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USING SESAME OIL TO TREAT THE INFECTION OF HEMORRHAGIC *E. COLI* O157 : H7 BACTERIA ISOLATION IN BAGHDAD : MOLECULAR AND HISTOLOGICAL STUDY

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Abstract

A total of 200 stool samples bloody diarrhea and150 urine samples were collected from children of both sexes, with age between 3 to10 years. Aims of study including Isolation *E coli O157:H7* from bloody diarrhea and urine, Detection of virulence gene *hly A*, *flic H7* and *rfbO157* by (PCR), Study the pathogenicity of the isolated of *E coli O157:H7* in mice model, Study overlap effect of sesame oil and silver nanoparticles with antibiotic on pathogenicity *E coli O157:H7*. All isolates were identified by culture using selective media , VITEK 2 and they were confirmed by latex agglutination test. Polymerase chain reaction (PCR) was employed to detect some virulence gene as *hly A - flicH7* and *rfbO157* in stool and urine isolates. The result revealed 11 isolates, 8 (4%) obtained from patients with bloody diarrhea and 3(2%) from patients with UTI. PCR amplification result revealed presence of *hlyA, flicH7*and *rfbO157*genes in all isolates. The pathogenicity of *E coli O157:H7* were study to recognize the alterations in some organs of mice after experimentally infected with this pathogen. The histopathological examination of intestine for infected mice after 24-48 hours of infection showed infilteration of inflammatory cells, focal lining epithelial stratification , then distention of villi appeared . The liver showed accumulation of lymphocyte, hemorrhage in central vein with sinuses expansion with increase nuclear size.

Trimethoprim, sesame oil, silver nanoparticles were used as treatment for infected mice. Sesame oil gave excellent result as treatment in comparison with antibiotic and silver nanoparticles, the result showed intestine degeneration with infiltration of inflammatory cells, at 2 nd day post treatment. At 4 th days post treatment the intestine showed slight inflammatory cells infiltration . when liver showed irregular chromatin distribution with bizarre looking some nuclei at 2 nd day post treatment. Complete intestine and liver recovery and appeared as normal organs after 7 days post treatment.

Key words : *Escherichia coli O157:H7*, antibiotic, silver nanoparticles, sesame oil.

Introduction

Enterohemorrhagic *E. coli* (EHEC) belong to a group of bacteria known as attaching and effacing (A/E) pathogens that cause disease by adhering to the lumenal surfaces of their host's intestinal epithelium (Robyn *et al*., 2013). Shiga toxin-producing Escherichia coli (*E. coli*) (STEC), also called verocytotoxin-producing *E. coli* (VTEC), have emerged as pathogens that can cause food-borne infections and severe and potentially fatal illnesses in humans, such as haemorrhagic colitis (HC) and haemolytic uraemic syndrome (HUS) which is the main cause of acute renal failure in children (Kargar and Homayoon, 2015). *E. coli*, *O157:H7* has increased importance worldwide as a public health problem in the past two decades. It is the predominant and most virulent serotype in a pathogenic subset of Vero toxin producing *E. coli* (Nateneal *et al*., 2016). The most prevalent EHEC serotype causing outbreaks in North America is *O157:H7* with cattle serving as the primary reservoir for this highly infectious organism, thus making EHEC contamination a major issue in food and water safety (Croxen and Finlay, 2010). There are several viru-lence factors that contribute to *E. coli* pathogenicity, such as pilli, enterotoxins (LT, ST), shiga-like toxins, endotoxin (lipopolysaccharide), hemolysin, aerobactin, cytonecro-tizing factor, intimin, and biofilm formation (Blanco *et al*., 1996 ; Wong *et al*.,

2012). Endotoxin lipopolysaccharide (LPS) and Vero toxin (VT) are released and absorbed cross the gut epithelium. LPS , VT and maybe other virulence factors lead to an increase in pro inflammatory cytokines from host cells and subsequent release of chemokines from inflammatory cells (Palermo *et al*., 2009).

Histopathological examination of tissue biopsies for the identification of infectious organisms is a very important diagnostic tool Specifically, in clinical medicine, histopathology has contributed to advances in our understanding of disease leading directly to new and more effective treatment. Some new infection involving histopathology in their discovery, have also lead to fresh diagnostic challenges. Histopathology has become increasingly common in recent decades (James, 2017).

Materials and Methods

Sample collection

A total of 200 bloody diarrhea and 150 urine samples were collected in the period from beginning of September to end of December 2016 in Al-Eskan pediatrics hospital and children safe hospital from children of both sexes aged of 3 - 10 years. They were all suffered from bloody diarrhea and UTI.

Isolation of bacteria

Full loop was taken and enriched in modified tryptic soy broth (mTSB) supplemented with vancomycin (4mg/L) according to (Sanderson *et al.,* 1995) and incubated in 37 °C for 24h. Then full loop was streaked onto CT-SMACto seek sorbitol non-fermenting bacteria (colorless colonies) after that onto Hicrome media and Eosin methylen blue (EMB) as differential Media. The bacteria were identified by VITEK2 (Biomerieux- france). The latex agglutination test (Oxoid-England) was done for confirmation the serotype.

Molecular detection

Bacterial DNA Extraction

DNA was extracted from 8 isolates from stool and 3 from urine by DNA extraction Kit (promega USA) and then detected by gel Electrophoresis. The extraction protocol carried out as recommended by the manufacture data sheet. DNA Purity and concentrations of all samples were determined by using Spectrophotometer 2600 uv/vis (Unico USA).

