Prevalence and comparison between antibiotic sensitivity, virulence factors production in *Escherichia coli* O157:H7 and other *Escherichia coli* strains isolated from urinary tract infections and Diarrhea cases

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Abstract

Escherichia coli O157:H7 is the most recognized strain responsible for numerous global foodborne outbreaks. As a Shiga toxin-producing *Escherichia coli* (STEC), it is notably hazardous due to its ability to produce potent Shiga toxins, *Stx1* and *Stx2*. The study included collection of 320 samples divided into: 60 patients with diarrhea and 260 from patients with urinary tract infections (UTI). The isolates which developed pink colony and green metallic sheen on MacConkey and Eosin Methylene Blue (EMB) respectively, were characterized using biochemical test and then growing on Cefixime-Tellurite Sorbitol MacConkey agar (SMAC-CT). The Sorbitol fermenting colonies were cultured on HiCrome *E. coli* O157:H7agar. The results showed that out of 92 indole positive isolates, only 23\92(33.3%) were ferment sorbitol on CefiximeTellurite Sorbitol MacConkey agar (SMAC-CT), compared to 69\92 (66.7%) of non- sorbitol fermenters. Further specificity was provided by appearing of all the 23 suspected *E. coli* O157:H7 as dark purple colonies on HiCrome agar. However, when specific serology kit for *E. coli* O157:H7 strain was applied on the 23 isolates the result showed that only 11 were having both the somatic and flagellar antigen O157:H7 respectively whereas 12 have not. This result was consistence with the sequenced amplified *16S rRNA* gene. The prevalence of five virulence genes was assessed in the 11 E. coli O157:H7 using Polymerase Chain Reaction (PCR) methods, which revealed a high frequency of virulence genes *FliC* (100%), *RfbE* (100%), followed by *stx1* and *stx2* (72.7%), and lastly *eae A* (54.5%). Antibiotics susceptibility test was applied on all 92 *E. coli* isolates against nine antibiotics showed that the most resistance in *E. coli* strains-other than O157:H7 was toward amoxicillin – clavulanate (82.6%) followed by tetracycline (62%) and then Azithromycin 55(59.8%), whereas, it was revealed the highest sensitivity rate toward Meropenem (79.4%), followed by Levofloxacin with a sensitivity rate of 65.2%, while piperacillin-tazobactam showed moderate efficacy (56.5%).

Among the 11 *E. coli* O157:H7 isolates, resistance levels were notably higher compared to the nonO157 group. These isolates exhibited particularly elevated resistance to tetracycline (85.7%, $p < 0.001$), followed by amoxicillin–clavulanate (81.8%) and azithromycin (63.6%, $p = 0.059$), as well as moderate resistance to piperacillin–tazobactam (54.5%), ceftazidime (50.0%), and gentamicin (45.5%). Regarding susceptibility, the highest sensitivity among O157:H7 isolates was recorded for meropenem (85.8%, $p < 0.0002$), followed by norfloxacin (72.7%) and levofloxacin (63.6%).

A significant relationship ($p < 0.001$) was found between bacterial isolates and their antibiotic sensitivity, the antibiotic kind and resistance pattern due to the connection intensity. The observed sustained sensitivity of both groups to carbapenems and fluoroquinolones reinforces the clinical relevance of these agents in managing severe *E. coli* infections, particularly those involving multidrug-resistant or highly virulent strains such as O157:H7. Furthermore, comparison between the eight O157:H7 isolates harboring both *stx1* and *stx2* genes and the remaining gene-negative

strains revealed no statistically significant differences in their antibiotic susceptibility profiles, suggesting that virulence gene carriage is not directly linked to resistance phenotypes.

Keywords: Shiga toxin-producing *E. coli* O157:H7, UTI, antibiotic resistance, virulence factor, serological test

Introduction

Diarrhea, which defined as three or more loose or watery bowel movements within a 24-hour period, remains a major global health concern, affecting all age groups and leading to complications such as dehydration (1). Among bacterial causes, *Escherichia coli* is a key pathogen, with specific strains particularly Shiga toxin-producing *E. coli* (STEC) being responsible for severe gastrointestinal diseases and large-scale foodborne outbreaks (2).

