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Molecular confirmation of some virulence genes of *E. coli* isolated from lambs' colibacillosis

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Abstract

Colibacillosis is an infectious disease caused by pathogenic strains of E. coli, which are considered the most common and significant bacteria that cause dysentery or diarrhea in lambs. The objective of the present inquiry was to determine whether the bacterial isolates under examination possessed the principal virulence genes. The study entailed the cultivation of 65 E. coli isolates, identified through growth on chromogenic agar. Following a 24-hour culture on EMB agar at 37°C, all isolates were subjected to DNA extraction. In the present study, four genes *uidA*, *stx1*, *stx2*, and *eaeA* were amplified by the polymerase chain reaction using gene-specific primers. The current study's findings verified that all 65 isolates were *E. coli* based on the *uidA* gene. Sixty isolates (92.3%) had the stx1 gene, while 47 isolates (72.3%) had the stx2 gene. At the same time, the *eaeA* gene was detected in 17 isolated bacteria (26.15%). A total of 26.15% of the isolates incorporated all three virulence factors. Of the 65 *E. coli* isolates, 64 (98.5%) were enterohemorrhagic *E. coli* (EHEC). The enteropathogenic *E. coli* (EPEC) isolates did not exist in the current study. To conclude, colibacillosis is the most frequent and significant cause of dysentery and diarrhea in lambs in Mosul. The isolated bacteria exhibited a high level of virulence, represented by the presence of genes encoding Shiga toxins and Intimin. The highlight must be attention to the possibility of implicated of lambs as a reservoir for these zoonotic bacteria that cause several syndromic illnesses in humans.

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Introduction

Neonatal diarrhea and enteritis are the most common gastrointestinal disorders in newborn lambs, especially in lambs within the first month of age. Weakness, dehydration, and frequent watery stools are their hallmarks. In severe and advanced cases, high mortality is more common (1). Colibacillosis is the most significant disease in the first few weeks of lambs' age, caused by pathogenic *Escherichia coli*; this leads to neonatal diarrhea and/or septicemia and, finally, a high mortality rate, particularly in conventional farms. It deforms growth, health disorders, and immunity, resulting in great economic losses due to cost and less effective treatment (2). Enteropathogenic *E. coli* (EPEC) and Enterohemorrhagic (EHEC) are the main types of *E. coli* that cause colibacillosis in lambs, kids, and other ruminants (3). Enterohemorrhagic strains produce highly potent Shiga toxins, so these types of *E. coli* are named Shiga toxin-producing *E. coli* (STEC). Enterohemorrhagic *E. coli* produces at least one kind of Shiga toxin, differentiated biochemically and molecularly and called *Sxt1* and *Sxt2* (3,4). STEC is the most prevalent bacterial population that can infect humans. Humans can contract STEC through fecal-oral routes by consuming tainted food or water, as ruminant animals are the pathogen's natural reservoirs (5). Small ruminants are a significant carrier of STEC for many hosts, including humans (6), as such, recognition of STEC in sheep is crucial due to their role in human infections. EHEC

and EPEC strains can also both harbor the intimin gene (*eae*A), which is one of the virulence factors implicated in attachment and biofilm production (7).

Because there has been little molecular research on colibacillosis and the molecular diagnosis of its causative agent in lambs in Iraq in general, and particularly in Mosul city, the aim of this study was the molecularly diagnose *E. coli* causing colibacillosis and investigate some of its virulence genes responsible for the production of disease in lambs within the region.

Materials and methods

Ethical approve

The Institutional Animal Care and Use Committee issued numbered UM.VET.2024.041 dated 9/7/2024 licensed this study.

Conventional diagnosis of isolates

In a prior investigation conducted by the same researchers (8), 65 *E. coli* isolates were derived from 67 rectal swabs of lambs from diverse localities that were obtained and analyzed for bacterial isolation. The isolated bacteria were identified by another study through their growth on some selective culture media, specifically MacConkey's broth, MacConkey's agar, and Eosin Methylene Blue. Then, the detection of *E. coli* was done depending on different biochemical tests, including Indole, Methyl Red, Voges-Proskauer, and Citrate (IMVC) (9). The diagnosed *E. coli* isolates were cultivated on the chromogenic agar (Hicrom^{TM} *E. coli* agar-Himedia, India) to differentiate Shiga toxin-producing from non-producing *E. coli* colonies by making greenish-blue colonies (according to manufacturing instructions).