Polymerase Chain Reaction (PCR)

Oligonucleotide primers were identified genes design according to Paton and Paton(1998) and Gannon *et al*. (1997) and list as in table 1.

PCR tubes were transferred to the thermalcycler to start the amplification reaction according to a specific program for each gene.

PCR program of rfb_{O157} including Initial denaturation at 94°C for 4minuts in 1 cycle, Denaturation at 94°C for 30 second, Annealing at 55°C for 30 second and Extention at 72°C for 1 minute in 35 cycle, final extention at72°C for 4min. in 1 cycle.

PCR program of *flic H7* gene including Initial denaturation at 94°C for 30 sec. in 1 cycle, Denaturation at 94°C for 30 second, Annealing at 55°C for 30 second and Extention at 72°C for 1 minute in 35 cycle, final extention at72°C for 4min. in 1 cycle.

PCR program of *hlyA* gene including Initial denaturation at 94°C for 1 min. in 1 cycle, Denaturation at 94°C for 1 min., Annealing at 55 °C for 2 min. and Extention at 72°C for 2.5 min. in 35 cycle , final extention at 72°C for 5min. in 1 cycle.

Histopathological study

Animals grouping

For 48 mice BALB/c body weight (22-25gram) (age 9-15 weeks) that provided by animal house of AL-RAZI center in Baghdad which are used in basic research, were housed in previously sterilized and cleaned plastic cages. Eight groups of mice were used in this study each group consist of 6 mice :

1- The first group used as control was administered by normal saline only 0.2 ml.

2- The second group infected orally with 0.2ml *E coli O157:H7* of $1.8x10^6$ cfu/ml (does lower than LD_{50} $1.8x10^7$) and sacrificed at two intervals (1^{st} day and 2^{nd}day post infection) .

3- The third group infected administered 0.2 (ml/Kg) antibiotic (trimethoprime) after one day post infection .

4- The fourth group administered 0.1ml Sesame oil as treatment after one day post infection.

5- The fifth group administered both 0.1ml Sesame oil and 0.2 ml antibiotic (trimethiprime).

6- The sixth group administered 0.1 ml of 0.5mg/kg silver nano-particles 80nm for seven days after infection.

7- The Seventh group administered 0.1 ml of 0.5mg/kg silver nano-particles 80nm and 0.1ml Sesame oil after infection.

8. The Eighth group administered 0.1ml of 0.5 mg/kg silver nano-particles 80nm and 0.2ml antibiotic after infection.

All mice in each group were sacrificed at three

Table 1 : Oligonucleotide primers sequences used for PCR amplification.

Primer	Sequence (5'-3')	Amplicon size(bp)	Reference
rfbO157	F: CGGACATCCATGTGATATGG R: TTGCCTATGTACAGCTAATCC	**259**	Paton and Paton (1998)
fliCH7	F: GCGCTGTCGAGTTCTATCGAGC R:CAACGGTGACTTTATCGCCATTCC	**625**	Gannon *et al*. (1997)
HlyA	F:GCATCATCAAGCGTACGTTCC R:AATGAGCCAAGCTGGTTAAGCT	**534**	Paton and Paton (1998)

Table 2 : Frequency of Non sorbitol fermenting (NSF) and CT-SMAC confirmed *E. coli* in Human stool and urine.

Isolates Source	Total samples	Sorbitol fermenting *E coli*	No. of E coli O157 positive isolates for CT-SMAC
Human stool	200	120(60%)	8(4%)
Human urine	150	87(58%)	3(2%)
Total	350	207	11(6%)

Table 3 : Frequency of latex agglutination positive *E. coli O157:H7*.

Isolates source	Isolates No.	*O157* positive
Human stool	200	8 (4%)
Human urine	150	3 (2%)
Total	350	11

intervals (2^{nd} days post treatment , 4^{th}days post treatment and 7^{th} days post treatment).

Histological examination

The intestine removed from control and infected mice and cut in long pieces, then fixed in 10% neutral buffered formalin. Further routine processing for hematoxylin and eosin staining was performed in the biotechnology research center at the AL-Nahrain university according to Musa (2017).

Results

The result revealed that 120 out of 200 stool sample were positive to *E. coli*. only 8 isolates were diagnosed as *E. coli O157:H7* from stool and 3 isolates from urine that appeared on SMAC as small, circular and colorless colonies with smoky center and (1-2) mm in diameter and on Hicrome media that appeared dark purple to magenta colored moiety (table 2).

Vitek2 gave the same results with culture

Serological identification of *E. coli 0157:H7*

Latex agglutination test is sensitive and specific in identification of *E. coli* O157:H7 but is expensive compared to ordinary conventional agglutination tests (Kimura *et al*., 2000). All of the *E. coli* isolates was confirmed by latex agglutination test for the somatic O157 antigen (table 3).

Detection of virulence factors genes

All isolates (11) were investigated for some virulence factors that responsible for pathogenicity of this pathogen and found that all isolates have these genes as showing in table 4 and figs. 1,2,3,4,5,6.

Pathogencity investigation of *E coli O157 : H7* isolates by experimental Infection of mice (*in vivo* study)

The symptoms appeared in all mice with morbidity (100%) after 24-48 hrs post infection. The clinical sings represented by bloody diarrhea, raised fur, shivering labored breathing. This finding also described by Ko *et al*. (2005) and Musa (2017). No signs of disease or mortality were observed in animals of control group that administrated with normal saline.