Pathogenic *Escherichia coli* strains are clinically significant due to their ability to harbor virulence factors that enhance colonization, immune evasion, and disease severity (3). Molecular detection and characterization of Shiga toxin (*stx1* and *stx2*) and intimin (*eae A*) genes in *Escherichia coli* isolated from fecal samples of cattle, sheep, and humans in Basrah Governorate: A cross-sectional study (4). Strains harboring *stx2*, especially subtypes *stx2a* and *stx2b*, are more frequently associated with severe disease including hemolytic uremic syndrome (HUS), endothelial injury, and renal failure than strains producing other toxin subtypes (5). The *eae A* gene encodes the outer membrane adhesion protein intimin, which is essential for the formation of attaching and effacing (A/E) lesions on intestinal epithelial cells (6). This mechanism disrupts the host cytoskeleton, causing villus effacement and loss of absorptive surface, thereby aggravating diarrheal severity (7). Furthermore, *eae A* has been recognized as a critical virulence factor that differentiates enterohemorrhagic *E. coli* (EHEC) from less pathogenic strains (8). The *RfbE* gene is responsible for the synthesis of the O157 antigen, a component of the bacterial lipopolysaccharide (LPS). This antigen is crucial not only for serological identification of O157 strains but also for bacterial survival within the host by evading innate immune responses (9). Detection of *RfbE* is widely used as a molecular marker in confirming the O157 serogroup (10). Complementing this, the *FliC* gene encodes flagellin, the structural protein of bacterial flagella, which defines the H antigen in *E. coli* O157:H7. Beyond conferring motility and chemotaxis, flagellin is involved in biofilm formation and interaction with host immune receptors such as Toll-like receptor 5, triggering inflammatory responses (11). Its dual role in colonization and immune modulation makes *FliC* an important determinant of pathogenicity (12). Beyond gastrointestinal illness, *E. coli* is the primary causative agent of urinary tract infections (UTIs), affecting approximately 150 million people annually worldwide (13). The pathogenic potential of *E. coli* in UTIs also depends on strain-specific virulence factors, with toxin-producing strains posing significant therapeutic challenges (14).

Several studies have highlighted notable differences in antimicrobial resistance profiles between O157:H7 and non-O157 *E. coli* strains. For example, research indicates that non-O157 isolates frequently exhibit higher resistance to commonly used antibiotics compared to O157 counterparts

(odds ratio 2.4; 95% CI, 1.43–4.05) This suggests that while O157:H7 strains are clinically significant due to their virulence, they may not always show greater antimicrobial resistance than other serogroups, emphasizing the need for tailored surveillance strategies (15). Moreover, the relationship between virulence factors and antimicrobial resistance is complex and remains a subject of ongoing investigation. Some studies have shown that certain virulence genes are associated with resistance traits, while others report no clear correlation. A global analysis revealed a complex relationship that varied across bacterial populations, reflecting both environmental and evolutionary influences. This underscores the importance of incorporating both phenotypic and genotypic considerations when assessing *E. coli* pathogenicity and resistance in clinical research (16). Therefore, the present study aimed to investigate the prevalence of *E. coli* O157:H7 among patients with diarrhea and UTIs in Basrah city, characterize their virulence genes (*stx1*, *stx2*, *eae A*, *RfbE*, *FliC*), and evaluate antimicrobial resistance profiles to determine effective therapeutic options.

Materials and Methods

1.Sample Collection and Biochemical Identification: A total of 320 urine and stool samples were collected from clinical settings during the period from 2023-2024. The samples were cultured on MacConkey agar then Eosin Methylene Blue (EMB) agar and incubated at 37 °C for 24 hours for each of them. Colonies displaying typical *E. coli* morphology were further subjected to biochemical identification, using conventional protocols, Preliminary identification was supported by Gram staining, followed by biochemical confirmation that included catalase, oxidase tests, as well as the indole test. For further differential identification, isolates were cultured on Cefixime-Tellurite Sorbitol MacConkey agar (CT-SMAC) (17). Additionally, HiCrome O157:H7 agar (HiMedia, India) (18) was used to differentiate β-glucuronidase–positive strains.

Genomic DNA Extraction and PCR Amplification:

Genomic DNA was extracted from all isolates using the Presto™ Mini gDNA Bacteria Kit (Geneaid, Korea) according to the manufacturer's protocol as followed (19), pure bacterial colonies were suspended in nuclease-free water, followed by cell lysis using the provided lysis buffer to release genomic DNA. The lysate was transferred to the silica-based spin column, where the DNA bound to the membrane. The column was then washed twice with the provided wash buffers to remove proteins and impurities. Finally, DNA was eluted in nuclease free water and stored at −20 °C until further use. Confirmation of *E. coli* identity was performed through molecular techniques targeting the *16S rRNA* gene via PCR amplification of the universal *16S rRNA* gene with primer pair 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-GGTTACCTTGTTACGACTT-3′) (20). PCR cycling conditions consisted of an initial denaturation at 94 °C for 30 seconds, denaturation at 94 °C for 30 seconds, annealing at 56 °C for 30 seconds, extension at 72 °C for 1 minute, and a final extension at 72 °C for 5 minutes. Amplification of the specific *E. coli 16S rRNA* gene was performed under the same conditions except for the annealing step, which was

adjusted to 58 °C for 30 seconds with primer as show table (1). Reaction mixture contained the 2µL template DNA, 2µL specific forward and reverse primers 1µL for each primer, 12µL Master Mix (Promega, USA), and 9µL nuclease-free water, following the manufacturer's instructions. Amplified PCR products were analyzed by electrophoresis on a 1.5% agarose gel, stained with ethidium bromide, and visualized under UV transillumination. Sequencing PCR products were purified and sequenced at Macrogen Company (Seoul, South Korea) (21), and chromatograms were analyzed using Chromas software to confirm bacterial identity (22).

