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Molecular characterization and phylogenetic analysis tree of Shiga toxin-producing *Escherichia coli* (STEC) from local Basturma in Mosul city, Iraq

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Abstract

Escherichia coli (*E. coli*) stands out as the primary microorganism transmitted through food, having a variety of virulence factors, including *uidA*, *Stx1*, *Stx2*, and *Rfb*, which can cause food poisoning in people. One serotype of the bacterial species *E. coli* that is well-known for its capacity to generate a toxin similar to Shiga is *E. coli* O157:H7. It is a source of illness, frequently linked to foodborne infections brought on by consuming tainted and uncooked food, like raw milk and undercooked ground beef. Basturma is a traditional meat product crafted to preserve meat in Iraq. Forty-five samples of locally produced Basturma were collected from a variety of local stores across different regions in Mosul city. The study period extended from April 2023 to June 2023. The results of our study showed that the classical microbiology methods and PCR methods concurred in identifying *E. coli* in local Basturma samples at a rate of 6 out of 45 (13.4%). All *E. coli* isolates were found to possess the specific-species *uidA* gene (100%), with a molecular weight of 623 bp. The PCR analysis further revealed that 1 out of 6 isolates (16.7%) carried the *Stx1* gene with a molecular weight of347 bp, while none of the *E. coli* isolates harbored the *Stx2* and *rfb* genes. All isolates from Basturma were not *E. coli* O157:H7. There is a high similarity between *E. coli* isolated from Bab Al-Tob and Ras Al-Jada. Furthermore, the E. coli isolated showed a close relationship with *E. coli* isolated from different countries in the world.

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Introduction

Escherichia coli (*E. coli*) stands out as the predominant microorganism transmitted through food, causing various diseases that can result in human fatalities (1). *E. coli* contains a range of virulence factors, including genes like *uidA,* Shiga toxin 1 and 2 (*Stx1*, *Stx2*), and *Rfb* (2). The *uidA* gene in *E. coli* encodes the β-glucuronidase enzyme, which plays an important role in hydrolyzing glucuronides into glucuronic acid, while Shiga toxin-producing *Escherichia coli* (STEC) is known as a minor global problem worry among foodborne pathogens associated with meat, this is due to reported outbreaks and isolated instances of STEC infections occurring worldwide (3). STEC has the potential to induce various health issues, including different types of diarrheas, hemolytic uremic syndrome, thrombotic thrombocytopenic purpura, and renal failure (4). *E. coli* O157:H7, which produces Shiga toxin, is a significant strain of *E. coli* that can cause a range of diseases in both humans and animals (5). *E. coli* O157:H7 has the specific-species *Rfb* gene, and *E. coli* O157:H7 can synthesize the Stx1 and Stx2 toxins, which are responsible for causing food poisoning in consumers (6). Beef meat products have been recognized as the primary sources of reported foodborne disease outbreaks associated with *E. coli* O157:H7 (7). The meat containing the pathogenic bacteria can exist at any stage of the food chain, spanning from the slaughtering process to the consumer (8). While healthy animals and their products are generally regarded as sterile under typical conditions, certain procedures conducted during the processing in the

slaughterhouse have the potential to introduce microbial contamination; these processes may lead to contaminated meats and instruments with pathogenic bacteria (9). In addition, beef meat products may be exposed to STEC contamination during the synthesis process and become the reservoir of STEC, which causes risks to humans (10). A common technique for attributing the human disease burden caused by food-borne illness to particular origins is the "microbiological approach." this method entails isolating the pathogen from both the food and infected humans (11). The safety of raw cured fermented sausages relies on several factors, including the incorporation of salt and nitrite, the process of drying that affects the amplification of bacteria on the meat, and storage at a cold or moderately low temperature for a specified period, allowing for the desired curing effect (12). The acknowledgment of dry fermented sausages as a probable hazard to food care has prompted certain lands to implement regulations aimed at reducing associated risks (13). Many studies emphasize the importance of fermented meats as a notable source of disease occurrences (14). Basturma, an Iraqi meat product, is extensively produced in various regions of the country, with notable production in Mosul city, located in the Nineveh Governorate. This product shares similarities with Turkish dry-fermented sausage, which is heavily smoked, and Spanish dry-fermented sausage. These products, whether partially dried or smoked, are recommended for consumption only after undergoing heat processing, such as frying or grilling (15). Numerous researchers have redirected their focus towards the utilization of additives, such as acidulants and other substances, in the processing of meat trimmings intended for ground meat. This approach serves as a method to prevent contamination of the final ground meat product (16).

