Isolation and Identification of E. Coli O157:H7 Strains Among Diarrheal Sample in Relation with the Presence of Stx1, Stx2, Hlya and Eaea Genes

Muna Abdulmohsen Abdulmajeed; Nehan Bahaaldden Jafar; Yasir Hamad Hamada
Department of Biology, College of Science, University of Kirkuk- Iraq
Email: nihan@uokirkuk.edu.iq

Abstract
Escherichia coli serotype O157:H7 is a human pathogen that has been linked to meat and meat products, dairy products, vegetables, and water all over the world. The current investigation sought to isolate and identify E. coli O157:H7 strains from diarrheal samples in relation to the presence of shiga toxin genes (stx1, stx2, hlyA and eaeA).A cross-sectional study was carried out in Kirkuk city from November 2021 to April 2022. The study included 327 children with diarrhoea who admitted to Paediatric wards in Paediatric Hospital and Gynaecological and Paediatric (Al-Nasr) Hospital in Kirkuk city. Samples were inoculated on the MacConkey agar media and subculture on Eosin Methylene blue then on sorbitol MacConkey agar to get the pure culture of the microorganisms. The bacteria growing were diagnosed on the basis of color, shape, size, edge and height of the growing colonies, ferment or non-ferment the sorbitol MacConkey agar and further secondary culture were done for isolated E. coli O157:H7 strains for antibiotic sensitivity tests as well as different virulence factors included biofilm formation as well as detection of the presence of shiga toxin genes (stx1, stx2, hlyA and eaeA) by PCR. From total of 327 samples used in this study, E. coli O157:H7 represented 1.83% (6 of 327) and E. coli represented 57.80% (189 of 327). The study showed that all E. coli O157:H7 strains isolated from diarrheal samples were biofilm producers. The study showed that all E. coli O157:H7 strains isolated from diarrheal samples have eaeA and hlyA genes. The study showed that 83.33% E. coli O157:H7 strains isolated from fecal samples have STX1 gene and 16.67% E. coli O157:H7 strains have STX2 gene. The study demonstrated that all E. coli O157:H7 isolated strains were sensitive to Imipenem, Meropenem and Amikacin (100%) and 83.33 sensitive to gentamycin while all isolates were completely resistant to ticarcillin, Aztreonam, Piperacillin and 91.7% of them were resistant to ceftazidime.
Keywords: E. coli; O157:H7; Shiga toxin; Diarrhoea Kirkuk

1. Introduction

The human pathogen Escherichia coli O157:H7 has been linked to a variety of foods and environments around the world, including meat and meat products, dairy products, plants, and water (Abbasi et al., 2020). It has been determined to be a bacterium, and research has shown that it can cause hemorrhagic colitis (Olorunshola et al., 2000). Diarrheal diseases continue to be one of the primary causes of morbidity and mortality among infants and young children, and they are especially common in less developed countries (Abu-Ali et al., 2019). It has been linked to infections caused by E. coli O157:H7, which manifest themselves with symptoms including blood, cramping stomach pain, fever, nausea, and vomiting (Abd Al-Rubaey et al., 2016). Shiga toxin E. coli (STEC) strains consist of multiple serotypes, among which the O157:H7 path type elaborates potent shiga-like toxins. These toxins are implicated in the development of haemorrhagic colitis (HC), which causes bloody diarrhoea and can result in fatal complications in children, the elderly, and people with compromised immune systems. An uptick in the number of Shiga toxin-producing E. coli (STEC) strains has been connected to an increase in the number of (Wang et al., 2013). E. coli O157:H7 produces either one or two distinct types of Shiga-like toxins, stx1 and stx2, which are responsible for the majority of the clinical symptoms associated with these diseases. The production of Shiga-like toxins is one of the most important virulence factors associated with the pathogenicity of E. coli O157:H7. Stx1 is related genetically and structurally to the Shiga toxin, which is produced by strains of Shigella dysenteriae that belong to serotype 1. Despite this, the amino acid sequence of Stx2 is only 56% identical to the sequence of Stx1, which was discovered earlier (Ibarra et al., 2013). The Stxs are AB5 toxins, and the A subunit of each of these toxins is an enzyme. This enzyme depurinates the 28S rRNA, which ultimately results in the death of the target or goal cell. Through the pentameric B moiety, which functions as a mediator, the globotriaosylceramide (Gb3) receptor is linked to the holotoxin (Abdullah et al., 2014). There are multiple strains of E. coli O157:H7, and each one can cause symptoms that range from mild to severe. Differential expression of stx2, which may result in H7 strains, appears to be more responsible for serious difficulties in HUS than strains that simply generate stx1. This may be the case because differential