DNA extraction

All isolates were cultured on EMB agar for 24 hours at 37°C, and then the bacterial DNA was extracted depending on the manufacturer's protocol of the extraction kit (AddBio/Korea). The extracted DNA was kept at -80°C, and used for subsequent steps.

Determining the DNA purity and concentration:

The concentration and purity of DNA of the isolates were analyzed by Implen NanoPhotometer (model N50, Germany). The purity of the DNA was evaluated at A260/A280 and A260/A230 (10-12).

Primers used in PCR

Four pairs of gene-specific primers (*uidA, stx1, stx2,* and *eaeA*) produced by Macrogen- Korea were used in the present study (13). The *uidA* primer pair was used for the molecular confirmation of *E. coli* isolates (14) with an amplicon size of 623bp. The three other primer pairs were used to differentiate between EPEC and EHEC (15,16) (Table 1).

Table 1: Sequences of primers used in the study

Primer name	Primer sequences (5 to 3)	Amplicon size	Source
uid A	F: CCA AAA GCC AGA CAG AGT R: GCA CAG CAC ATC AAA GAG	623 bp	(17)
stx1	F: AAA TCG CCA TTC GTT GAC TAC TTC T R: TGC CAT TCT GGC AAC TCG CGA TGC A	366 bp	(18)
stx2	F: CGA TCG TCA CTC ACT GGT TTC ATC R: GGA TAT TCT CCC CAC TCT GAC ACC	282 bp	
eaeA	F: TGC GGC ACA ACA GGC GGC GA R: CGG TCG CCG CAC CAG GAT TC	629 bp	(19)

Amplification program

The amplification mixture consisted of master mix (12.5 µL Promega, USA), F-primer (10 pmol) (2.5 µL), R-primer (10 pmol) (2.5 µL), DNA template (5 µL), and PCR water (up to 25 µL). Amplification processes were done according to the specific program for each gene (Tables 2-4).

Agarose gel electrophoresis

The agarose gel was prepared as described by Green and Sambrook (20). Briefly, 100 ml of 1X TBE (Promega, USA) was transferred into a beaker, and 2g of the agarose powder was mixed with the buffer and heated in a microwave oven for complete dissolution. The mixture was allowed to cool to 50-60°C, and 5 microliters of safe dye (AddBio) was added. The mixture was poured into the casting tray and the comb was placed at its place to make wells. The agarose solution was allowed to solidify at room temperature. Then, the comb was removed carefully, and the gel tray was put into an electrophoresis chamber, filled with 1X TBE buffer until the buffer level reached up to 3-5 mm above the surface of the gel. Afterward, a mixture of 3µl of amplified DNA (PCR product) and 9µl of Promega loading dye was loaded into all wells except one of them, which was loaded with a 100bp DNA marker (Promega, USA). Then, the agarose gel electrophoresis was run for 1 hour at 80V until the DNA bands were migrated, and finally, the bands were visualized and photographed by using a UV transilluminator.

Table 2: Amplification program of the *uidA* gene

Stage	Step	No. of cycles	Time	Temperature
First	Initial denaturation	1	3 min	94
Second	Denaturation	30	1 min	94
	Annealing		40 sec	57
	Extension		1 min	72
Third	Final extension	1	3 min	72

Table 3: Amplification program of the *stx1* and *stx2* genes

Stage	Step	No. of cycles	Time	Temperature
First	Initial denaturation	1	4 min	94
Second	Denaturation	35	30 min	94
	Annealing		45 sec	56
	Extension		1 min	72
Third	Final extension	1	5 min	72

Table 4: Amplification program of the *eaeA* gene

Stage	Step	No. of cycles	Time	Temperature
First	Initial denaturation	1	3 min.	94
Second	Denaturation	35	1 min.	94
	Annealing		45 sec.	57
	Extension		1 min.	72
Third	Final extension	1	3 min.	72

Results

Bacterial isolates

Of the 65 pure E. coli isolates cultivated on HiCrome™ E. coli agar, 52 isolates (80%) exhibited a greenish-blue coloration, while 13 isolates (20%) displayed a white coloration (Figure 1).

Figure 1: Isolates of *E. coli* grown on chromogenic agar.

Concentration and purity of DNA samples

The concentrations of DNA measured for 65 samples ranged from 23.85 ng/µl to 241.60 ng/µl. The samples had a 260/280 absorbance ratio between 1.89- 2.087. Whereas at A260/A230, the samples ratio ranged from 1.92-2.086 (Figure 2).