Virulence Gene Detection

Detection of virulence genes *stx1, stx2, eae A, RfbE,* and *FliC* was carried out using gene-specific primers as show in table 1. For amplification of the *stx1* and *stx2* genes, PCR reactions were performed with an initial denaturation at 94 °C for 3 minutes, followed by 35 cycles consisting of denaturation at 94 °C for 30 seconds, annealing at 56 °C for 45 seconds, and extension at 72 °C for 1 minute, with a final extension at 72 °C for 5 minutes. Amplification of the *eae A* gene was performed under the same conditions except for the annealing step, which was adjusted to 57 °C. For *RfbE* and *FliC* genes, the annealing temperature was maintained at 59 °C under the same cycling conditions mentioned above. In each PCR reaction, a total volume of 25 µL was prepared, consisting of 2 µL of template DNA, 2 µL of specific primers (1 µL each for the forward and reverse primer), 12 µL of Master Mix (Promega, USA), and 9 µL of nuclease-free water, following the manufacturer's instructions to ensure optimal amplification conditions and reaction accuracy. Amplified PCR products were analyzed by electrophoresis on a 1.5% agarose gel, stained with ethidium bromide, and visualized under UV transilluminatio. The annealing temperature for each gene in the present study was optimized using gradient PCR with different annealing temperature.

Table (1): The specific primer Sequence for *stx1*, *stx2*, *eae A*, *fliC*, *rfbE* and *E16s* genes.

Genes	Name of the primer	Primer sequence (5-3)	Size of the Product (bp)	Length (bp)	Ref.
stx1	Reverse	5'-TCTCAGTGGGCGTTCTTATG-3"	366bp	25	
	forward	5'-TACCCCCTCAACTGCTAATA- 3"		25	
stx2	Reverse	5'-GCGGTTTTATTTGCATTAGC-3"	282bp	24	(23)
	forward	5'-TCCCGTCAACCTTCACTGTA-3"		25	
eae A	Reverse	5-'TGCGGCACAACAGGCGGCGA- 3"	629bp	20	
	forward	5'-CGGTCGCCGCACCAGGATTC- 3"		20	
E16s	Reverse	5'- CCCCCTGGACGAAGACTGAC- 3"	401bp	20	(24)
	forward	5'- ACCGCTGGCAACAAAGGATA- 3"		20	
fliC	Reverse	5'TACCATCGCAAAAGCAACTCC- 3"	247bp	20	
	forward	5'GTCGGCAACGTTAGTGATACC- 3"		20	
rfbE	Reverse	5'-CTACGGTGAAGGTGGAATGG- 3"	327bp	20	
	forward	5'-ATTCTTCTCTTTCCTCTCTGCGG- 3"		20	

Serological Testing

Serological test to detect the somatic and flagellin of *E. coli* O157:H7 was performed using latex agglutination kits following the manufacturer's instructions (25). Latex reagents and controls (positive and negative) were being to room temperature. A sterile stick or loop was used to emulsify 1–2 colonies from SMAC agar. One drop of (20–30 μL) of latex reagent was added to a test card circle and mixed with emulsified colony by the stick. The sample was mixed and rocked gently for 1–2 minutes. Positive result was read by appearing visible agglutination (clumping) within 1–2 minutes. Negative result no agglutination appeared within 1-2 minutes.

Antibiotic Susceptibility Test

Antibiotic susceptibility test was carried out using the standard Kirby Bauer disk diffusion method with nine antibiotics toward 92 *E. coli* including *E. coli* O157:H7 and *E. coli* non O157:H7, as bellow (26): a bacterial suspension was prepared and adjusted to match the 0.5 McFarland turbidity standard, using a sterile swab, the suspension was evenly spread over the entire surface of Mueller-Hinton agar, antibiotic discs were then placed onto the agar surface using sterilized forceps or a disc dispenser, the inoculated plates were incubated at 35°C for 16 to 18 hours, after incubation, the diameter of the inhibition zones surrounding each antibiotic disc was measured in millimeters, and the results were analyzed according to the Clinical and Laboratory Standards Institute (CLSI) (27) guidelines, and categorized as Sensitive (S), Intermediate (I), or Resistant (R).

Results

The study was included 320 samples, comprising urine and stool specimens, 143 samples (44.7%) demonstrated microbial growth; (42.0%) were from stool samples and (58.0%) were from the urine samples. The remaining (55.3%) showed no growth Phenotypic and Characterization of *Escherichia coli* O157:H7 strain. Preliminary characterization of 143 bacterial isolates was based on cultural morphology. On MacConkey agar, pink colonies with bile salt precipitation indicated Gram-negative lactose fermenters. When these isolates were cultivated on Eosin Methylene Blue (EMB) agar, they exhibited a characteristic green metallic sheen. Out of 143 samples cultured on EMB and MacConkey agar, 92 (64.3%) were identified as lactose-fermenting isolates. Among the 143 isolates, 92 (64.3%) tested positive for indole production, in addition to catalase-positive and oxidase-negative, providing strong evidence for the presumptive identification of *E. coli.* However, when these isolates cultured on Cefixime-Tellurite Sorbitol MacConkey agar (SMAC-CT), 23(25%)were shown as pink colonies which assigned as a sorbitol fermenter. indicating their classification as the O157:H7 serotype. Similarly, when cultured on HiCrome™ *E. coli* agar, 23 isolates (25%) developed violet colonies reflecting positive β-glucuronidase activity, while the remaining isolates (69 isolates; 75%) formed blue colonies, demonstrating enzymatic variability among the tested strains. These combined cultural and biochemical characteristics provided an effective basis for distinguishing typical *E. coli* O157:H7 from atypical *E. coli* isolates during the preliminary screening process.

