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A molecular analysis of *Escherichia coli* O157:H7 strains isolated from cow and buffalo milk by the use of PCR in Karbala province

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Abstract--This study was conducted at Karbala governorate through the months of November 2021 until March 2022. Total number of 240 sampling was carried out from street vender and local store (milk, yogurts and cheese) from cow and buffalo. These samples were collected from different locations in Karbala in the Iraqi province. *Escherichia coli* O157H7 serotype prevalence rate from cow and buffalo was the major focus of this study of the research was conducted. 120 cow products and 120 buffalo products with different Karbala regions (local shops and street vendors) consisting a total of 240 samples. After initial enrichment, samples were streaked on Chromagar TM E. coli O157:H7 and sorbitole Maconkey agar. *Escherichia coli* O157h7 isolates were serotyped for to be detectable of the o157 and h7 antigens non-sorbitol fermenting (NSF) Isolates. Additionally, the virulent factor was examined. (rfbO157 and fliCH7) genes by PCR procedures. 14 isolates from 120 *Escherichia coli* O157:H7 in cow 11% and 18 from 120 (15%) buffalo. All fourteen samples isolates from produced milk became observed to be positive (rfbO157andfliCH7) (100%) genes, but just 13 from 18 isolates from buffalo.

Keywords---Escherichia coli O157:H7, rfbO157 gene, fliCh7 gene, Kerbala.

Introduction

Foodborne diseases have emerged as one of the major global public health issues and are highly common in undeveloped countries (Abdissa et al., 2017; Beyi et al., 2017; Saleem et al., 2021). As an important zoonotic pathogen of food-borne illness, *E. coli* serotype O157:H7 frequently causes diarrhea as well as human hemorrhage diarrhea and is a major cause of uremia associated hemolysis, which causes severe kidney failure in children (Al-Saadi et al., 2018). An important foodborne pathogen that can contaminate foods and risk people's health is *Escherichia coli* O157:H7. Thus, the purposes for guaranteeing the safety and quality of food, it is essential to identify this disease early and quickly in foods such raw milk ground beef and vegetables (Lim et al., 2010; Page and Liles, 2013).

Milk is a healthy and fulfilling nourishment for newborn mammals and humans, but it is also an excellent substrate for a variety of microorganisms (Leedom, 2006; Gharban and Yousif, 2021). *E. coli* O157:H7 has the ability to survive for days or even weeks in milk and dairy products (Dineen et al., 1998, 1998; Hudson et al., 1997). Because to microorganisms' involvement in the fresh milk that using to make cheese and their subsequent survival during the cheese-making process, cheeses can get contaminated (Donnelly, 2004) Pathogens can be spread by poorly cooked or processed foods. Ruminants, particularly cattle, buffalos, and sheep, are natural hosts for *E. coli* O157:H7 (Ismail et al., 2021). Recent findings have found that *E. coli* O157:H7 may survive for 30–40 days in low pH yogurt and cheese cooked in salt (Govaris et al., 2002).

Aim of study

Evaluation of PCR primer set designs was done to determine a conventional PCR method for identifying *E. coli* O157:H7 genes with specificity in cow and buffalo (milk, yogurt, and cheese) sample were collected.

Materials and Method

Collection of samples

Total number of 240 sample was carried out from milk, cheese and yogurt samples from street vendors and shops in various areas of the Holy Karbala Governorate that divided into: 120 samples cow milk and 120 samples buffalo milk which involve 60 sample from street vender and 60 sample local store that divided into 20 sample raw milk, 20 sample cheese and 20 sample yogurt. The sample was taken during sampling and preserved in a sterile screw-capped vial that was kept in an ice-filled ice box packets and transported right away to a bacteriological microbiology lab analysis. Isolation and bacterial identification was carried out using the methods Quinn et al. (2002) make a recommendation.

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E. coli O157 H7 Isolation and Cultivation

Agar medium preparation

In conformity with the manufacturer's instructions, all mediums were prepared and sterilized.

Blue Eosin-Methylene Agar

It was prepared, sterilized in conformity with the manufacturer's guidelines Oxoid (USA), poured onto petri plate within a sterilized hood, after that kept in a fridge at 4 °C .which used to identify lactose-fermenting enteric bacteria from non-lactose-fermenting enteric bacteria, as well as to isolate *Escherichia coli*.

MacConkey Sorbitol Agar (Sorbitol Agar)

Selective and differentiating medium to facilitate an identification of *E. coli* O157:H7 following preparations in accordance with the company guidelines, Himedia (India). *E. coli* O157:H7 appeared as a colorless colony after being cultured at 37°C for 18 hours (non sorbitol fermenter)

Modified HiCrome TM EC O157:H7 Selective medium

In order to identify and isolate *E. coli* O157:H7 , use chromogenic medium When the media is prepared in accordance with the manufacturer's specifications, bacteria appear as various colonies in various colors (Fadhil and Yousif, 2009).