doi.org/10.31838/hiv22.02.521 Received: 10.06.22, Revised: 23.07.22, Accepted: 20.08.22

expression of stx2 may result in H7 strains. In spite of the fact that there are a lot of in-depth research accessible for the O157 serotype, relatively little is known about the O26, O111, O118, and O121 serotypes (Islam et al., 2016), is a human disease-causing agent that has been traced back to beef and beef products, dairy products, plant life, and water sources all around the world (Abbasi et al., 2020). It has been determined to be a bacterium, and research has shown that it can cause hemorrhagic colitis (Olorunshola et al., 2000). Diarrheal diseases continue to be one of the primary causes of morbidity and mortality among infants and young children, and they are especially common in less developed countries (Abu-Ali et al., 2019). It has been linked to infections caused by E. coli O157:H7, which manifest themselves with symptoms including blood, cramping stomach pain, fever, nausea, and vomiting (Abd Al-Rubaey et al., 2016). Shiga toxin E. coli (STEC) strains consist of multiple serotypes, among which the O157:H7 path type elaborates potent shiga-like toxins. These toxins are implicated in the development of haemorrhagic colitis (HC), which causes bloody diarrhoea and can result in fatal complications in children, the elderly, and people with compromised immune systems. An uptick in the number of Shiga toxin-producing E. coli (STEC) strains has been connected to an increase in the number of (Wang et al., 2013). E. coli O157:H7 produces either one or two distinct types of Shiga-like toxins, stx1 and stx2, which are responsible for the majority of the clinical symptoms associated with these diseases. The production of Shiga-like toxins is one of the most important virulence factors associated with the pathogenicity of E. coli O157:H7. Stx1 is related genetically and structurally to the Shiga toxin, which is produced by strains of Shigella dysenteriae that belong to serotype 1. Despite this, the amino acid sequence of Stx2 is only 56% identical to the sequence of Stx1, which was discovered earlier (Ibarra et al., 2013). The Stxs are AB5 toxins, and the A subunit of each of these toxins is an enzyme. This enzyme depurinates the 28S rRNA, which ultimately results in the death of the target or goal cell. Through the pentameric B moiety, which functions as a mediator, the globotriaosylceramide (Gb3) receptor is linked to the holotoxin (Abdullah et al., 2014). There are multiple strains of E. coli O157:H7, and each one can cause symptoms that range from mild to severe. Differential expression of stx2, which may result in H7 strains, appears to be more responsible for serious difficulties in HUS than strains that simply generate stx1. This may be the case because differential expression of stx2 may result in H7 strains. In spite of the fact that there are a lot of in-depth research accessible for the O157 serotype, relatively little is known about the O26, O111, O118, and O121 serotypes (Islam et al., 2016). The current study aimed to isolate and identify E. coli O157:H7 bacteria among samples of diarrhea in connection to the presence of Shiga toxin genes. This was accomplished by collecting samples of diarrhea (stx1, stx2, hlyA and eaeA).