The intestines of infected mice were examined macroscopically after two days post infection, it appeared congested and sticky in comparison with intestine of control group (figs. 7,8). The liver macroscopically appeared congested after 2 days post infection in comparison with control .

Histopathological examination

The pathogenicity of the stool recovered isolates were appeared in the infected animals as different lesions in different organs especially intestine and liver that attributed to virulence factors of this isolates which leads to the development of these histological alterations.

Histological examination of intestine

The macroscopic and microscopic examination of intestinal section of control group animals showed normal intestinal architecture (fig. 9).

Histopathological examination of Intestine of experimentally infected mice showed that intestine was

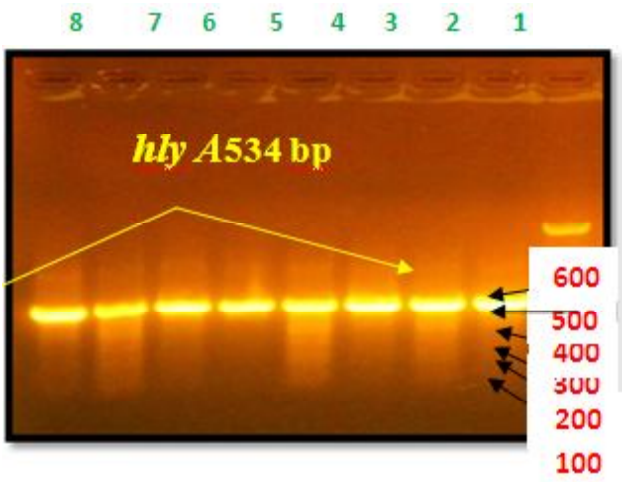

Fig. 1 : The PCR product of bacterial isolates from human stool (1,2,3,4,5,6,7,8), the amplified of *hly A* gene(534 bp) electrophoresed in 2% agarose gel.

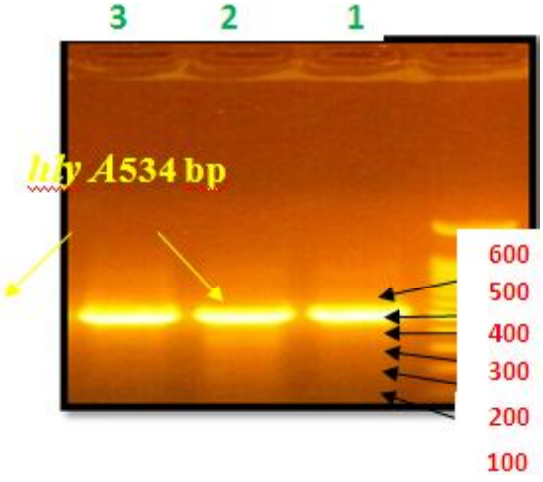

Fig. 2 : The PCR product of bacterial isolates from human urine (1,2,3), the amplified Of *hly A* gene (534 bp) electrophoresed in 2% agarose gel.

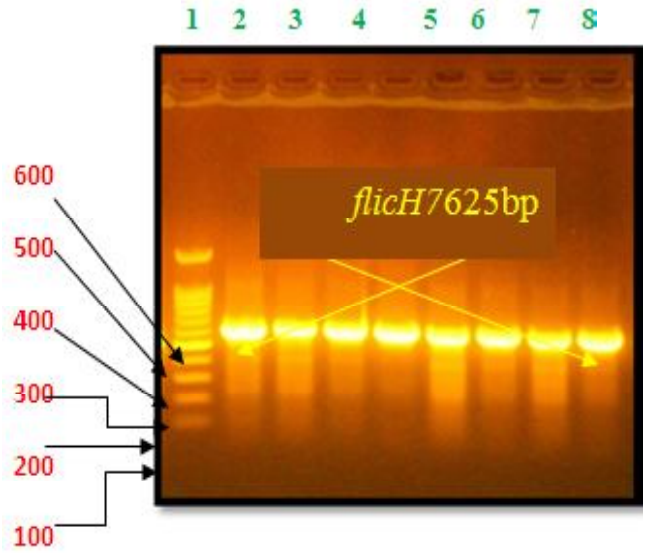

Fig. 3 : The PCR product for bacterial isolates from human stool (1,2,3,4,5,6,7,8), the amplified of *flic* gene (625 bp) electrophoresed in 1.5% agarose gel.

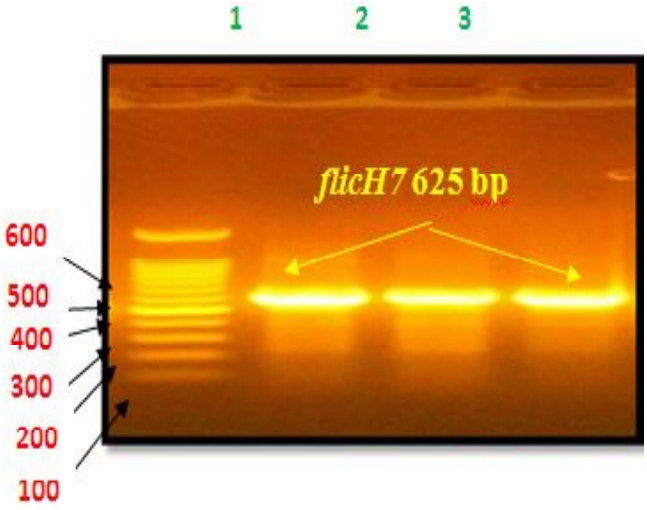

Fig. 4 : The PCR product for bacterial isolates from human urine (1,2,3), the amplified of *flic* gene (625 bp) electrophoresed in 1.5% agarose gel.