The goals of this study included isolating and identifying *E. coli* from Basturma, emphasizing the specific risks linked to raw cured fermented meats and other primary product kinds., and detecting the presence of the *Stx1* and *Stx2* genes, which cause food poisoning using the molecular biology technique.

Materials and methods

Ethical approval

The study was conducted in accordance with the ethical standards set by the Institutional Animal Care and Use Committee at the University of Mosul, College of Veterinary Medicine. All samples were collected with the owners' consent under the approved ID: UM.Vet.2024.066.

Samples collection

The primary focus of our research centered on the city of Mosul in Iraq, encompassing various neighborhoods in Mosul city, over the period spanning from April 2023 to June 2023. A total of 45 samples of locally produced Basturma were collected from a range of local stores, including 8 samples from each region (First Qadsia, Ras Al-Jada, Bab Al-Tob, and Al-Zahra) and 5 samples from Al-Tahrer area). These Basturma were meticulously gathered in sterile containers and subsequently placed in containers containing peptone water. Following this, a pre-enrichment process was carried out at 37°C for 18-24 hours to prepare samples for analysis. Subsequently, these samples were carefully transported in an icebox to the Research Center and Laboratories at Mosul University.

Isolation and identification *of E. coli*

The Basturma samples underwent a rigorous analysis to isolate and identify *E. coli*. All samples were cultured for 24 hours at 37°C in a nutritional broth that was purchased from LAB (United Kingdom). A tiny amount of nutrient broth was then placed over EMB agar and MacConkey agar, which were also obtained from LAB (UK) in accordance with conventional laboratory methods. These agar plates were incubated for 24 h at 37°C to facilitate growth and identification. Additionally, Oxoid's (UK)Brilliance *E. coli*/coliform Agar was used to distinguish between coliform and generic *E. coli* bacteria. Numerous biochemical studies provided additional confirmation of the probable *E. coli* isolates. In order to preserve the *E. coli* isolates, they were stored at -80ºC in Nutrient broth that contained 15% glycerol until they were required for additional analysis.

DNA Isolation

The following careful procedures were carried out in order to extract and analyze the potentially hazardous *E. coli* isolates. The samples were first grown on Brilliance *E. coli*/coliform medium and incubated at 37°C for 24 hours. The purpose of this specific agar medium is to encourage the growth and differentiation of *E. coli* and coliform bacteria. Performing the extraction process as directed, we isolated DNA from the *E. coli* isolates using the Qiagen (Germany) DNeasy Blood and Tissue Kit. The Nanodrop (Jenway, UK) instrument was subsequently employed to measure the isolated DNA, providing an accurate measurement of the DNA content. The *E. coli* DNA was measured, isolated, and then delicately stored at -20°C in order to maintain its stability and cleanliness for later research. The DNA is protected in this condition of storage and is readily accessible for additional study and experimentation.

Reaction of PCR

Table 1 displays how particular sequences of the *E. coli* genes *uidA*, *Stx1*, *Stx2,* and *rfb* have been produced employing the polymerase chain reaction (PCR) technique. A PCR reaction totaling 25 µl was prepared. The reaction mixture comprised 12.5 µl of Promega Corporation's (USA) 2× GoTaq Green Mix Master, 1 µl each of primers 1 and 2, 6.5 µl of Promega Corporation's (USA) DNeasy-free water, and 4 µl of the *E. coli* DNA template. A 1.5% agarose gel

from Peqlab (Erlangen, Germany) was prepared for gel electrophoresis, and the amplicons were placed into the wells together with a 100 bp ladder DNA marker. The amplified DNA fragments have been divided and seen using electrophoresis, and their sizes were determined by comparing them to the DNA ladder. Under suitable heat cycling conditions, the PCR amplification was carried out. These parameters, which include denaturation, annealing, and extension temperatures and durations, were tuned for the primer set and DNA template under investigation and were customized to the PCR method being employed.

DNA sequencing

To purify and sequence six PCR amplicons derived from isolates of Basturma, each previously identified as *E. coli*-positive through classical PCR, the samples were outsourced to Macrogen, a commercial sequencing company based in South Korea. The target gene for sequencing was *uidA*. Subsequently, the obtained *uidA* gene sequences were subjected to a comparative analysis against previously published *E. coli* sequences available on GenBank, utilizing the NCBI BLASTn tool accessible at http://www.ncbi.nlm.nih.gov. The alignment and comparison of these sequences were further examined through online multiple sequence alignment using CLUSTALW from GenomeNet [available at].

