilte D, Rumi MV, Carbonari CC, Chinen I *et al.*, 2010. Characterization of non -Shiga toxin-producing *Escherichia coli* O157 strains isolated from dogs. Rev Argent Microbiol, 42: 46-48.

[5] Kiliç A, Ertafi H, Muz A, Özbey G and Kalender H, 2007. Detection of the eaeA Gene in *Escherichia coli* from Chickens by Polymerase Chain Reaction. Turk J Vet Anim Sci, 31(4): 215-218

[6] Rodrigues J, Thomazini C, Lopes CA and Dantas LO, 2004. Concurrent Infection in a Dog and Colonization in a Child with a Human Enteropathogenic *Escherichia coli* Clone. JCM, 42(3):1388–1389

[7] Souza J, Wang L and Reeves P, 2002. Sequence of the *Escherichia coli* O26 O antigen gene cluster and identification of O26 specific genes. Gene, 297: 123–127.

[8] Aktan I, Woodward M and Ragione RM, 2009. Interaction between attaching- effacing *Escherichia coli* O26:K60 and O157:H7 in young lambs. Res Vet Sci, 87(1):13-5.

[9] Bancroft J and Gamble M, 2008. Theory and Practice of Histopathological Techniques. 6th edition, New York, London and Madrid, Churchill Livingstone, pp: 69-174.

[10] Fairbrother J and Nadeau É, 2006. Escherichia coli: on-farm contamination of animals. Rev Sci Tech Off Int Epiz, 25 (2): 555-569.

[11] Johnson J, Stell A and Delavari P, 2001. Canine Feces as a Reservoir of Extraintestinal Pathogenic Escherichia coli. Infect Immun, 69 (3): 1306-1314.

[12] Wang J, Wang S and Yin P, 2006. Haemolytic–uraemic syndrome caused by a non-O157:H7 *Escherichia coli* strain in experimentally inoculated dogs. J Med Microbiol, 55(1): 23-29

[13] Al-Jeboori K and Kezar M, 2014. Study on bacterial dissemination and pathological findings of Enteropathogenic *Escherichia coli* in white albino mice. IJAR, 2 (4): 462-467

[14] Bugarel M, Martin A, Fach P and Beutin L, 2011. Virulence gene profiling of enterohemorrhagic (EHEC) and enteropathogenic (EPEC) *Escherichia coli* strains: a basis for molecular risk assessment of typical and atypical EPEC strains. BMC Microbiol, 11:142

[15] AL-Zamely H and falh S, 2011. The Effect of Experimental *Escherichia coli* Infection on Some Blood Parameters and Histological Changes in Male Rats. The Iraqi J Vet Med, 35 (2): 22-27