2. Materials and Method

2.1 study design

A cross-sectional study was carried out in Kirkuk city from November 2021 to April 2022. For the purpose of the study, there were a total of 327 children with diarrhea who were hospitalized to paediatric wards in Kirkuk city's Paediatric Hospital and Gynecological and Paediatric (Al-Nasr) Hospital. This work has been carried out in the laboratories of the Women's and Children's Hospital (Al-Nasr), the Children's Hospital, and the Public Health Laboratory. In order to obtain a pure culture of the bacteria, samples were first inoculated onto media consisting of MacConkey agar, and then subcultured first onto Eosin Methylene blue and last on sorbitol MacConkey agar. Further secondary culture were done for isolated E. coli O157:H7 strains for antibiotic sensitivity tests as well as different virulence factors included biofilm formation as well as detection of the presence of shiga toxin genes (stx1, stx2, hlyA, and hlyB). The bacteria that were growing were diagnosed based on the color, shape, size, edge, and height of the growing colonies. In addition, fermentation or non-fermentation of the sorbito with the use of a genomic DNA purification kit, the DNA of twelve different E. coli O157:H7 bacterial isolates was successfully extracted and purified. The isolates were acquired by culturing the bacteria on chromogenic agar medium. After subjecting the sample to electrophoresis on agarose with a concentration of 1.5 percent and exposing it to ultraviolet light, the results were analyzed. The DNA was observed as tightly packed bands. Every step of the method was carried out in accordance with the manufacturer's instructions and the standard operating procedures of microbiology laboratories.

2.2 Stool sampling

Three hundred and twenty-seven stool samples were taken from children under the age of five who were suffering from acute diarrhea. These children were of both genders. A diarrheal illness is regarded to be acute if it has lasted for less than 14 days, regardless of whether or not it has been accompanied with blood, fever, or vomiting.

2.3 Characterization and Isolation of E. coli O157:H7

All of the samples that were collected were put through an isolation process for E. coli O157:H7 using a conventional culture method (MacConkey agar, Eosin Methylene Blue (EMB), Sorbitol MacConkey agar (SMAC), and chromogenic agar medium with Cefaxime- Tellurite). This process took between 24 and 48 hours and was carried out at a temperature of 37 degrees Celsius (pink pale color). Initial tests for identification were carried out, including Gram staining, catalase, oxidase, and

motility testing. The results of the other identification tests, including the indole test, hemolysis test, citrate utilization test, and urease test, were verified. (Paresh et al., 2013)

2.4 Molecular detection of STEC

E. coli O157:H7 isolates were processed for further confirmation by the detection of virulence factors of E. coli O157:H7 (stx1 (coding shiga toxin1), stx2 (coding shiga toxin2), eaeA (coding intimin), and hlyA (coding hemolysis) by the Polymerase Chain Reaction (PCR) method. This was done after the isolation, biochemical, and serological characterization of (Franz, et al., 2007). Purification of DNA Colonies of E. coli O157:H7 that were negative for sorbitol were chosen for further examination utilizing the PCR method. In order to accomplish this goal, DNA extraction was carried out making use of the DNA extraction kit (ABIOpure, USA) in accordance with the protocol supplied by the company. Quantus Fluorometer (Promega, USA) was used to detect the concentration of extracted DNA. The qualification and of extracted DNA was evaluated by 1.5 percent agarose gel (Promega, USA), and Quantus Fluorometer (Promega, USA) was used to measure the concentration of extracted DNA. The isolated DNA was kept at a temperature of -20 degrees Celsius until the PCR reaction. In this particular investigation, the following primer, which was designed for use with genes, was utilized (AL-saadi;2018). PCR Protocol, Procedure, the stx1, stx2, eaeA, and hlyA genes, each of which has 347, 592, 376, and 167 base pairs (bp), respectively, were amplified by utilizing one pair of specialized primers manufactured by Macrogen in Korea (Table 2). A final volume of 20 L containing approximately 50-100 ng of template DNA was used for the PCR reaction. Following the PCR amplification, an agarose gel electrophoresis was used to verify the existence of the amplified product. The extracted DNA served as the sole basis for determining whether or not PCR was successful. PCR products were put into the system directly. We used a thermal cycler from Thermo Fisher Scientific in the United States to execute the temperature cycling necessary for the PCR reaction. For the PCR product, l.5 % agarose was put directly into each well. After waiting for an hour, the electrical power was turned on at 100 volts and 100 milliamps. DNA travels from the cathode pole to the positive anode pole. Gel imaging technology was used to visualize the bands that were stained with ethidium bromide in the gel.

