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Molecular detection of E.coli O157: H7 from diarrhea of sheep in Baghdad city

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***Abstract*---**This study was based on the importance of the pathogens that cause diarrhea in sheep and their impact on livestock, *E.coli* O157:H7 which are considered one of the most important pathogens, were studied and isolated with sensitivity test during the period (October 2021 to December 2021), samples were taken from infected sheep in fields around Baghdad (Abu-ghraib , AL-mahmoudia and AL-yosifiya), All samples were cultured on MacConkey agar , Eosine Methylene Blue and Sorbitol MacConkey agar for *E.coli* isolation according the isolates were identified by biochemical tests.out of 101 diarrhea samples, 100 isolates gave positive *E.coli* results and was taken three isolates appeared more resistance to antibiotics , and one isolate very sensitive to antibiotics. These isolates were selected to be confirmed by using molecular detection PCR. Among the three isolates most resistant to antibiotics showed a molecular diagnosis two belonging to the *E.coli* O157-H7 , in addition to the isolate most sensitive to antibiotics , which gave a positive *E.coli* O157-H7 result, but isolates are more resistance antibiotics appeared similar to the result of isolate appear more sensitive antibiotics because bacteria can evolve rapidly to adapt to environmental change., this evolution can turn harmless bacteria into harmful bacteria.

***Keywords*---**PCR, laboratory technique, molecular detection.

Introduction

Escherichia coli (*E. coli*) is a normal flora in the intestine of most animals and human. Some intestinal and extra-intestinal diseases could be caused by *E. coli* strains. Diarrhea, urinary tract infections, septicemia, mastitis, and newborn

meningitis are some of the symptoms(Tenaillon *et al.*, 2010). This bacterium is a rod-shaped, Gram-negative bacterium, and classified as a member of the family Enterobacteriaceae within the Gammaproteobacteria class Escherichia coli is among one of the well-studied bacteria. (Tenaillon *et al* .,2016).

The *E. coli* strains that produce diarrhea due to intestinal infections are known as diarrheagenic *E. coli* (DEC), which is divided into pathotypes, according to the combination of virulence factors (VFs) that determine virulence, colonization sites, and the signs and symptoms generated in the host. The DEC pathotypes are represented by: enteropathogenic *E. coli* (EPEC), Shiga toxin-producing *E. coli* (STEC), enterotoxigenic *E. coli* (ETEC), enteroaggregative *E. coli* (EAEC), enteroinvasive *E. coli* (EIEC), and diffusely adherent *E. coli*(DAEC).(Gomes *et al.*,2016).The Shiga toxin-producing *Escherichia coli* O157: H7 has become an important food and waterborne pathogen that causes diarrhea, hemorrhagic colitis, and hemolytic-uremic syndrome (HUS) in humans. An enterohemorrhagic bacterial strain, *E. coli* O157: H7 infects the alimentary tract and induces abdominal cramps with hemorrhagic diarrhea. Transmission of *E. coli* O157: H7 occurs via the fecal-oral route after consumption of contaminated, undercooked liquids and foods. Alternatively, *E. coli* 0157: H7 can be transmitted by person-to-person through fecal shedding and accounts for an estimated 11% of infections. (Atnafie *et al.*,2017; Erickson *et al.*,2019).

Material and Methods

Samples collection from sheep rectum

A total of 101 samples were collected from infected sheep diarrhea of both sex were obtained from the sheep , located in three areas (Abu-ghraib , Al – mahmoudia and Al-yousifiya) in aperiod of three months from (October 2021-December 2021). Samples were collected from sheep lamb (70) and adult (31) suffering with clinical signs : (anorexia, depression and diarrhea).

Isolation and identification of *E.coli*

Subsequently, a loop of each broth was streaked on surface of Blood agar, MacConkey agar,Sorbitol MacConkey agar and incubated at 37C for 24 hr ,as well Biochemical tests(IMVC) and TSI (siddique,2018).

Microscopic examination

Cell arrangement, size and shapes were detected under light microscopic using oil immersion lens (100X) (Murray et al., 2020).