Identification using *16S rRNA* gene

After extracting the DNA, the *16S rRNA* gene was amplified using the PCR technique, the results visualized in the agarose gel electrophoresis and then visualized by UV transilluminator. Comparing the findings with a standard DNA ladder shows the product as a single band with a size of around 1500bp. Ninety-two amplified 16s rRNA gene using PCR were sent to Macrogene company south Korea, and the upcoming resultant data were analyzed via Chromas software. Out of them, 85 isolates were successfully processed, while the others were discarded owing to mixed sequencing. The result of 77 interpretable sequences, 11 isolates were matched *Escherichia coli O157:H7* and 66 isolates belonging to other *E. coli* strains. In addition to, eight isolates were classified as distinct bacterial species *Klebsiella spp*. 4, *Raoultella spp*. 3, and *Serratia spp*. 1.

The constructed phylogenetic tree clustered the 42 analyzed isolates—including the 11 *E. coli* O157:H7 strains, other *E. coli* isolates with high sequence similarity, and 7 non-*E. coli* strains. Most *E. coli* isolates grouped tightly together in a monophyletic clade, reflecting their high genetic relatedness and supporting their accurate species-level identification. In contrast, the non-*E. coli* isolates formed distinct outgroups, which validates the discriminatory power of the 16S rRNA gene in differentiating between genera within Enterobacteriaceae.

The phylogenetic tree shows the relation *E. coli* stains with other closely related genus. Using Mega 12 (28) for unconcatenated sequence (42) of *E. coli* and other G-ve bacteria. The culture and biochemical characterization of 143 bacterial isolates revealed a diagnostic profile consistent with *Escherichia coli.* Which conformed by 16s rRNA identify.

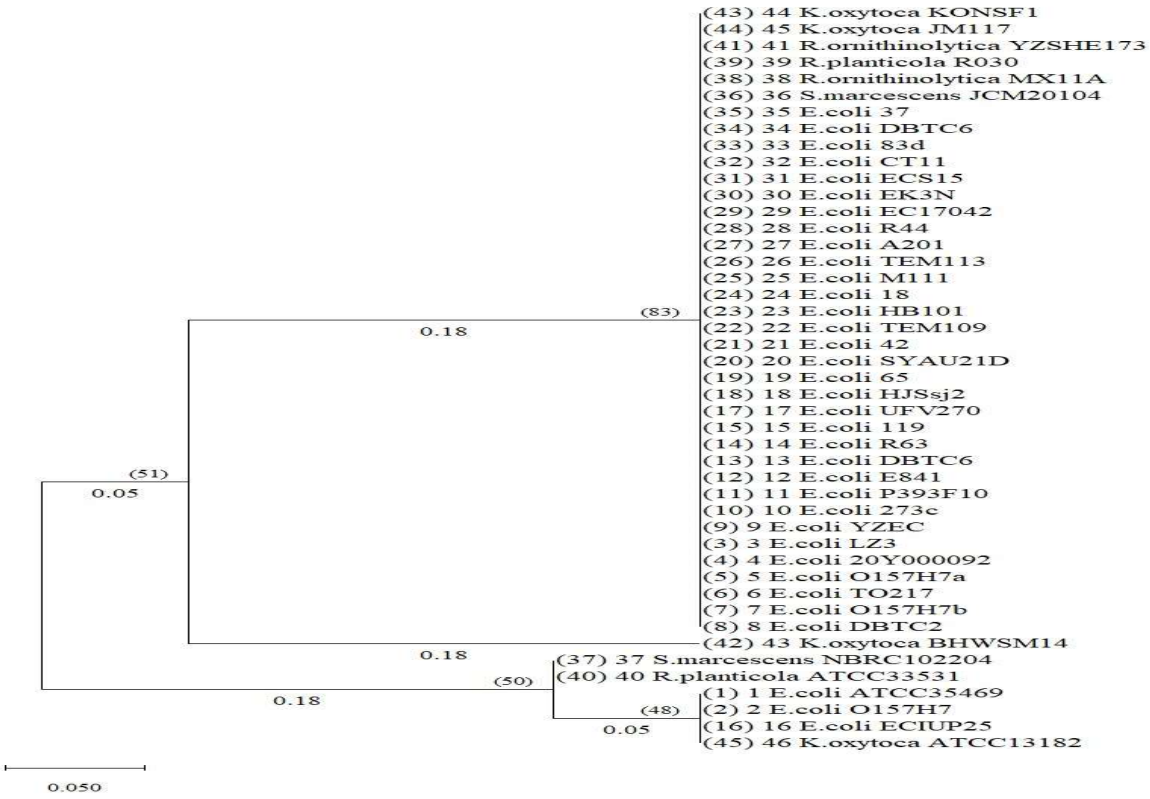

Figure 1. The constructed phylogenetic tree using MSA program with UPGMA from clustering the 42 isolates—including the 11 *E. coli* O157:H7 strains, other *E. coli* isolates with high sequence similarity, and 7 non-*E. coli* strains.