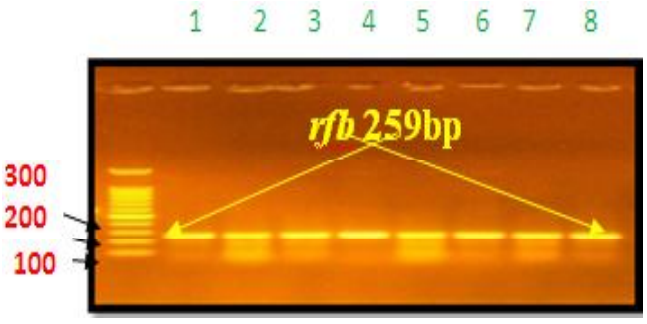

Fig. 5 : The PCR product for bacterial isolates from human stool (1,2,3,4,5,6,7,8) ,the amplified of *rfb* gene (259 bp) electrophoresed in 1.5% electrophoresed agarose gel.

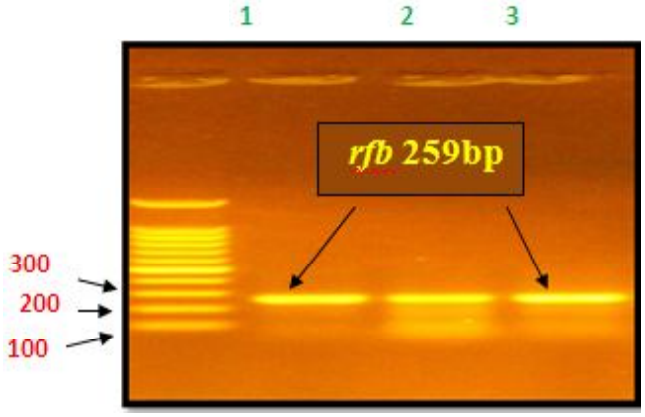

Fig. 6 : The PCR product for bacterial isolates from human urine (1,2,3), the amplified of *rfb* gene (259 bp) electrophoresed in 1.5% agarose gel.

Fig. 7 : Small intestines of mice appear congested were examined macroscopically after two days post infection.

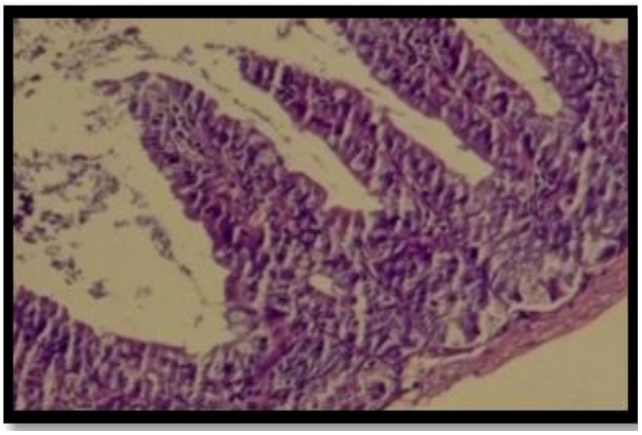

Fig. 9 : Histopathological section of intestine of control group mice shows normal histology of intestine (H&E stain 10X).

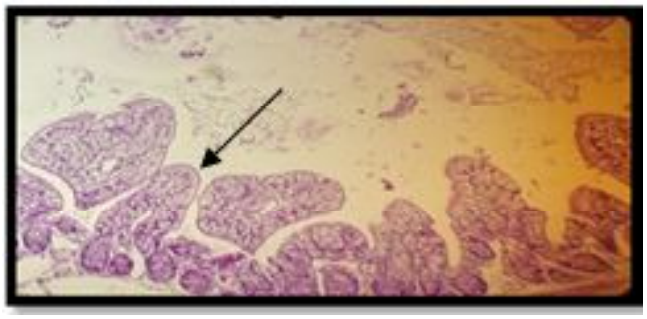

Fig. 11 : Histopathological section in the intestine of infected mice at day 2nd post infection show distention of villi (H&E stain 10X).

affected with variable lesions with infilteration of inflammatory cells, focal lining epithelial stratification and basal lamina degeneration, fig. 10 at one day post infection. This agreement with Jennifer *et al*. (2010) who found symptoms appeared on all mice after one day post infection with *E coli O157:H7* that administered orally and changes appeared in the intestine when they examined histopathology. On the other hand (Romina *et*

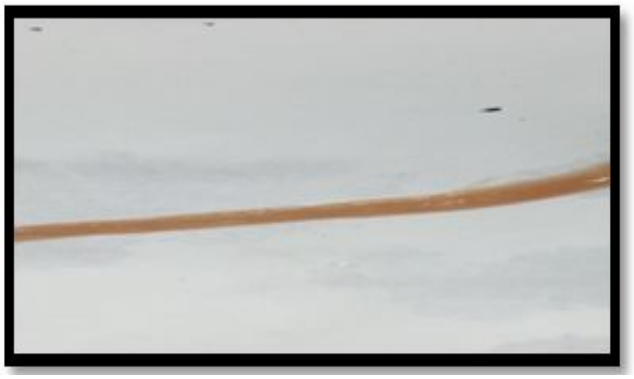

Fig. 8 : Small intestine of mice was appeared normal when examined macroscopically after administered with de-ionized water.