3. Results

3.1 Distribution of isolated E. coli

A total of 327 samples used in this study, *E. coli* O157:H7 represented 1.83% (6 of 327) and *E. coli* represented 57.80% (189 of 327), Table 1

Table 1: Distribution of isolated E. coli and other culture results of the studied samples

Culture results	Stool samples	
	No.	%
E. coli	189	57.80
E. coli O157:H7	6	1.83
mixed bacteria	125	38.23
No growth	7	2.14
Total	327	100
P. value: 0.001		

The cultural profile is described E. coli O157:H7 growth on sorbitol MacConkey agar, Escherichia coli of the serotype O157:H7 does not ferment sorbitol and as a result generates colorless colonies on MacConkey Sorbitol Agar (plate1). The consequences of development on Chromogenic agar base medium with cefixime tellurite supplement were used, and the results showed that E. coli O157:H7 was present E. coli O157:H7 strains isolates appeared to be a pale pink to mild red tint in their colony appearances (plate2).

Plate 1: E. coli O157:H7 on SMAC agar Plate 2: E. coli O157:H7 on CHROM

3.2 Detection of Biofilm formation

The study showed that all *E. coli* O157:H7 strains isolated from diarrheal samples were biofilm producers as done Congo Red Agar (CRA) method, Plate 3.

plate 3: Congo Red Agar (CRA) showing biofilm of E. coli O157:H7.

3.3 Molecular detection genes (stx1, stx2, eaeA, hlyA) of E. coli O157:H7

The study showed that all E. coli O157:H7 strains isolated from diarrheal samples have eaeA and hlyA gene, 83.33% with STX1 and only 16.67 with STX2 genes, Table 2,3.

Table 2: Detection of gene in of E. coli O157:H7 strains isolated from diarrheal samples.

Genes	Present		Absent		Total	
	No.	%	No.	%	No.	%
eaeA	6	100	0	0	6	100
hlyA	6	100	0	0	6	100
STX1	5	83.33	1	16.67	6	100
STX 2	1	16.67	5	83.33	6	100

Table 3. Specific Primers for Detection of *E. coli* O157:H7 Using PCR.

Primer Name	Sequence 5`- 3`	Annealing Temp. (°C)	Product size (bp)
Stx1-F	AGTTAATGTGGTGGCGAAGG	58	347
Stx1-R	CACCAGACAATGTAACCGC		
Stx2-F	TTCGGTATCCTATTCCCGG	58	592
Stx2-R	CGTCATCGTATACACAGGAG		
eaeA-F	CACACGAATAAACTGACTAAAATG	55	376
eaeA-R	AAAAACGCTGACCCGCACCTAAAT		
hlyA-F	ACGATGTGGTTTATTCTGGA	50	167
hlyA-R	CTTCACGTGACCATACATAT		