Molecular detection of *E.coli* isolated from fecal samples of sheep by PCR Quantitation of DNA

According to protocol of ABIO pure , Quantus Fluorometer was used to detect the concentration of extracted DNA in order to detect the quality of samples for downstream applications. For 1 µl of DNA, 199 µl of diluted Quantifluor Dye was

mixed. After 5min incubation at room temperature, DNA concentration values were detected .

Primer preparation

These primers were supplied by Macrogen Company in a lyophilized form. Lyophilized primers were dissolved in a nuclease free water to give a final concentration of 100pmol/µl as a stock solution. A working solution of these primers was prepared by adding 10µl of primer stock solution (stored at freezer -20 C) to 90µl of nuclease free water to obtain a working primer solution of 10pmol/µl.

Agarose Gel Electrophoresis

After PCR amplification, agarose gel electrophoresis was adopted to confirm the presence of amplification. PCR was completely dependable on the extracted DNA criteria.

DNA loading

According to protocol of ABIO pure, PCR products were loaded directly. For PCR product, 5µl was directly loaded to well Electrical power was turned on at 100v/mAmp for 60min. DNA moves from Cathode to plus Anode poles. The Ethidium bromide-stained bands in gel were visualized using Gel imaging system.

Standard Sequencing

PCR product were sent for Sanger sequencing using ABI3730XL, automated DNA sequencer, by Macrogen Corporation – Korea. The results were received and analyzed using geneious software.

Application of discs

According to Kirby-Bauer test for antibiotic susceptibility, The plates were inverted and placed in an incubator at 37 C° for overnight. Clear zones made via antimicrobial inhibition of bacterial growing were measured in mm using a measuring caliper. The interpretation of values of disc diffusion method were performed and recorded as susceptible, intermediate and resistant according to the guidelines of the Clinical and Laboratory Standard Institute (CLSI), (Weinstein *et al.*, 2019).

Discussion and Results

Isolation and identification of *Escherichia coli* from sheep suffering diarrhea

One hundred one samples from sheep suffering diarrhea were examined during the period from(October 2021- December 2021). The bacterial isolation revealed that (100) of the samples were related to the *Escherichia coli* (Table 4-1).

Table (4-1): The numbers of *Escherichia coli* isolated from diarrhea in sheep

Source	No. of total samples	No. of *E.coli* isolates
Diarrhea samples	101	100

Isolation *E.coli* on Blood agar

The isolates *E. coli* were appear into blood agar for detection of the hemolysis after 24 hr of incubation at 37 C. For all isolates colonies that produced (gamma-hemolysis). This result agrees with(Altan *et al.,*2007) .

Isolation of *E.coli* on MacConkey and EMB agars

All isolate of *E.coli* gave pink color on MacConkey agar due to lactose fermentation were produced acidic by products that lower the pH, and this turns the pH indicator to pink. Second stage was done for *E.coli* isolates on Eosin Methylene Blue (EMB) agar which was used distinguishing *E.coli* from other Gram negative bacteria.it was used to identify *E. coli*, which produced a metallic green colony on the media, Fig (4-1). The results agree with (Alaa 2019).

Figure (4-1): *E.coli* on EMB agar appears as green metallic sheen color colonie

Isolation of *E,coli* on sorbitol MacConkey (SMAC) agar

The positive isolates on EMB agar that were selected and cultivated on SMAC agar appeared as colourless colonies, which indicate on *E.coli* O157 did not ferment sorbitol by Enterohemorrhagic *E.coli* (EHEC) Fig. (4-2). These result an agreement with (Al-wgaa and Alwan.,2014 ;Sukhumungoon, 2015) when they found Some EHEC serotypes cannot ferment D-sorbitol and appear as colorless colonies on SMAC as E. coli O157.

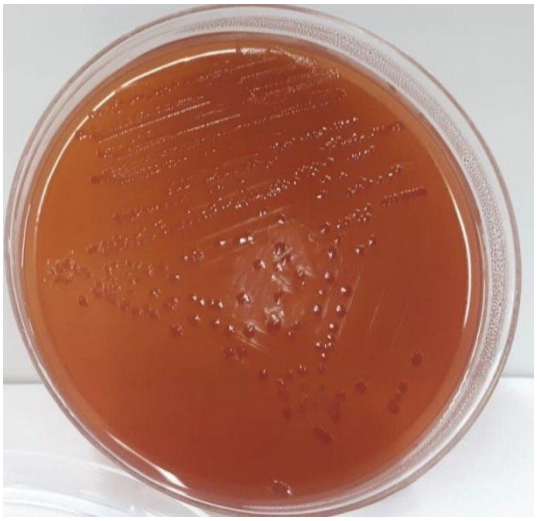

Figure (4-2): *E.coli* O157-H7 on (SMAC) agar appears as colorless due to unable to ferment sorbitol

Microscopic examination

The Isolate of *E.coli* appeared as Gram- negative, short rod shape, no-spore forming , single cell and in pairs under light microscope.