Detection of *E. coli* specific 16s rRNA gene

The *E. coli* specific 16s rRNA gene was amplified using PCR specific priers mentioned in material and methods section. The presence of the specific *16s rRNA* gene of *E. coli* for 92 isolates was searched. The agarose gel electrophoresis results for *16S rRNA* specific gene detection in *E. coli*. Positive bands (~401bp) detection indicating successful amplification of the *16S rRNA* gene in these samples. Which confirm the identification of *E. coli* as a selected isolate.

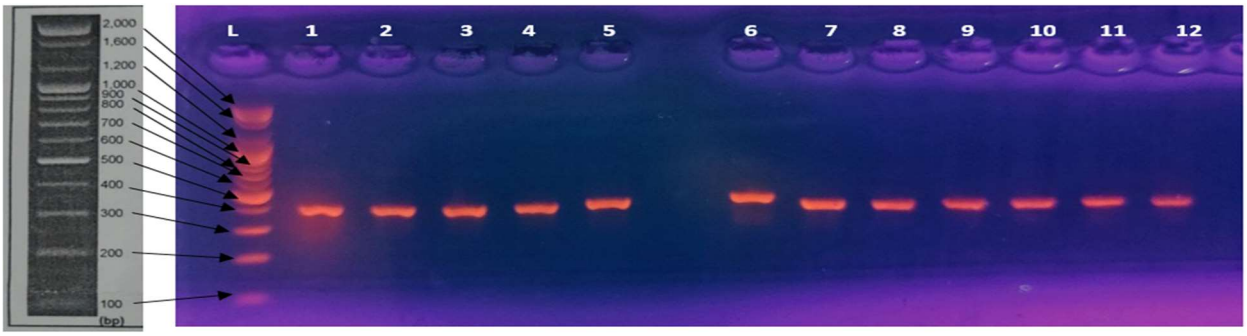

Figure (2): The detection of *E. coli* specific *16s rRNA* using 1.5 % agarose for 60 minutes at 70 V, stained with ethidium bromide, L: DNA ladder 100 basis points, lane 1 ,2, 3 ,4 ,5 ,6, 7, 8, 9, 10, 11 and 12 *16S rRNA* at ~401 bp. As a selected isolate

Among the 11 confirmed *E. coli* O157:H7 isolates, all were positive for the serotype-specific *RfbE* and *FliC* genes, confirming their O157:H7 identity. Eight isolates (72.7%) tested positive for both stx1 (~366 bp) and *stx2* (~282 bp), while three were negative for both genes. Furthermore, six isolates (54.5%) carried the *eaeA* (intimin) gene. Gel electrophoresis confirmed the distinct amplicons of stx1 and *stx2* in the positive isolates, as shown in Figure 3A and 3B. Out of the 92 isolates tested in this study, only 8 yielded amplifications of both *stx1* and *stx2* genes, confirming the correct product size and accurate PCR amplification. These virulence gene clusters were identified in *E. coli* O157:H7 isolates recovered from UTI and diarrheal cases.

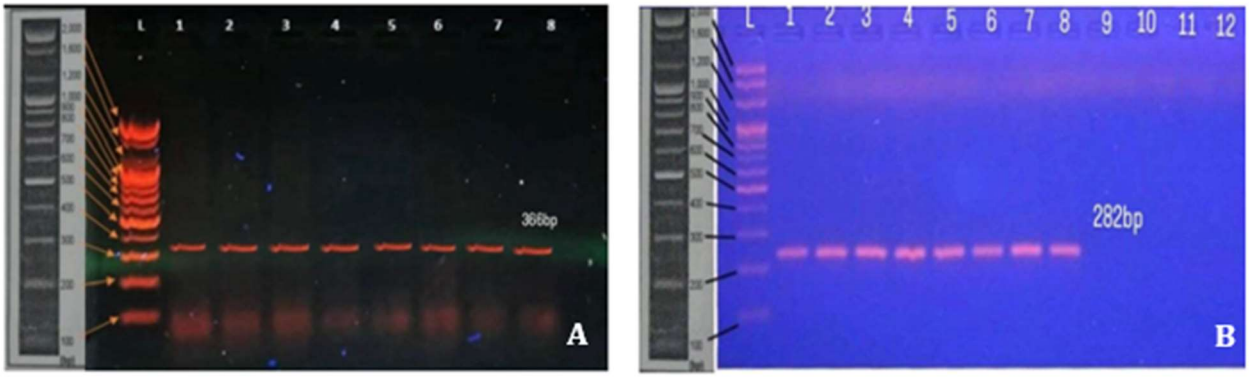

Figure 3. 1.5% agarose gel electrophoresis was performed for the PCR products for 60 minutes at 70 V. **A**: detection of *stx1* gene 366bp, stained with ethidium bromide, L: DNA ladder 100 basis points, lane 1, 2, 3, 4, 5, 6,7 and 8 for *stx1* gene ~366bp. **B**: The detection of *stx2* gene ~282bp using stained with ethidium bromide, L: DNA ladder 100 basis points, lane 1, 2, 3, 4, 5,6, 7 and 8 positive and lane 9, 10, 11, and 12 negative for gene