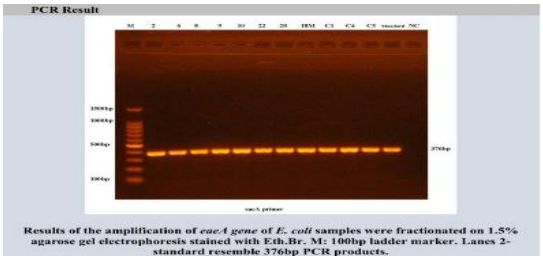

Figure 1: Detection of eaeA gene in of E. coli O157:H7 strains

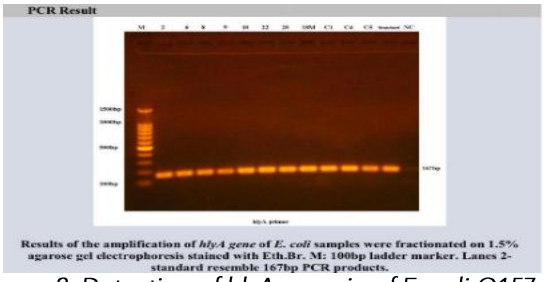

Figure 2: Detection of hlyA gene in of E. coli O157:H7 strains isolates

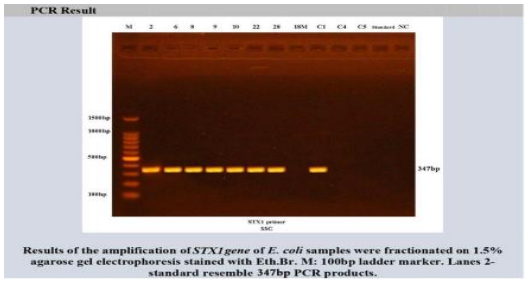

Figure 3: Detection of STX1 gene in of E. coli O157:H7 strains isolates

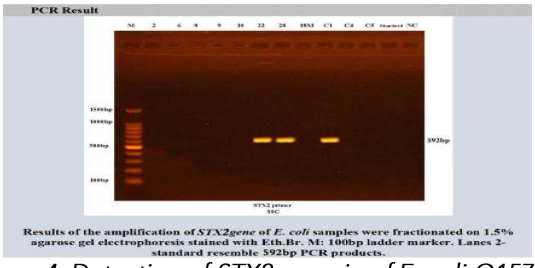

Figure 4: Detection of STX2 gene in of E. coli O157:H7 strains

3.4 Antibiotic susceptibility

The study demonstrated that all *E. coli* O157:H7 isolated strains were sensitive to Imipenem, Meropenem and Amikacin (100%) and 83.33 sensitive to gentamycin while all isolates were completely resistant to ticarcillin, Aztreonam, Piperacillin and 91.7% of them were resistant to ceftazidim, Figure 5,

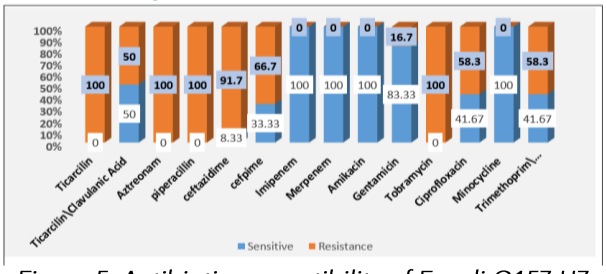

Figure 5: Antibiotic susceptibility of E. coli O157:H7 strains isolated from all samples.

4. Discussion

The majority of E. coli O157:H7 foodborne outbreaks have been connected to the intake of foods originating from cattle, namely foods contaminated with cattle feces. This is especially true for the outbreaks that have occurred in the United States. This is as a result of the fact that E. coli O157:H7 has been discovered in healthy cattle feces on numerous occasions, and cattle are known to be asymptomatic carriers of disease (Abdul-Hussein et al.,2018). The consumption of locally produced cheeses remains at a high level in Iraq, much like it is in other nations. These cheeses, on the other hand, are often produced with milk that has not been pasteurized, which does not meet adequate standards for sanitary quality. A major number of traditional cheeses are made using unpasteurized milk, and these cheeses are either consumed directly after manufacture, or they are aged in salt brine for a period of time before being consumed (Najim and AL-Isawi, 2017).