Biochemical tests

Indole test

After the peptone water tubes containing the colonies of known *e. coli* were inoculation and cultured at 37 ° c over 48 hours, also some drops of the Kovac's solution, made in accordance with the manufacturer's protocol, were added to the culture medium without mix. The bright red circle that appeared on the liquid medium's top was considered to be apositive.

Motility Analyze

For the purpose of determining the motility of bacteria, a medium designed motility was applied. By inserting bacteria into tube, these were still cultured for 24hrs at 37°C.

Confirmation with Vitek-2 system

Microscopic Appearance

The *E. coli* O157 H7 is a gram-negative bacterium that looks like a rod with a pointed tip under a microscope. It is widespread in the natural world (Buckle, 2014).

Molecular methods

DNA extraction

Using the Addprep® Bacterial Genomic DNA Extraction Kit (AddBio /Korea), all phenotypically identified bacterial isolates were submitted to DNA extraction. We next followed the manufacturer's recommended protocol for gram-negative bacteria.

The *rfbO157* and *fliCh7* genes were examined by (AddBio /Korea) using using the standard PCR method detect *Escherichia coli* O157h7. The fragment sizes of the genes (*rfb* O157, and *fliCh7*) were 420 as well as 625bp, respectively.

Detection of Applying PCR, detect *rfbO157* and *fliCH7*

Details were determination the pcr amplification (PCR) technique on *Escherichia coli* O157h7 genetic material (containing *rfbO157* as well as *fliCH7*).

Primer sets

For the (*rfbO157* and *fliCH7*) genes, two commercial primers (Addprep® Bacterial Genomic DNA Extraction Kit) were used (Table 1). Using a 1% agarose gel and the gel electrophoresis method, extracted DNA was performed using a Nanodrop measurement device, the extracted DNA's quality and density was determined.

Controlled temperature construct and techniques

Final amount of 20µl was used, of which 10µl were for PCR, 1 µl were used for each primer including forward also and reverses, and 4µl were used for nuclease-free water and 4 µl contain DNA template Table (2). The thermocycler programme for the *rfbO157* and *fliCH7* genes included the following steps:

- One cycle with a duration of three minutes at 95 °C used for denaturing the template.
- Every of the 35 cycles has 3 stages: denaturation at 95 °C for thirty seconds, annealing at 65 °C during thirty seconds, and elongation at 72 °C for 1 min.
- with a final extension step at 72 °C taking 5 min.

Agarose Gel Electrophoresis-Based Amplification Product

One percent electrophoresis of agarose gels was used to analyse the PCR positive outcome. and a UV transilluminator following ethidium bromide staining use at a concentrations of 0.5 g/mL.

Table 1
Lists the nucleotide sequences and amplicon sizes for each PCR primer

Target gene	Primer name	Primer sequence (5'-3')	Amplification size (bp)	Reference
<i>rfbE</i>	O157AF	AAGATTGCGCTGAAGCCTTTG	497	(Desmarchelier et al., 1998)
	O157AR	CATTGGCATCGTGTGGACAG		
<i>fliC</i>	FLICH7 F	GCGCTGTCGAGTTCTATCGAGC	625	(Gannon et al., 1997)
	FLICH7 R	CAACGGTGACTTTATCGCCATTCC		

Table 2
Primary PCR Thermocycler Conditions

PCR step	Temp.	Time	Repeat
Initial Denaturation	95°C	3min.	1
Denaturation	95°C	30sec.	35 cycle
Annealing	63 °C	35sec.	
Extension	72 °C	1min.	
Final extension	72 °C	5min.	1
Hold	4 °C	Forever	-

Results

E. coli O157:H7 Isolation

E. coli colonies formed in many morphological forms and colors on various mediums. The colonies displayed a reddish colour on MacConkey agar. Eosin Methylene Blue has a metallic shine. Gram stains of colonies thought to be *E. coli* colonies showed negative, non-spore producing rod. Biochemical experiments on the isolated bacteria produced varied results. Simmon citrate and Voges-proskuar tests came back negative, however indole and motility tests showed positive. Yellow with/without gas production was seen in the Triple Sugar Iron test (TSI). On SMA-CT, the isolated *E. coli* colonies showed up as tiny, round, colorless rounds with smoky centres (1-2 mm in diameter). The *E. coli* O157 colonies on Chrome agar were mauve in color.