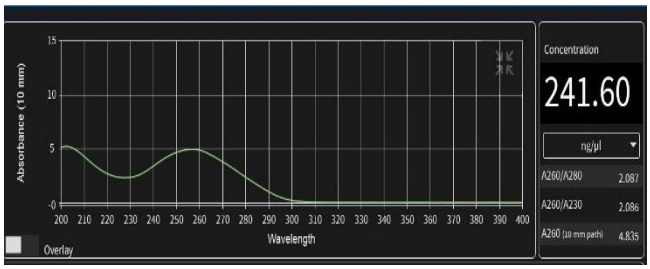

Figure 2: Determination of DNA samples concentration and purity.

Confirmation of diarrheagenic *E. coli* in lambs using PCR

All 65 isolates were confirmed as *E. coli* by detection of the *uidA* gene using the PCR technique. The size of products was 623bp (Figure 3).

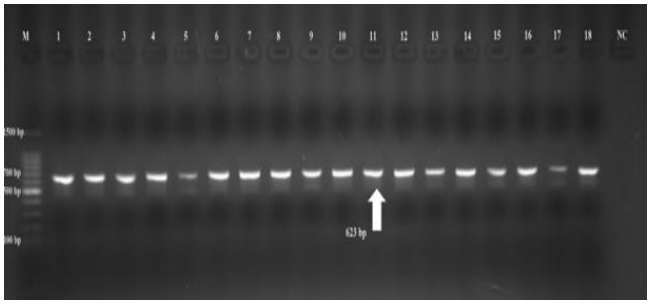

Figure 3: Confirmation of *E. coli* isolates by amplification of the *uidA* gene. Agarose gel electrophoresis (2%) confirms the *E. coli* isolates and shows bands of approximately 623 bp as the expected size of the *uidA* gene partially amplified by the PCR. M: 100 bp DNA marker, lanes 1-18 positive PCR products, and lane NC: negative control.

Detection of virulence genes

All *E. coli* isolates that were confirmed molecularly were tested to detect the presence of virulence genes in this study, which are *stx1*, *stx2,* and *eaeA* genes, using molecular techniques. Sixty *E. coli* isolates 92.3% bore the *stx1* gene with a size of PCR products of 366 bp (Figure 4), and 47 (72.3%) isolates had the *stx2* gene with a size of products of 282 bp (Figure 5). At the same time, 17 isolates (26.15%) showed the existence of the *eaeA* gene with a size of products of 629 bp (Figure 6). According to the above results, 64 out of 65 isolates of *E. coli* were EHEC 98.5% due to the presence of *stx1* or *stx2*, or both of them, with or without the *eaeA* gene. In contrast, one isolate 1.5% lacked the three virulence factor genes (Table 5). According to the results, EPEC isolates did not exist in the current study (Table 5).

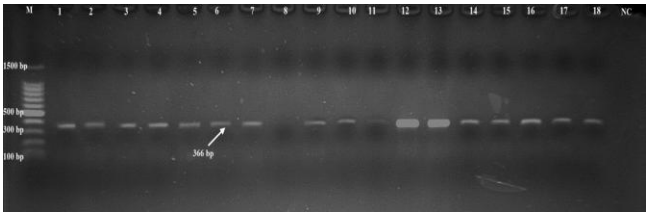

Figure 4: Confirmation of the presence of the *stx1* gene. Agarose gel electrophoresis 2% confirms the *E. coli* isolates and shows bands of approximately 366bp as the expected size of the *stx1* gene partially amplified by the PCR. M: 100 bp DNA marker, lanes 1-7, 9-10, 12-18 positive PCR products; 8, 11 negative PCR products, and lane NC: negative control.

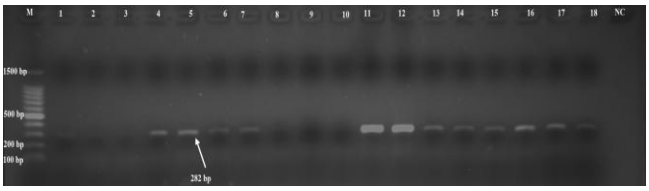

Figure 5: Confirmation of the presence of the *stx2* gene. Agarose gel electrophoresis 2% confirms the *E. coli* isolates and shows bands of approximately 282bp as the expected size of the *stx1* gene partially amplified by the PCR. M: 100 bp DNA marker, lanes 4-7, 11-18 positive PCR products; 1-3, 8-10 negative PCR products, and lane NC: negative control.