Table (2): Shows whether five virulence-associated genes (*stx1, stx2, eaeA, RfbE*, and *FliC*)

Bacteria species	*stx1*	*stx2*	*eaeA*	*RfbE*	*FliC*
Escherichia coli O157:H7 (6)	+	+	+	+	+
Escherichia coli O157:H7 (2)	+	+	-	+	+
Escherichia coli O157:H7 (3)	-	-	-	+	+
Escherichia coli other strain (66)	-	-	-	-	-

Detection of the *E. coli O157:H7* strain using serotyping kit

A positive result is indicated by an apparent agglutination in a clear fluid, whereas a negative response is indicated by a homogeneous, turbidity. Eleven out of 23 *E. coli* O157:H7 isolates (47.8%) culture on Sorbitol MacConkey Agar gave a positive result for serotyping. Confirmed the agglutination for (O157:H7).

Antibiotic sensitivity test

antibiotic susceptibility testing Profile of 92 *Escherichia coli* isolates was interpreted according to CLSI 2023 guidelines (27), revealed a clear variation in the response of the isolates to the tested antimicrobial agents, highlighting the extensive effort undertaken to evaluate the resistance and sensitivity profiles. Among non- O157:H7 *E. coli* isolates, the highest resistance was exhibited against amoxicillin–clavulanate (82.6%), followed by tetracycline (62.0%) and azithromycin (59.8%), indicating widespread resistance to these commonly used antibiotics. In contrast, these isolates demonstrated the greatest sensitivity to meropenem (79.4%), followed by levofloxacin (65.2%) and piperacillin–tazobactam (56.5%), underscoring the effectiveness of these agents against most strains. Statistical analysis showed a strong significance for resistance to tetracycline ($p = 0.007$) and amoxicillin–clavulanate ($p < 0.0001$), as well as significant sensitivity to meropenem ($p < 0.0001$), levofloxacin ($p < 0.00001$), and norfloxacin ($p = 0.0001$), while gentamicin, ceftazidime, and piperacillin–tazobactam exhibited non-significant associations. Regarding the 11 *E. coli* O157:H7 strains, resistance patterns were more pronounced. The bacteria exhibited a notably high resistance to tetracycline (85.7%, $p < 0.001$), emphasizing its limited therapeutic utility in these cases, while azithromycin resistance approached statistical significance ($p = 0.059$). On the other hand, *E. coli* O157:H7 showed the highest sensitivity toward meropenem (85.8%, $p < 0.0002$), confirming its robust activity against these strains followed by levofloxacin, while gentamicin, ceftazidime, and piperacillin–tazobactam demonstrated variable but non-significant responses. Further analysis of the 11 *E. coli* O157:H7 isolates harboring the *stx1* and *stx2* genes, including a comparison between the 8 isolates positive for both genes and the remaining gene-negative isolates, revealed no statistically significant differences in resistance or sensitivity patterns across the tested antibiotics.

Discussion

The laboratory analysis of 320 clinical samples revealed a bacterial growth rate of 44.7% (143/320), which is within the globally expected range for suspected infections (30–50%) (29). *Escherichia coli* was the predominant pathogen, representing 64.3% (92/143) of isolates, with a markedly higher recovery from stool (95.0%) than urine samples (42.2%), reflecting its enteric nature and established role in both diarrheal disease (30) and urinary tract infections **(31)**, The lower recovery from urine may also be linked to prior empirical antibiotic use. Preliminary identification was based on culture and biochemical assays: all isolates produced pink lactose-fermenting colonies on MacConkey agar and a metallic-green sheen on EMB, with positive indole and catalase but negative oxidase reactions, consistent with standard diagnostic features (30, 32). While classical assays remain reliable for preliminary screening, they cannot differentiate pathogenic O157:H7 from commensal *E. coli*, necessitating the use of selective and chromogenic media. In this regard, CT-SMAC enhances selectivity through the addition of cefixime and tellurite, which suppress competing Enterobacteriaceae and *Proteus spp.*, thereby improving O157:H7 recovery (17). However, sorbitol-fermenting O157:H7 variants (SF-O157) have been reported and may appear as pink colonies on CT-SMAC, potentially escaping detection if sorbitol fermentation is the sole criterion. A large outbreak in France was attributed to SF-O157 strains (33), and further characterization demonstrated that such strains may harbor virulence genes comparable to classical non-fermenting isolates (34). To further improve diagnostic accuracy, HiCrome *E. coli* O157:H7 agar provided distinct chromogenic differentiation, with O157:H7 isolates appearing pink to mauve and non-O157 producing bluish-green colonies. Studies have confirmed its superior performance compared with conventional media, enhancing presumptive identification and reducing laboratory workload (35, 36). Nonetheless, limitations have been noted: some a typical or stressed O157:H7 strains may display unusual colony colors or be masked by heavy background flora, leading to false negatives (37, 38). Collectively, our findings support a layered diagnostic strategy that integrates MacConkey, EMB, CT-SMAC, and HiCrome agars. This stepwise approach maximizes sensitivity and specificity, minimizes misclassification, and ensures that only phenotypically distinct colonies are subjected to serological and molecular confirmation, thereby increasing the overall reliability of detecting *E. coli* O157:H7. The 16S rRNA sequencing of 84 out of 92 selected bacterial isolates from a total of 143 clinical isolates revealed that 77 belonged to the genus *Escherichia coli*, while 7 were identified as non-*E. coli* species, indicating a limited microbial diversity within the examined clinical samples. This finding underscores the diagnostic efficiency of the culture-based and biochemical screening methods used, which accurately recovered *E. coli* isolates while minimizing misidentification of unrelated taxa. Based on the sequencing results, a representative subset of 42 isolates was selected, comprising: All 11 confirmed O157:H7 isolates**,** the most genetically similar *E. coli* isolates based on 16S rRNA sequences**,** 7 non**-***E. coli* isolates, and a reference O157:H7 strain for phylogenetic comparison. Multiple sequence alignment (MSA) was performed on the 16S rRNA sequences of these 42 isolates alongside with reference strain *E. coli* using Mega 12, to identify nucleotide polymorphisms and improve the accuracy of phylogenetic reconstruction. This step ensured that observed variations in the phylogenetic tree reflected true genetic differences rather than alignment artifacts, thereby strengthening the reliability of the evolutionary analysis.