In total *E. coli* O157:H7 was isolated from 27 (11.25%) of 240 sample Isolates were biochemically typical of *E. coli* O157:H7 (sorbitol non-fermenting and confirmation with Vitek-2 system) and motile expressing the H7 antigen according

to culture methods, biochemical tests and PCR using *rfbE* O157 gene and *FLICh7* that amplified from raw milk cheese yogurt isolates via using conventional PCR a product size was approximately 497 bp. and using *fliC* FLICh7 gene that amplified a product size was approximately 625 (fig. 1 and 2) as shown in the table (1)

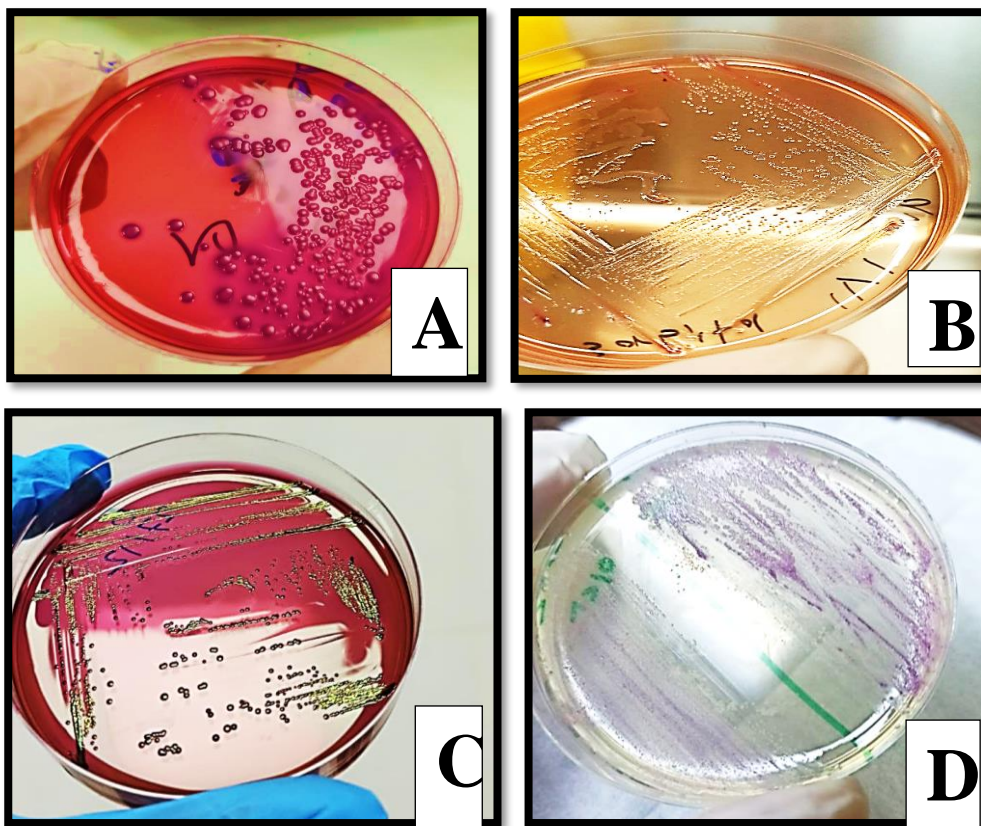


Fig 1. (A) *E. coli* colonies a reddish color on MacConkey agar (B) *E. coli* colonies colourless On SMA-CT agar (C) Eosin Methylene Blue has a metallic shine (D) Hichrom agar coloration from pale pink to mauve

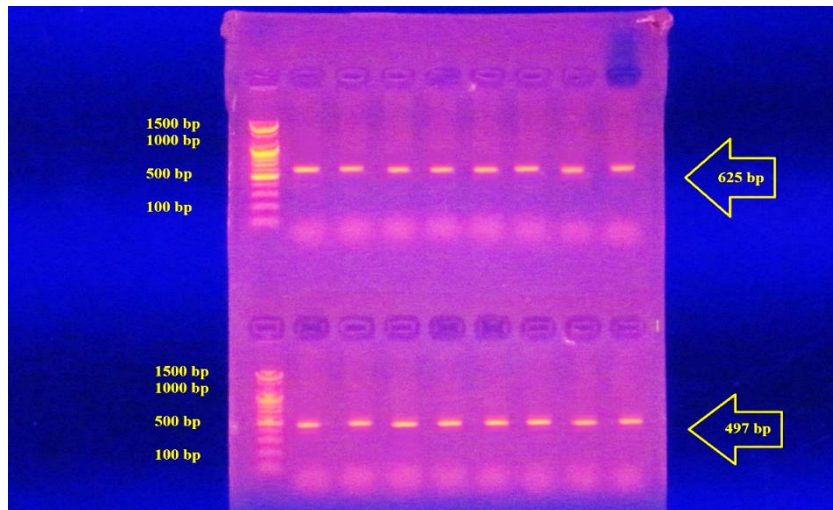


Fig 2. Agarose gel electrophoresis shows amplification product of 497bp and 625 bp fragments of (*rfbO157* and *fliCh7*) genes by PCR