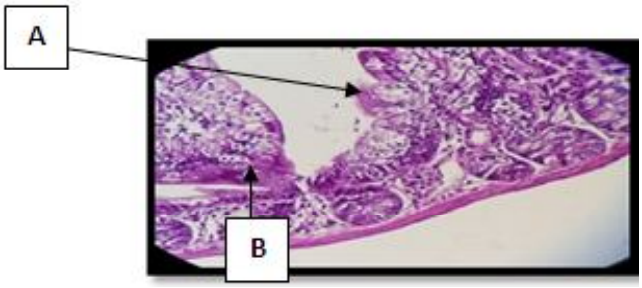

Fig. 10 : Histopathological section in the intestine of infected mice at one day post infection shows focal lining epithelial stratification (A), basal lamina degeneration (B) (H&E stain 10X).

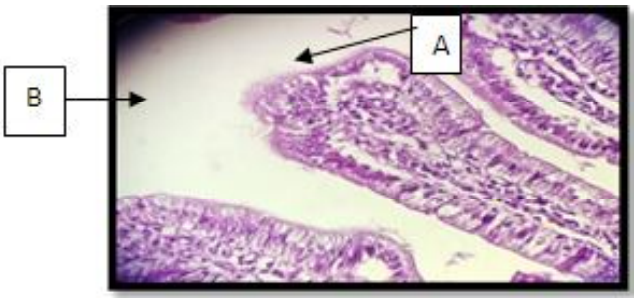

Fig. 12 : Histopathological section in the intestine of infected mice at day 2nd post infection shwos reactive epithelial atypia secondary to inflammation (A) with multiple layer (B) (H&E stain 10X).

al., 2012) recorded sticky, considerably empty, flimsy, of miceintestine when macroscopic examination after 1 day post infected with *E coli O157:H7*.

At, 2th day post infection, intestine of infected mice showed distention of villi with increase inflammatory cell infiltration, fig. (11) multiple areas show stratification with mixed inflammatory cell(lymphocyte , plasma and eosinophil cell). Stratification of lining epithelial cell with destruction of basal lamina, reactive epithelial atypia secondary to inflammation with multiple layer (fig. 12).

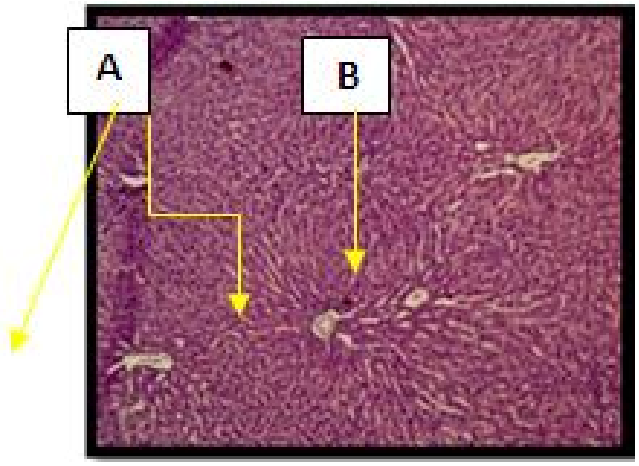

Fig. 13 : Histopathological section in liver of control group mice, shows normal liver histology, (A) Normal centrilobular area, (B) Normal hepatocyte (H&E 10X).

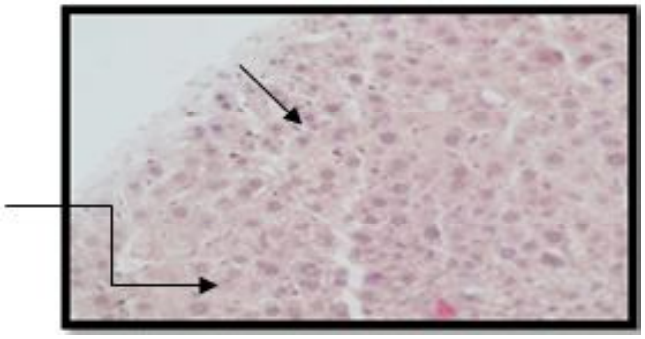

Fig. 15 : Histopathological section in liver of infected mice at second day post infection shows degeneration cells and sinuses expansion (H&E stain 10X).

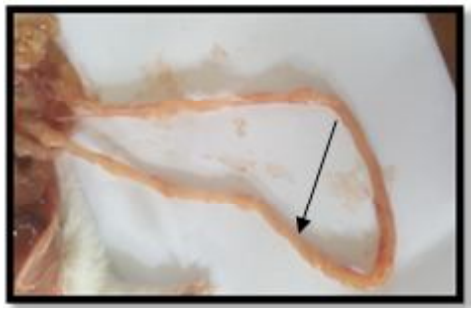

Fig. 18 : Small intestine of mice experimentally infected, appear with few congestion when administrated trimethoprime for 7 days.

Histological examination of liver

Microscopic examination of liver section of mice in control groups presented in fig. 13.