The resulting phylogenetic tree showed that the 11 clinical O157:H7 isolates clustered closely with the reference strain, sharing more than 99% sequence similarity, confirming their strong genetic relatedness. In contrast, the non-O157:H7 *E. coli* isolates were distributed across several subclades, while the non-*E. coli* isolates formed clearly distinct clades.

Molecular profiling highlighted the genetic diversity within the O157 serogroup. Amplification of the *E. coli*-specific 16S rRNA gene (401 bp) verified bacterial identity and served as an internal control for PCR specificity and DNA integrity (39,40). Assessment of virulence gene in *E. coli* PCR verification utilizing *E. coli* specific *16S rRNA* and variable distribution among isolates. During the initial molecular screening, several isolates yielded no amplification products for the target virulence genes (*stx1* and *stx2*), raising the possibility of PCR failure or insufficient DNA template. Therefore, the *E. coli*-specific 16S rRNA gene was used as an internal control to verify PCR efficiency and DNA extraction quality. All 92 isolates produced positive amplification for the *E. coli*-specific *16S* rRNA gene, confirming the integrity of the bacterial DNA and the validity of the PCR conditions, and indicating that the absence of virulence gene products in some isolates likely reflects their true absence rather than technical errors. Among the O157:H7 isolates, eight (72.7%) harbored both *stx1* and *stx2* genes, known to encode Shiga toxins associated with hemorrhagic colitis and hemolytic uremic syndrome, particularly *stx2* due to its strong cytotoxicity (41, 42). Six isolates (54.5%) carried the *eaeA* gene encoding intimin, an adhesion factor critical for epithelial attachment, while its absence in other isolates underscores intra-serogroup variation and emphasizes the importance of molecular surveillance for accurate risk assessment (43). All 11 O157:H7 isolates tested positive for *rfbE* (327 bp) and *fliC* (247 bp), confirming their serotype identity and supporting the use of these genes in multiplex PCR for accurate strain differentiation (45,46). These results reaffirm that while 16S rRNA is reliable for species-level identification, serogroup- and virulence-specific markers such as *rfbE, fliC, stx1, stx2*, and *eaeA* are indispensable for precise characterization and minimizing false negatives (44).

Finally, the close evolutionary relatedness between *E. coli* and *Shigella flexneri* presents significant diagnostic challenges. The reliance on additional genetic markers—particularly *rfbE, fliC,* and Shiga toxin genes—remains essential for strain-level discrimination in both clinical and epidemiological contexts (45). Serological agglutination remains the confirmatory gold standard for differentiating *Escherichia coli* O157:H7 from other strains. In this study, 11 out of 23 sorbitol-positive isolates (47.8%) showed visible agglutination with commercial antisera targeting O157 and H7 antigens, confirming their identity as O157:H7. This finding aligns with recent evidence that emphasizes the importance of combining selective and chromogenic media with confirmatory serological tests to achieve accurate identification of enterohemorrhagic *E. coli* strains. Bielaszewska *et al*. (46) highlighted that while chromogenic media improve the specificity and speed of O157:H7 detection, they should be complemented by confirmatory assays such as latex agglutination to avoid misidentification. Similarly, Wang *et al*. (47) reported that selective media like CT-SMAC and HiCrome greatly enhance the recovery of Shiga toxin-producing *E. coli* (STEC), but accurate pathotype-level classification requires integration with serological