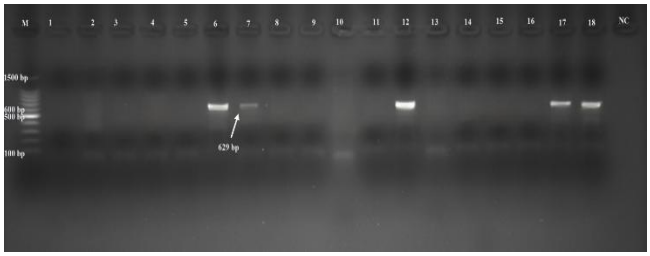

Figure 6: Confirmation of the presence of the *eaeA gene*. Agarose gel electrophoresis 2% confirms the *E. coli* isolates and shows bands of approximately 629bp as the expected size of the *stx1* gene partially amplified by the PCR. M: 100 bp DNA marker, lanes 6-7, 12, 17-18 positive PCR products; 1-5, 8-11, 13-16 negative PCR products, and lane NC: negative control.

Linking between molecular diagnosis and phenotypic diagnosis of EHEC (STEC) and EPEC isolates

The phenotypic results based on the growth on HiCrome™ *E. coli* agar did not relate to the molecular results, except in diagnosing the isolates as *E. coli*. The second clear benefit was the identification of isolates carrying the gene *Stx2* alone or with other genes (Table 6).

Table 5: Classification of EHEC isolates according to the presence of the *stx1*, *stx2*, and *eaeA* genes

No. of isolates	*Stx1*	*Stx2*	*EaeA*	EHEC %	EPEC %
26	X	X	-----	40%	-
17	X	X	X	26.15%	-
17	X	-----	-----	26.15%	-
4	-----	X	-----	6.2%	-
1	-----	-----	-----	-----	-
Total ratio/65	60 (92.3%)	47 (72.3%)	17 (26.15%)	64 (98.5%)	0 (0.0%)

X letter meaning the isolate bearing the gene.

Table 6: Linking between molecular diagnosis and phenotypic diagnosis of EHEC

Virulence genes	Isolates (n)	%	Greenish blue	%	White	%
Stx1+Stx2	26	40%	26	100	0	0
Stx1+Stx2+eaeA	17	26.15%	17	100	0	0
Stx1	17	26.15%	5	29.4	12	70.6
Stx2	4	6.2%	4	100	0	0
Without virulence genes	1	1.5	0	0	1	100

Discussion

Diarrhea in lambs is caused by several diseases, which lead to great economic losses. Colibacillosis is the most common and important cause of dysentery or diarrhea, and it is a bacterial disease caused by pathogenic E. coli in newborn animals, mainly lambs, within 1-3 days after birth, and may also occur in older ages and around the time of weaning (20,21). The results of the existing study revealed that the extraction process was fantastic since concentrations

of measured DNA samples were from 23.85 ng/µl to 241.60 ng/µl, with a range of 87.36 ng/µl, and consistent with the scientific articles, the concentration up to 20 ng/µl is very satisfied for amplification (22). Kapa Biosystems (23) indicated that isolated DNA quantities ranging between 10 and 100 ng/µl are necessary for PCR investigation. The absorbance ratio A260A280 for purity indicated that the samples had a ratio ranging from 1.89 to 2.087, classifying them as pure samples. Whereas the A260/A230 ratio of the samples varied from 1.92 to 2.086, indicating exceptional purity. In general, the results of DNA extraction are considered satisfactory if the purity value falls between 1.8 and 2.0, and the concentration exceeds 20 ng/µl (24,25). One of the first steps in DNA testing that is crucial to the effectiveness of PCR testing is extraction. This supports the assertion made by Fulton et al. (26) and Joshi et al. (27) that the success of PCR analysis depends on DNA extraction. Ibrahim (28) states that in order to move on to the amplification stage of PCR analysis, high-quality and high-quantity DNA extraction findings are required, because in the PCR amplification process, the enzyme functions slowly when contaminating substances like polysaccharides and polyphenols are present in the extracted DNA (29).

All isolates under investigation were confirmed as *E. coli* according to the amplification results of the *uidA* gene, which is a typical species-specific gene sequence and unique to *E. coli*, so it is the perfect gene for the diagnosis of *E. coli* (30,31). Numerous studies, local and universal, have relied on the *uidA* gene for molecular confirmation of *E. coli* isolates associated with various infections in animals, humans, and foodborne illnesses (30-32).