At second day post infection, liver sections showed accumulation of lymphocyte, hemorrhage in central vein with sinuses and Hepatocyte cells appeared, degenerative cells with sinuses expansion (figs. 14, 15). Increase nuclear size and increase hyperchromasia with irregular chromatin distribution), with bizarre looking some nuclei

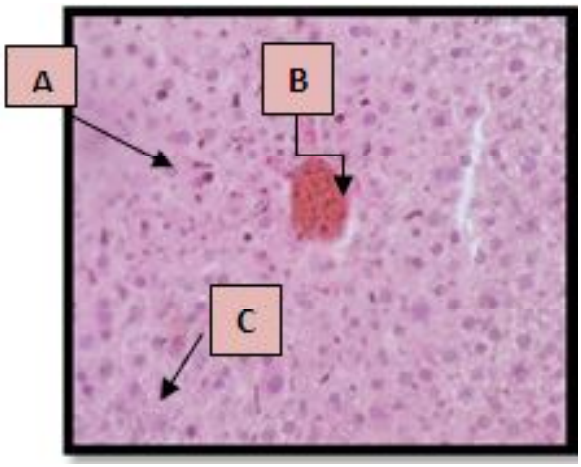

Fig. 14 : Histopathological section in liver of infected mice at second day post infection shows hemorrhage in central vein (A) with sinuses (B) and Hepatocyte cells (C) (H&E stain 10X).

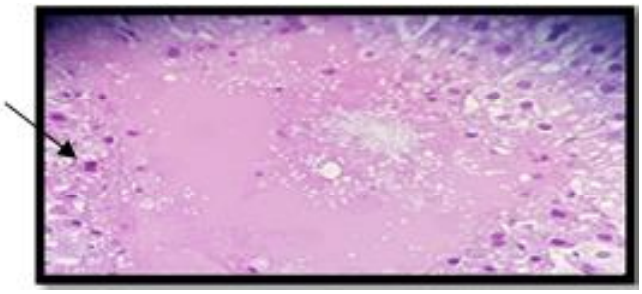

Fig. 16 : Histopathological section in liver of infected mice at second day post infection shows area of necrosis (H&Estain 10X).

and some have prominent nucleoli. increase N/C ratio with area of necrosis (fig. 16). The congestion and hemorrhage noted in this study accounted for activation of endothelial cells by inflammatory mediators.

Treatment

Trimethoprime

Experimental treatment by trimethoprime (0.2ml/kg) for the third group (n=6) after the symptoms showed clearly on mice at day1 post infection. Two mice were dead after two days post treatment, while other animals recovery was recorded after 4 days of treatment until the end of experiment. Similarly no morbidity or mortality was observed in control group animals throughout experiment. After seven days of treatment with antibiotic the intestine of scrified mice appeared response to this treatment with few congested (fig. 18).

Histological examination of intestine treatment with trimethoprime

At 2 nd day post treatment with antibiotic , intestine showed stratification epithelial cell, cell infiltration with

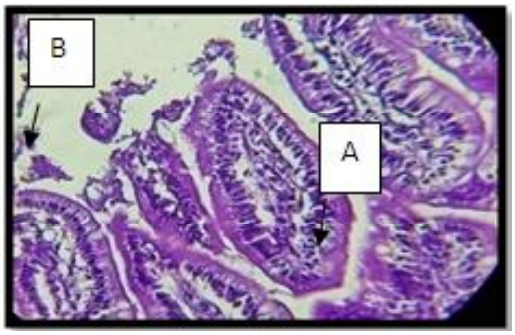

Fig. 19 : Histopathological section in the intestine of infected mice after 2 nd day of treatment with trimethoprime shows cell infiltration with degeneration of basal lamina and stratification epithelial cell (H&Estain 10X).

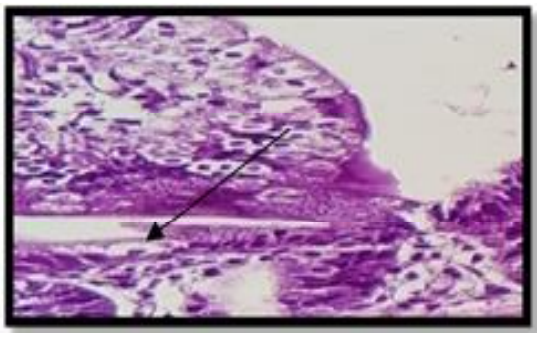

Fig. 20 : Histopathological section in the intestine of infected mice after 4th and 7th day of treatment with trimethoprime shows inflammatory cells infiltration with degeneration of basal lamina (H&Estain 40X) .

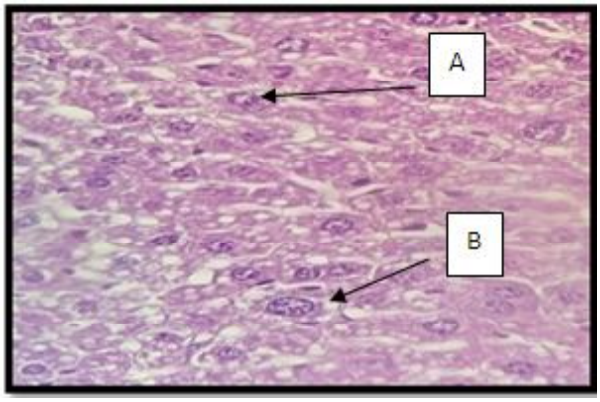

Fig. 21 : Histopathological section in the liver of infected mice after 2nd day of treatment with trimethoprime shows irregular chromatin distribution (A) and bizarre with nuclear enlargement (B)(H&Estain 40X).