Biochemical test results

The results of biochemical identification of IMViC test gave positive results for the indole, methyl red, so indol gave red ring and methyle red appear red while it gave negative results for voges prosker appear of colourless , simmon citrate gave negative result, Figure(4-3). Which agree with (Fotadar *et al.*,2005; Dustin *et al.*, 2016 ; Mahe *et al.*,2021). When they found the same result .

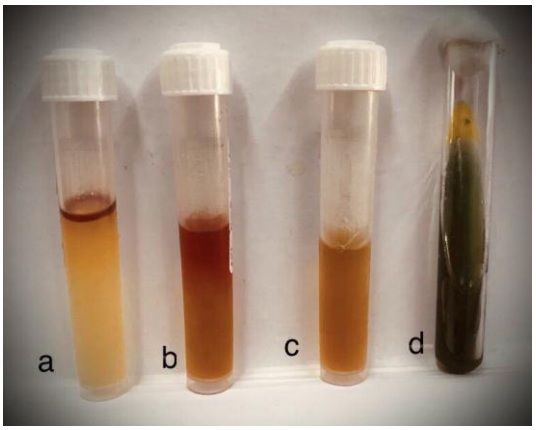

Figure (4-3) Biochemical test (IMViC) of *E.coli* .(a) indol ,(b) methyl red ,(c) voges-proskauer ,(d) simmon citrate.

Molecular diagnosis of pathogenic *E.coli* isolates from sheep suffering diarrhea

Polymearse chain reaction (PCR) and DNA Sequence analysis for *E.coli*

100 isolates gave positive E.coli results and was taken three isolates appeared more resistance to antibiotics , and one isolate very sensitive to antibiotics. These isolates were selected to be confirmed by using small subunit ribosomal PCR gene (16S rRNA gene) amplified by a specific primer. Then, these isolates were sent to Korea for identifying the sequence and comparing with similar sequence of the 16Sr RNA gene of the reference strains of *E. coli* in the Gen Bank. The Results of the amplification of using 16srRNA gene, which amplified a product size of approximately 1500 bp figure (4-4). Also it was performed as a method of confirmation of presumptive E. coli isolates that expected to produce a 1500 bp PCR product.The detection of 16S ribosomal RNA (16S rRNA) genes through polymerase chain reaction (PCR) amplification is a widely accepted method for the identification of bacteria (Sarvari *et al.*,2018).

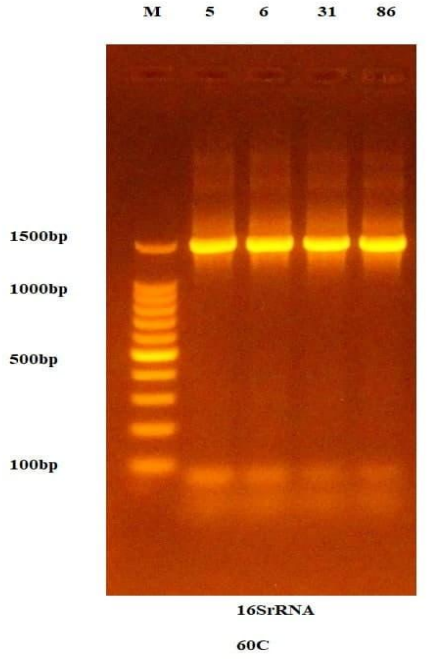

Figure (4-4): Results of the amplification of 16SrRNA gene of Unknown bacterial species were fractionated on 1.5% agarose gel electrophoresis stained with Eth.Br. M: 100bp ladder marker. Lanes 1-4 resemble 1500bp PCR products