This work indicates that the distribution of virulence fac tors among *E. coli* isolates is intricate, with a notably high i ncidence of Shiga toxins, highlighting significant public he alth risks and potential economic losses. Depending on the current results, 92.3% of the isolates contained the *stx1* gene, and 72.3% of the isolates exhibited the *stx2* gene. At the same time, 26.15% of the isolates showed the existence of the *eaeA* gene. In contrast, 1.5% of isolates were negative to the three virulence genes. The presence of *stx1*, *stx2* alone or together, and with or without the presence of *eaeA*, is the leading marker for virulence in STEC, which plays a crucial role in the occurrence of colibacillosis in lambs, so that 98.5% of the isolated *E. coli* in the present study were classified as EHEC isolates because any *E. coli* strain that is positive for *stx1* and/or *stx2* and for *eaeA* is referred to as EHEC (33,34). EHEC isolates are the main causes of colibacillosis in lambs (30-35). Concerning EPEC isolates, they did not exist in the current study.

Shiga toxins affirm the significant pathogenicity of *E. c oli*, resulting in intestinal damage that causes diarrhea and e nteritis in infected lambs. This injury mainly damages the endothelium of the blood vessels, leading to thrombosis and pathological activation of intravascular coagulation by preventing endothelial and other cells from synthesizing proteins (36-38). The two forms of Shiga toxins have different levels of toxicity and clinical effects. For example, in animals, Shiga toxin 2 was found to be about 100 times more toxic than Stx1. For instance, studies revealed that Stx2 is 40-400 times more effective than *Stx1* in infected mice. This high potency is linked to *Stx2*'s A1 subunit, which exhibits more ribosome attraction and catalysis than *Stx1* (39,40). Thus, the existence of both virulence factors makes the strain more virulent.

The *eaeA* gene encodes Intimin, which promotes attachment to enterocytes and produces attaching and effacing (A/E) lesions in the intestinal mucosa (7,41). Therefore, even though not all isolates harbored both *Stx1* and *Stx2* genes and the majority of them lost the *eaeA* gene, the data point to the high frequency of Shiga toxin-producing *E. coli* in lambs in Mosul city. Consequently, excessive attention should be paid to these high rates and their prevalence among lambs, which represent one of the most important food sources for humans in this city, in particular, and across the country in general. For instance, but not exclusively, to illustrate the significance of these STEC isolates' transmission through food sources, 4,769 people were impacted by 466 documented STEC outbreaks between 2010 and 2017, of which 3,353 cases were deemed foodborne in the US (42).

Compared to a previous study conducted in Nineveh Governorate (43), where they found that the *stx1* and *stx2* genes were present in all isolates, the present study showed that a high percentage 40% of the isolates carried both the Shiga toxin genes (*stx1* and *stx2*), a large percentage of the isolates 26.15% expressed *stx1* only, with a low prevalence of isolates 6.2% expressed *stx2* gene alone. There are also differences between the results of the existing study and another local study that was carried out in Kirkuk province, where the *stx1*, *stx2*, and *eaeA* genes were identified in 64.28%, 67.85%, and 100% of the isolates, respectively (44). The findings of the current study revealed the prevalence of *stx1* in the isolates, followed by *stx2* and *eaeA*. The observed discrepancies are ascribed to sample variability; the current study focused on colibacillosis cases in lambs, whereas the previously mentioned local studies utilized human feces and food samples, and other research encompassed general diarrhea cases, as well as samples from sheep pens, workers' hands, and animal products.

Numerous global studies (45-47) recorded the Shiga toxin and Intimin separately or together as virulence factors in the isolated *E. coli* strains. A study conducted in southern Brazil identified the *eae* gene and categorized the isolates as EPEC strains in 19.2% of the sheep investigated, with positivity observed exclusively in lambs (45). Another article in Argentina (46), after determining the presence of *stx* and *eaeA*, the isolated *E. coli* was classified as STEC and EPEC strains. EPEC carriers were detected in 50% of the farms evaluated, while STEC carriers were found in all of the farms. The risk associated with the farm-to-table food supply

should not be ruled out. In Iran, according to a study, 4.48% of the isolates were EPEC, while 40.34% of the isolates belonged to STEC. The high incidence of STEC suggested that diarrheal lambs are a significant human reservoir (47). Furthermore, in a Turkish study 48.7% of the *E. coli* isolates from diarrheal lambs and goat kids were identified as Shiga toxigenic, 30.8% as enterotoxigenic, and 20.5% as enteropathogenic. These three pathotypes were found to be highly prevalent in the western region of Turkey (48). The cause for the discrepancy in the findings of the aforementioned research papers could be the age of the animal, the sample size and source, geographic and environmental conditions, and local pathogen load (49-51).