Fig. 23 : Small intestine of mice appear as normal when administrated (sesame oil) after 7 days.

degeneration of basal lamina (fig. 19).

At 4th and 7th days post treatment with antibiotic, intestine showed inflammatory cell infiltration with degeneration basal lamina (fig. 20).

Histological Examination of liver treatment with trimethoprime

At 2 nd day post treatment with antibiotic, liver showed irregular chromatin distribution and bizarre with nuclear enlargement (fig. 21).

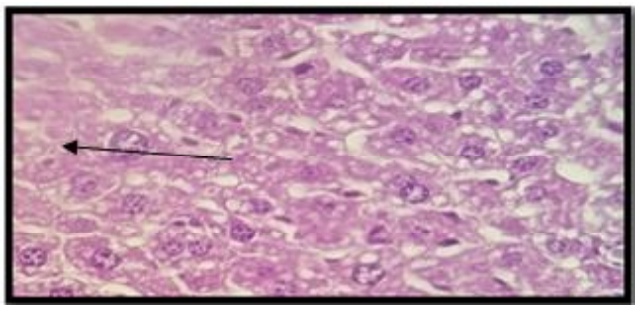

Fig. 22 : Histopathological section in the liver of infected mice after 4th and 7th day of treatment with trimethoprime shows nuclear enlargement (H&Estain 40X).

At 4 th and 7th day post treatment with antibiotic , liver showed nuclear enlargement (fig. 22).

Treatment by sesame oil

The fourth group consist of six mice (n=6). After 1 day of administered by pathogen no one of mice were dead. Sesame oil used when symptoms appeared after 1 day post infection. No morbidity was recorded after day 3 post infection until the end of experiment. After 5 days of treatment the mice return active. When mice killed after seven days of treatment with oil the intestine appeared great response to this treatment that return without congested as normal (fig. 23).

Histological examination of intestine treatment with sesame oil

At 2 nd day post treatment with oil, intestine showed focal lining stratification epithelial cell, degeneration with infiltration of inflammatory cells (fig. 24).

At 4 th days post treatment with oil , intestine showed slight inflammatory cells infiltration (fig. 25).

At 7th days post treatment with oil , intestine showed normal (fig. 26).

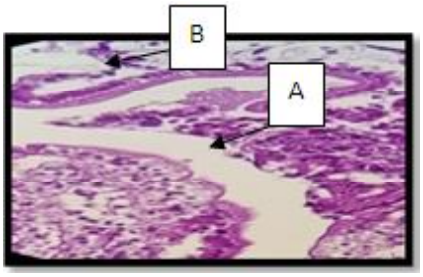

Fig. 24 : Histopathological section in the intestine of infected mice after 2nd day of treatment with oil shows focal lining stratification epithelial cell (A), degeneration with infiltration of inflammatory cells(B) (H&Estain 10X).

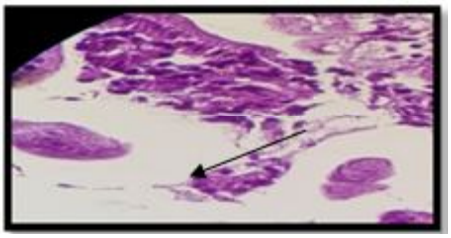

Fig. 25 : Histopathological section in the intestine of infected mice after 4 th day of treatment with oil shows slight inflammatory cells infiltration (H&Estain 40X).

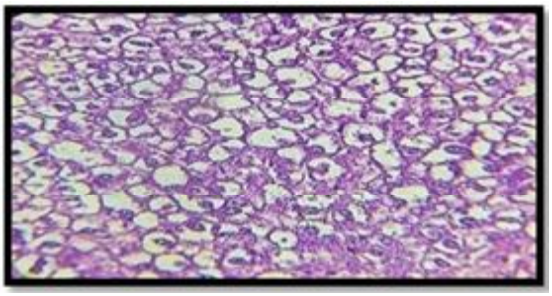

Fig. 28 : Histopathological section in liver of infected mice after 4th and 7th day of treatment with oil show normal hepatocyte cells (H&E stain 10X).

Histological examination of liver treatment with sesame oil

At 2nd day post treatment with oil, liver showed irregular chromatin distribution with bizarre looking some nuclei (fig. 27).

At 4th and 7th days post treatment with oil, liver showed normal hepatocyte cells (fig. 28).

Discussion

Escherichia coli O157:H7 is well documented Shiga toxin–producing (STEC) serotype, it is one of hundreds of *E. coli* strains. Although, the most strains are harmless and live in the intestine of healthy human and animals, STEC strain produces a powerful toxin and can cause severe illness (Baqir *et al.,* 2008; JiYoun *et al.,* 2010). The primary reservoir of this strain is the

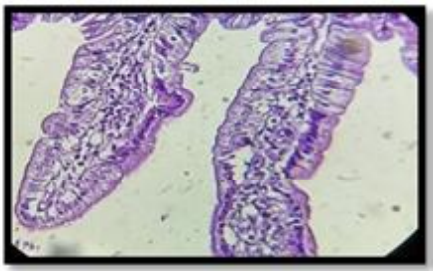

Fig. 26 : Histopathological section in the intestine of infected mice after 7th day of treatment with oil shows normal cells (H&Estain 10X).