Among the three isolates most resistant to antibiotics showed a molecular diagnosis two belonging to the *E.coli* O157-H7 , in addition to the isolate most sensitive to antibiotics , which gave a positive *E.coli* O157-H7 result ,but isolates are more resistance antibiotics appeared similar to the result of isolate appear more sensitive antibiotics because bacteria can evolve rapidly to adapt to environmental change., this evolution can turn harmless bacteria into harmful

bacteria. Full sequences (1500 bp) of the 16S rRNA gene of the strains have been registered in GenBank with the accession number *Escherichia coli* O157:H7 strain AJ1996 and Escherichia coli strain AJ1996. These results are propped by the ribosomal RNA sequencing as a more powerful technique for identification of bacteria, and the 16S rRNA gene is large enough for informatics purposes. These results agree (Manaka *et al.*,2017).

Antibiotics susceptibility of *E.coli* isolates

Antibiotics susceptibility of *E.coli* isolates by disc diffusion method

By using the disks diffusion method , one hundred isolates of *Escherichia coli* were tested for their antibiotics susceptibility toward ten antibiotics disc. All of the examined *Escherichia coli* showed high resistance (55) isolates against Tetracyclin, ,(42) Ampicillin, (34) Nalidic acid, (33) Nitrofurantion, respectively, while high sensitive (78) isolates against Gentamicin,(77) Ciprofloxacin ,(63) Chloramphenicol ,(59) Cefitraxon , respectively ,while high intermediate (71) isolates against Cefotaxime, (55) Amikacin, (53) Nitrofurantion,respectively. Tetracyclin was appeared high significant difference of resistance among other antibiotics at level (**P≤0.01) an agreement with (Silva et al 2020). When they found identified a moderate to very high prevalence of AMR to different classes of antimicrobial agents (penicillins, aminoglyco-sides, phenicols, tetracyclines and the combination sulfamethoxazole- trimethoprim) in *E.coli* isolates from sheep. Table(4-2) and Figure (4-5).

Table (4-2): Susceptibility disk by using disc diffusion method against *Escherichia coli*

Antibiotic each 100 isolate µg	No.of *E.coli* isolate, Resistance (R)	No.of *E.coli* isolate, Sensitive (S)	No.of *E.coli* isolate, Intermediate (I)	P-value
TETRACYCLIN(TE10)	55	28	17	0.0008**
AMPICILLIN(AMP10)	42	50	8	0.0001**
NALIDIC ACID(NA30)	34	58	8	0.0001**
NITROFURANTION(F100)	33	14	53	0.0003**
CEFOTAXIME(CTX30)	29	0	71	0.0001**
CHLORAMPHENICOL(C30)	28	63	9	0.0001**
CEFITRAXON(CRO10)	23	59	18	0.0002**
AMIKACIN(AK10)	17	28	55	0.0008**
GENTAMICIN(GN10)	10	78	12	0.0001**
CIPROFLOXACIN(CIP5)	9	77	14	0.0001**
P-value	0.0001**	0.0001**	0.0001**	
**(P≤0.01).				

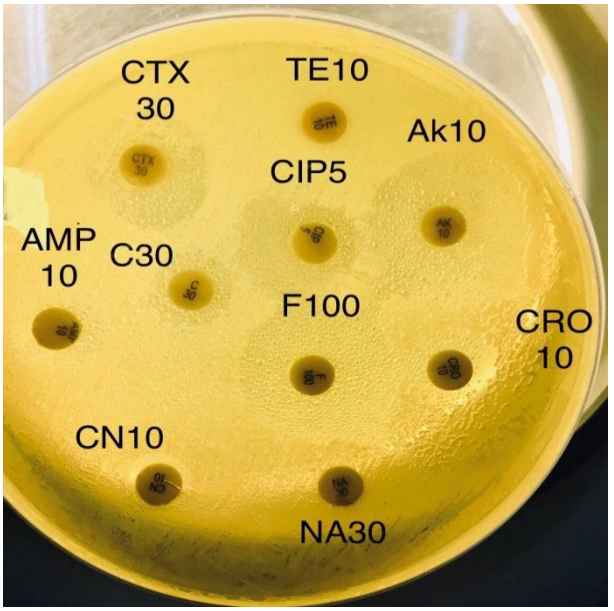

Figure (4-5): Antibiotic susceptibility test against Escherichia coli

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