The correlation between phenotypic identification on Chromo agar and molecular identification for differentiating STEC from EPEC isolates in the present study was inconclusive, as stx1 isolates exhibited both greenish-blue and white colonies. Consequently, the cultivation of *E. coli* isolates on Chromo agar has not definitively distinguished between Shiga toxin-producing and non-producing isolates. The only notable outcome was with *stx2* isolates that appeared greenish blue colonies. The cultivation on chromo agar is unreliable for differentiating *E. coli* isolates from other Enterobacteriaceae, as 20% of isolates confirmed molecularly as *E. coli*, based on the presence of the *uidA* gene, manifested as white colonies. This phenomenon may be due to the lack of the β-D-glucuronidase (GUD) enzyme, which imparts a greenish-blue hue to colonies and is produced by 94-96% of *E. coli* strains (52).

Conclusions

In conclusion, colibacillosis is the most common and important cause of diarrhea or dysentery in lambs in Mosul city. The isolated bacteria are highly virulent, possessing virulence factors represented by the occurrence of the Shiga toxin genes in addition to the presence of Intimin as an adhesion factor in some isolates. This raises the likelihood that lambs serve as a reservoir for these zoonotic germs, which cause many syndromic disorders in people, particularly in youngsters. This study confirms colibacillosis as a major disease in lambs, primarily caused by pathogenic *E. coli* isolates carrying virulence genes. EHEC (STEC) strains dominated in lambs in Mosul city, with Shiga toxin genes (*stx1*/*stx2*) as key virulence factors, while the *uidA* gene proved ideal for species confirmation. The *eaeA* (intimin) gene contributed significantly to pathogenicity, though its absence in some isolates suggests alternative mechanisms.

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Conflicts of interest

Regarding the research data and instruments utilized in this work, the authors declare that there is no conflict of interest.

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التأكيد الجزيئي لجينات ضراوة الإيشريكيا القولونية المعزولة من داء العصيات القولونية في الحملان

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الخلاصة

داء العصيات القولونية، وهو مرض جرثومي تسببه جراثيم الإيشريكيا القولونية الممرضة، وهو المسبب الأكثر شيوعًا وأهمية للزحار أو الإسهال لدى الحملان. هدف هذا البحث إلى تحديد ما إذا كانت العزلات البكتيرية قيد الدراسة تمتلك جينات الضراوة الرئيسية. تضمنت الدراسة زراعة ٦٥ عزلة من الإيشريكيا القولونية، تم تحديدها من خلال نموها على أجار كروموجيني. بعد زراعتها لمدة ٢٤ ساعة على أكار EMB عند درجة حرارة ٣٧ درجة مئوية، خضعت جميع العزلات لاستخراج الحمض النووي. في هذه الدراسة، تم تضخيم أربع جينات *eaeA* و *stx1* و *stx2* و *uidA* بواسطة تفاعل البوليميرات المتسلسل باستخدام بادئات خاصة بالجين. وقد أكدت نتائج الدراسة الحالية أن جميع العزلات ٦٥ كانت للإيشريكيا القولونية. اعتمادا على الكشف عن جينات الضراوة، احتوت ستون عزلة (٩٢,٣%) على جين*stx1* ، بينما احتوت ٤٧ عزلة (٧٢,٣%) على جين *stx2*. وفي الوقت نفسه، احتوت ١٧ عزلة (٢٦,١٥%) على جين *eae*A. واحتوت ٢٦,١٥% من العزلات على عوامل الضراوة الثلاثة. ومن بين ٦٥ عينة من الإيشريكيا القولونية، كانت ٦٤ عزلة (٩٨,٥%) مصنفة كعترات الإيشريكيا القولونية النازفة للأمعاء. ولم تكن عترات الإيشريكيا القولونية الممرضة للأمعاء EPEC موجودة في الدراسة الحالية. وباختصار، فإن داء العصيات القولونية هو المسبب الأكثر شيوعًا وأهمية للزحار أو الإسهال في الحملان بالموصل. وأظهرت الجراثيم المعزولة مستويات عالية من الضراوة، والتي تمثلت في ذيفانات الشيغا والإنتيمين. ويجب تسليط الضوء على إمكانية تورط الحملان كخازن لهذه الجراثيم التي تعتبر حيوانية المنشأ وتسبب العديد من الأمراض المتلازمة لدى البشر.

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