According to the findings of the research that we carried out on stool samples, E. coli O157:H7 accounted for 1.8 percent (6 of 327), while E. coli accounted for 58 percent of the total (189 of 327). According to Ahmed (2014), who was the one who carried out the research and conducted the study, E. coli O157 was detected in 0.7 percent of the children who presented themselves to a local hospital in Libya with diarrhea. Najma and colleagues (2021) found that the prevalence of E. coli O157 among cases of acute diarrhea in Tunisian children was 1.6 percent. This information was gleaned from their research. While Getaneh et al. (2021) found that the prevalence of diarrhea caused by E. coli O157:H7 was 15.3 percent, we came to different conclusions based on our own observations. The mismatch may have been caused by a number of variables, including differences in the sample size, the source of the research population, and the method used to collect the samples. In general, the relatively high prevalence of the organism obtained in the current finding might be because of the cosmopolitan and high level of animal-to-human interaction in the study subjects than the countries mentioned above, indicating that E. coli O157:H7 is an important diarrhea causing pathogen in the study population. This was determined by comparing the prevalence of E. coli O157:H7 in the study subjects to the prevalence of E. coli O157:H7 in the countries mentioned above. This was established by making a comparison between the prevalence of E. coli O157:H7 among the people who participated in the study and the prevalence of E. coli O157:H7 in the nations that were listed. It was discovered that ETEC and EAEC were the most prevalent infections that could be isolated from children who experienced diarrhea. In point of fact, the ETEC pathotype is recognized as being one of the most important diarrheagenic E. coli in developing countries. This is because it can cause severe bloody or watery diarrhea. In the past, high prevalence rates of ETEC have been found in North Africa, specifically in Egypt, where it has been proven that there is a substantial association with diarrhea (Shaheen et al., 2004). On the other hand, the vast majority of other studies have not revealed any significant connection between ETEC and diarrhea; in fact, ETEC strains have been found among children who are otherwise healthy (Viboud et al., 1999). It has been demonstrated that these differences can be attributed to the fact that ETEC infection rates throughout children vary not only geographically but also over the course of time (Qadri et al., 2005). The findings of our research shed light on the importance of using Chrom agar media in the process of diagnosing E. coli O157:H7. Additional research has produced findings that are in line with those of your study. Bettelheim (1998) made the discovery that the strain of E. coli O157:H7 that causes mauve-colored colonies employs one of the chromogenic substrates. The presence of colonies on Chrom agar O157 that have a mauve colour is viewed as strong proof that the organism being investigated is E. coli O157:H7. Colonies of non-E. coli O157:H7 bacteria might have a natural color if they do not use any of the chromogenic substrates. This is because natural colors are produced by bacteria when they do not use any of the chromogenic substrates. However, if they do use one of the chromogenic substrates, the colonies may have a huge ranging from blue-to-blue green depending on the intensity of the use. According to a number of studies, the percentage of biofilm development among E. coli in general ranges from sixty to seventy percent, whereas the rate of biofilm formation in E. coli O157:H7 can reach more than eighty percent. [Citation needed] (Nejma et al., 2014; Wang et al., 2014). In addition to this A correlation has been shown between the development of biofilm and an increase in the disease-causing capacity of some strains. According to the findings of several investigations, between fifty and seventy percent of the isolates obtained from patients who suffered from recurring infections were biofilm producers (Trabulsi et al., 2002). On Congo Red medium Agar, a single strain of E. coli that is responsible for diarrhea was cultured, as indicated by the results of the qualitative study. This was observed across the board for all E. coli strains (CRA). According to the findings of the research that was carried out by Pragyanet et al. (2016), the CRA method led to the production of inaccurate conclusions regarding the creation of biofilm by Escherichia coli in vitro. STEC infections can now be detected and verified utilizing PCR-based techniques that target the virulence genes (stx1 and stx2). As a result, detection rates have increased in both speed and accuracy (Noll et al., 2015). The aforementioned findings were in line with those discovered in the research that Aljanaby et al., (2017) carried out at Al-kufa Hospital during the months of October 2015 and April 2016 in order to compile their findings. Out of a total of 50 E. coli isolates with severe diarrhea, these results revealed that the (stx1 and stx2) genes were only present in diarrheal specimens, namely in two isolates (11.1 percent) and three isolates (16.6 percent) respectively. Escherichia coli O157:H7, which produces the Shiga toxin: H7 strains are foodborne infectious agents that cause a number of life-threatening diseases, including hemorrhagic colitis and hemolytic uremic syndrome equally in male and female children (Fujii et al., 2015), its pathogenicity is usually linked to a shiga toxins, it producing two phage-encoded cytotoxins called shiga toxins (encoded by the shiga toxin phage), and its pathogenicity is usually linked to Maldonado et al. conducted a study in 2005 that used multiplex PCR for screening clinical and diarrheal E. coli isolates. The researchers found that 84 percent of E. coli isolates tested positive for all four genes. This finding was based on the fact that the researchers screened clinical and diarrheal E. coli isolates (stx1, stx2, eaeA, and hlyA). E. coli is a very active organism; it is capable of horizontal gene transfer, which enhances the genetic diversity of the

organism. This capacity contributes to E. coli's propensity for antibiotic resistance. This can, under certain circumstances, lead to the development of novel pathogenic strains, which have been linked to human gastrointestinal sickness as well as other significant problems (Samie et al., 2017).