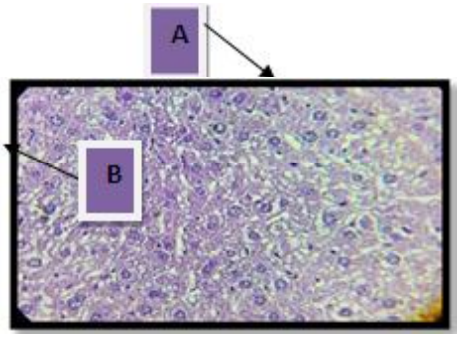

Fig. 27 : Histopathological section in liver of infected mice after 2nd day of treatment with oil shows irregular chromatin distribution (A) with bizarre looking some nuclei (B)(H&E stain10X).

cattle. Cattle feces are a major source of contamination of beef, other food products, and water**.** The direct contact with animals and material contaminated with animal feces, will increase the chances of infection.

In the present study, the virulence of *E. coli* O157:H7 was assessed in bloody diarrhea and urine samples using three genes including: *fliCH7*, *rfbO157* and *hly*A.

Patients selection was restricted to those children suffered from bloody diarrhea and UTI because *E. coli O157:H7* is mostly associated with this clinical features. In the current study 8 (4%) of Non Sorbitol Fermenting (NSF) revealed from bloody diarrhea samples which is similar to the study conducted by Al- awwadi *et al.* (2013). *E. coli O157:H7* isolated from 3(2%) samples of urine, this result less than Wong *et al*. (2012).

The confirmation of *E. coli O157:H7* was done by latex agglutination which confirmed identification of 8 isolated from stool and 3 from urine.

Many studies done about this bacteria in Iraq and world, that isolated it from different sources such as Ibrahim *et al*. (2014) isolated *E coli O157:H7* from Tigris River and children stool, also Baqer (2013), who detected this bacteria in food and patients in Baghdad, while sheikh *et al*. (2013) isolated of *Escherichia coli O157:H7* from beef hamburgers in Khuzestan Province, Iran.

Table 4 : Frequency of *hly A, fliCH7* and *rfbO157* genes of isolates in the samples.

Isolates source	No.of positive isolates with SMAC media	*hly A* gene	*flic H7* gene	*rfbO157*gene
Human stool	8	8(100%)	8(100%)	8(100%)
Human urine	3	3 (100%)	3(100%)	3(100%)
Total	11	11	11	11

The differences between the current result and other studies depending on site of collection ,season, number of hospitals surveyed and medication especially exposure to antibiotics, in addition to the use of significant parameters and methods to detect these pathogens. High percentage result in some study may be due to the life style, rural individuals were more contact with source of infections such as carrier animals and their dairy or meat products (Khanjar and Alwan, 2014).

The molecular study was done by PCR for detection of 3 genes (*hlyA – rfb*O157 – *flic* H7) responsible for some virulence factors and the result revealed that all isolates (11) were harbored these genes through amplification the specific fragments of these genes. The *hly A* was chosen in this study as enterohemolysin protein that cause lysis of red blood cells by destroying their cell membrane that lead to cause bloody diarrhea when *rfb* O157 gene choose as lip polysaccharide the O antigen and *flic* H7 gene (encoding fimbria) that help bacteria to attachment. These results confirmed that this pathogen considered as an important causes of gastrointestinal tract impairment and urinary tract infection in human.

The serotype *O157:H7* positive for gene (*hly* A) isolated from urine, may indicated that this pathogen play crucial role in urinary tract infection in the humans and cause renal failure in the infected individuals. This result similar with Samy *et al.* (2017), who identified 2 *E coli O157:H7* isolates positive for *hly* A from patients with UTI, when less than Al-wgaa (2014), who reported 8 isolates from urine have *hly* gene in the urinary tract infections of humans in Baghdad.

In this study, intestinal sections shwoed lymphocyte aggregation below the mucosa and distention of villi with increase inflammatory cell infiltration. multiple areas shows stratification and destruction of basal lamina. Most of these finding are described by other researchers as (Mansour *et al*., 2014) and (Longa *et al*., 2008). The intestinal histopathological changes observed in this study could in part be explained by the effect of endotoxin (lipopolysaccharides), the destruction of epithelial layer of mucosal glands and infiltration of inflammatory cells observed in this study also mentioned by (Milan *et al*., 2012), who inject lipopolysaccharides of *E coli O157:H7* intra peritoneally into mice.

Histopathological changes of liver observed in this study were similar to that mention by Das *et al*. (2012) who found congestion in liver of mice that infected by *E coli O157:H7* that isolated from rectal swab of pet dogs and cats in West Bengal.

Pankaj *et al*. (2013) showed necrosis in liver of pigeons after infected by *E. coli O157:H7* in India. Hepatic paranchymal necrosis has been reported a consequence of tissue anoxia resulting from vasoconstriction or by extracellular bacterial toxin (Brenden and Huizingat, 1986).

Treatment *E coli O157:H7* with trimethoprim appeared good result due to inhibits bacterial growth by inhibiting the synthesis of dihydrofolic acid of *E coli O157:H7* (Tarun and Chi, 2017).

Sesame oil appeared as treatment more effective and quicker than trimethoprime because contain vitamin k – proteins and antioxidant materials that may inhibition all biological activity of bacteria that lead to prevent production protein and inhibit the effect of lipopolysaccharide.

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