Discussion

The routine identification of pathogenic forms of *E. coli* in agricultural samples is complex. It takes a lot of effort and time to routinely identify harmful *E. coli* strains in agricultural samples. Although there are ways to isolate EHEC O157:H7, the process is typically more challenging with agricultural samples due to the variety of bacteria, which frequently leads to cross reactions. Due to their lack of the metabolic defect and antibiotic resistance present in O157:H7 strains, other EHEC serotype identification is much more difficult. Unaffected by serotype, one method for estimating the possibility of contamination of a sample by pathogenic forms of *E. coli* is the use of PCR for the identification of virulence genes associated with pathogenicity in *E. coli*. EHEC strains often have the locus of enterocyte effacement, which includes *eaeA*, and one or both of these genes *rfbE* O157 gene and *FLiCh7* together with *stx1* or *stx2* in a milk sample.

The might be described as suggesting the presence of a pathogenic strain of *E. coli*. The PCR analyses only identify the presence of each gene within this population and cannot determine whether the genes are present in a single, highly pathogenic form of *E. coli* because the survey detailed here was conducted on enrichments of the general coliform population in each sample. But the discovery of these genes in this population should to make it possible to evaluate the risks posed by these organisms in agricultural products. The most common method of identifying *Escherichia coli* O157:H7 is by cultivating the bacteria on various media. On sorbitol MacConkey agar supplemented with cefixime and potassium tellurite (CT-SMAC), García et al. (2010) found that typical *E. coli* O157:H7 appeared as colourless colonies and did not ferment sorbitol on SMAC agar, whereas the majority of non-O157 strains showed. The sorbitol-MacConkey agar plate is enriched with potassium tellurite (2.5 mg/L) and the variation cifaxime (0.05 mg/L) by (Yousif & Hussein, 2015). As compared to other *E. coli* serotypes, the *E. coli* O157:H7 on SMAC agar O157 colonies look clear because they cannot ferment sorbitol. sorbitol-MacConkey agar SMAC plates with cifaxime

(0.5mg) were also constructed by (Laegreid et al., 1999). for the isolation of *E. coli* O157:H7 from calves, and potassium tellurite (2.5 mg), The colonies that are sorbitol negative after an 18-hour incubation period at 37°C are colourless.

Our results showed that Hichrom agar was extremely helpful in identifying *E. coli* O157:H7. In contrast to MacConkey and EMB agar, Hichrom medium contains sorbitol and a chromogenic combination instead of lactose and indicator dyes, making it a more appropriate medium for the isolation and detection of *E. coli* O157:H7. The inclusion of the chromogenic substance X-glucuronide in this medium made it easier to identify the *E. coli* cells that produce glucuronidase when exposed to the X-glucuronide. The colonies produced by the released chromophore ranged in coloration from pale pink to mauve (Jenkins et al., 2020). The current study's results are in line with those of (Klaif & Saleh, 2019) who use Chrom agar greatly helped in the identification of *E. coli* O157:H7 (Klaif & Saleh, 2019) Our findings are in agreement with Tavakoli et al. (2008), who noted that the use of chrome agar allowed presumptive identification of *E. coli* O157:H7 from the primary isolation plate and differentiation from other organisms. Non-*E. coli* O157:H7 organisms may use chromogenic substrates, resulting in blue to blue green colour colonies. According to a related research by Yousif and Al-Taii (2014), *Escherichia coli* O157:H7 may be diagnosed using Chrom agar.

The current study's finding of positive findings by PCR confirmed that *E. coli* O157:H7 was discovered by using virulence genes (*rfbO157*, *flicH7* genes). In Plateau State, Nigeria, Itelima and Agina found 2.9% prevalence of STEC O157 in fermented dairy samples (Itelima & Agina, 2010) which is less than the 4.1% prevalence found in the current study. raw cow's milk samples tested in Egypt and 3% of the samples tested in Austria. However, the findings of a research conducted in the European Union showed that from 1 to 13% of *E. coli* O157:H7 presence. The frequency of *E. coli* varies in different countries due to the similarities and variances in environment and cleanliness around the globe. Nevertheless, based on the data that have been recorded by the According to recent studies, *E. coli* O157:H7 is present in By the way, the amount of this pathogen in milk is low. Iraq places a huge amount of attention on the milk samples. The findings of the current study are similar to those of a research carried out in Iran by (Rahimi et al., 2012) showed that 7% of the samples

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