Schouten et al. (2004) demonstrated that the eaeA gene is present in all Escherichia coli O157 isolates found on Dutch dairy farms. The results of our study were consistent with those obtained by other researchers who had obtained a high percentage in the past (100 percent). Hassan, (2015) demonstrated that E. coli O157: H7 had resistance to gentamicin, ampicillin, nalidixic acid, and co-trimoxazole; high resistance to ticarcillin, Aztreonam cefotaxime, and ceftazidime; and moderate-to-low resistance to ciprofloxacin, amikacine, ceftriaxone, and imipenem. Additionally, the strain had high resistance The results of this study are consistent with those found in Israa et al. (2014) and Schroeder et al (2002). Antibiotics to which E. coli O157:H7 displayed the following patterns of resistance include cefotaxime (54 percent), amikacine (67.5 percent), ceftriaxone (40.5 percent), ceftazidime (48.6 percent), and ciprofloxacin. (45.9 percent). Antibiotic-resistant strains are the primary source of the formation of antibiotic-resistant strains and the transmission of antibiotic-resistant strains to humans through the food supply chain. Antibiotic-resistant strains are exceedingly difficult to treat with frequently used medications. The improper use of antimicrobial drugs in farming and medicinal procedures involving animals and people is the primary factor contributing to the development of antibiotic-resistant strains as well as their spread (Miryala and Ramaiah, 2019). The development of antibiotic resistance in the bacteria that live in the intestines is becoming an issue that is becoming increasingly relevant for the health of the public all over the world. Because of the extensive use of antimicrobials, the selection of bacterial strains that are resistant to antimicrobials is encouraged, which makes it more difficult to treat bacterial illnesses (Mashak, 2018).

It's possible that the high level of resistance to this drug is due to the fact that it's the antibiotic that's most readily available, and that it's also the one that's most frequently prescribed for treating gastrointestinal infections. Both of these factors contribute to the spread of resistant strains of bacteria. In addition, during the past few years, there has been a growing trend among people purchasing it over the counter in order to self-medicate with it. During the course of this specific experiment, a considerable proportion of E. coli O157:H7 isolates demonstrated multidrug resistance to a number of different antibiotics at variable percentages. This conclusion is in line with the results that were obtained by other researchers, who discovered that E. coli O157:H7 isolates demonstrated multidrug resistance. This conclusion is consistent with the data that were obtained (Abbasi et al., 2021). The rise of antimicrobial resistance in E. coli O157:H7 isolates is a significant obstacle in both human and veterinary medicine. This is due to the extensive use of these antimicrobials in both human and veterinary medicine. This is because these medications are widely used in the treatment of both human patients and animals in veterinary medicine (Stephan et al., 2000). Both imipenem and meropenem were shown to have a high level of sensitivity in this investigation, as the data showed. There is a possibility that this high level of sensitivity is connected to the relatively recent use of this drug in Iraq for the treatment of diseases in both people and animals.

5. Conclusions

The study demonstrated that all *E. coli* O157:H7 isolated strains were sensitive to Imipenem, Meropenem and Amikacin and resistant to ticarcillin, Aztreonam, Piperacillin, The study showed that all *E. coli* O157:H7 strains were biofilm producers as done CRA method. The study showed that all *E. coli* O157:H7 strains have *eae*A gene, 83.33% *E. coli* O157:H7 strains isolated from fecal samples have *STX*1 and that 16.67% *E. coli* O157:H7 strains have *STX*2 gene

6. Acknowledgements

The authors are thankful to staff of the Department of Biology, Faculty of science, Kirkuk University and Public Health Laboratories, IRAQ, for providing necessary research facilities.

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