confirmation. Moreover, Bocanegra-Ibarias *et al*. (48) noted that misclassification of *E. coli* strains can affect the clinical interpretation of Shiga toxin 2-producing isolates, which underscores the necessity of confirmatory steps to guide patient management and outbreak control. This approach is further supported by Paton and Paton (44), who stated that accurate detection of O157:H7 relies on combining culture-based screening with serological and molecular assays to ensure diagnostic precision. Notably, the 11 isolates identified as O157:H7 by serological agglutination were the same isolates confirmed as O157:H7 through 16S rRNA sequencing and phylogenetic analysis, providing strong concordance between phenotypic and molecular approaches. This agreement reinforces the reliability of the stepwise diagnostic strategy applied in this study. However, some studies have highlighted potential limitations of using agglutination as the sole confirmatory method. Gilmour *et al.* (49) reported that certain non-O157 *E. coli* strains expressing cross-reactive surface antigens may yield false-positive agglutination reactions, emphasizing the need to pair serological tests with molecular assays to ensure accuracy (50). Incorporating the agglutination assay in this study minimized such risks by restricting its use to sorbitol-positive colonies and subsequently confirming them with molecular markers, thereby enhancing the diagnostic precision of the overall workflow. This confirmatory step is crucial because accurate detection of O157:H7 is directly linked to implementing effective infection control measures and preventing potential outbreaks (51). Therefore, incorporating the agglutination assay in this study provided a critical confirmation layer that reduced false positives and ensured reliable identification of true O157:H7 strains, directly supporting infection control and epidemiological surveillance efforts.

The antimicrobial susceptibility patterns observed in this study revealed a pronounced divergence between *E. coli* O157:H7 and non-O157:H7 isolates, highlighting an emerging clinical threat. The non-O157:H7 isolates exhibited alarmingly high resistance rates to first-line oral antibiotics, particularly amoxicillin–clavulanate (82.6%), tetracycline (62.0%), and azithromycin (59.8%). These findings are consistent with previous reports from Basrah hospitals documenting widespread resistance among Enterobacteriaceae recovered from diarrheal and urinary tract infections (3, 52). Such high resistance levels are likely driven by the dissemination of plasmid-mediated extended-spectrum β-lactamases (ESBLs) and AmpC β-lactamases, which are increasingly reported in Iraq and the surrounding region (53). Despite this, non-O157:H7 isolates retained high susceptibility to meropenem (78.8%), norfloxacin (67.9%), levofloxacin (65.4%), and piperacillin–tazobactam (58.1%), supporting the notion that carbapenems and fluoroquinolones remain among the most reliable therapeutic options. Similar observations have been reported globally, where carbapenems continue to demonstrate potent activity even against multidrug-resistant Enterobacteriaceae (54). However, overreliance on these last-resort agents could accelerate the emergence of carbapenem-resistant *E. coli* (CRE), a phenomenon increasingly documented in Iraq and other countries (55), underscoring the urgent need for strict antimicrobial stewardship. Interestingly, the O157:H7 isolates showed even higher resistance rates than non-O157:H7 strains, with marked resistance to tetracycline (85.7%), amoxicillin–clavulanate (81.8%), and azithromycin (63.6%), and moderate resistance to piperacillin–tazobactam (54.5%), ceftazidime (50.0%), and gentamicin (45.5%). This suggests that O157:H7 isolates may have

acquired mobile genetic elements carrying both resistance and virulence determinants. Such co-selection mechanisms have been previously reported, where plasmids and pathogenicity islands facilitate horizontal transfer of resistance and virulence genes, promoting the emergence of highly pathogenic multidrug-resistant clones (56). Despite their elevated resistance levels, O157:H7 isolates remained highly susceptible to meropenem (85.8%), norfloxacin (72.7%), and levofloxacin (63.6%), mirroring the susceptibility profile of non-O157:H7 isolates. This finding reinforces the role of carbapenems as the most effective therapeutic option currently available for severe *E. coli* infections, although their use should be carefully restricted to prevent the spread of resistant lineages. Moreover, no statistically significant association was observed between the presence of stx1/stx2 virulence genes and antimicrobial resistance phenotypes among O157:H7 isolates. This supports previous studies showing that virulence and resistance traits can be independently distributed within *E. coli* populations (52, 54, 56). However, the potential future convergence of these traits on the same mobile genetic platforms remains a concern, warranting continuous genomic surveillance. Collectively, these findings reflect a concerning trend: first-line oral antibiotics such as amoxicillin–clavulanate and tetracycline are becoming increasingly ineffective, while carbapenems remain highly active but must be used judiciously. This underscores the urgent need for robust antimicrobial stewardship programs, rapid diagnostic screening, and molecular surveillance strategies to curb the dissemination of multidrug-resistant *E. coli*—particularly the highly pathogenic O157:H7 lineage.

Conclusion

The study confirmed the presence of virulence genes (*stx1, stx2, eaeA, RfbE*, and *FliC*) in *E. coli* O157:H7 isolates using both molecular and serological techniques. Antibiotic susceptibility testing revealed a high level of resistance to commonly used antibiotics, with significant associations between resistance patterns and susceptibility profiles. This finding underscores the urgent need for continuous monitoring and proper antibiotic stewardship. However, the frequency of O157:H7 isolates appears relatively low, which may be due to the limited focus on their routine detection in diagnostic laboratories or the possibility that these bacteria are currently under epidemiological control in the studied population.

Importantly, *E. coli* O157:H7 isolates displayed sorbitol fermentation on CT-SMAC agar, diverging from the classical non-fermenting phenotype typically used for presumptive identification emphasizes the necessity of confirmatory serological and molecular assays (*rfbE, fliC*) to avoid misclassification and to ensure accurate epidemiological surveillance of this highly pathogenic lineage.

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