For the construction of phylogenetic trees, the same CLUSTALW tool from GenomeNet, in conjunction with the Neighbor-Joining (NJ) program, was employed. The *uidA* gene sequences from *E. coli* served as an outgroup in the phylogenetic tree construction, involving 500 replicates to enhance robustness. This comprehensive approach aimed to elucidate the genetic relationships among the *E. coli* isolates from Basturma by leveraging purification, sequencing, and subsequent bioinformatics analyses, ultimately contributing to a better understanding of the phylogenetic context of these isolates.

Table 1: The sequence Primers and PCR program used for detecting the *uidA, Stx1, Stx2,* and *rfb* gene

Gene	Primer	Sequence (5- 3)	Amplicon Size [bp]	PCR Program*	Reference
uidA	uidA-1	5-AAAACGGCAAGAAAAAGCAG-3	623	I	(17)
	uidA-2	5-ACGCGTGGTTAACAGTCTTGCG-3			
Stx1	Stx1-1	5-AGTTAATGTGGTGGCGAAGG-3	347	II	(18)
	Stx1-2	5-CACCAGACAATGTAACCGC-3			
Stx2	Stx2-1	5-CCATGACAACGGACAGCAGTT-3	779	III	(19)
	Stx2-2	5-CCTGTCAACTGAGCAGCACTTTG-3			
Rfb	rfb-1	5-CGGACATCCATGTGATATGG-3	259	IV	(20)
	rfb-2	5-TTGCCTATGTACAGCTAATCC-3			

PCR program: I=35 times (94°C – 30s, 57°C – 30s, 72°C – 30s), II=35 times (94°C – 60s, 55°C – 60s, 72°C – 60s), III=35 times (94°C – 60s, 62°C – 60s, 72°C – 60s), IV=35 times (94°C – 60s, 52°C – 60s, 72°C – 60s).

Results

The classical microbiology methods for local Basturma found that 6/45 (13.4%) of isolates were *E. coli*. As indicated in Table 2, no E. coli was isolated from First Qadsia and Al-Noor. The percentage rate of *E. coli* recovered from Ras Al-Jada and Bab Al-Tob was 25% (2/8); from Al-Zahra it was 12.5% (1/8), and from Al-Tahrer it was 20% (1/5), as in table 2. All *E. coli* isolate was confirmed by using the PCR assay, which showed that all *E. coli* isolates have the *uidA* gene 100%, which has a molecular weight of 623 bp (Figure 1 and Table 3). The PCR technique revealed that 1/6 (16.7%) of isolates possessed the *Stx1* gene (Figure 2 and Table 3). Not one of the isolates of *E. coli* carried the *Stx2* and *rfb* genes (Figure 3 and 4).

The sequencing results of individual sequencing analysis (BLASTn) were performed on five novel *uidA* gene sequences, all of which were obtained from Basturma. The sequences of *E. coli* listed in the NCBI Genbank are indexed below the subsequent accession numbers (PP332803, PP332803, PP332805, PP332806, and PP332807) as shown in table 4. In addition, this study revealed that the individual sequencing analysis (BLASTn) for five sequences of the *uidA* gene comprises every item taken from Basturma. Using the maximum likelihood approach in MEGA11 software, the phylogenetic tree analysis showed that the local sequences of *E. coli* isolated from Ras Al-Jada (PP332803) and Bab Al-Tob areas (PP332806) were closely related (100% identity), while the local sequences of *E. coli* isolated from Ras Al-Jada and Bab Al-Tob areas (PP332805) was closely related (99% identity), the local sequences of *E. coli* isolated from Al-Tahrer areas was closely related with Ras Al-Jada and Bab Al-Tob areas (PP332805) (74% identity) (Figure 5).

Additionally, local *uidA* gene sequences showed a distinct relationship from those obtained from the GenBank database that was previously reported, based on a phylogenetic tree analysis utilizing the maximum likelihood technique in MegAlign software. There is a close relationship between our sequence types Seq E. coli-1, PP332804.1, PP332806, and PP332807 and the sequence type AP023205. 1 China, CP040269.1 Norway, and CP054564.1 USA was 99%. In addition, the sequence types

PP332803 and PP332805 showed similarity with CP019961. 1 Hong Kong, and CP057580. 1 UK was 96.3% (Figures 6 and 7).

Table 2: The number of samples and percentage of positive *E. coli* isolates

Areas	Samples (No.)	Positive N (%)
Al-Tahrer	5	1 (20)
First Qadsia	8	0
Al-Noor	8	0
Al-Zahra	8	1 (12.5)
Ras Al-Jada	8	2 (25)
Bab Al-Tob	8	2 (25)
Total	45	6 (13.4)

Table 3: The percentage of the uid A, Stx1, Stx2, and rfb genes in *E. coli* isolates

Gene	No. of positive *E. coli*	% of positive *E. coli*
uidA	6/45	13.4%
*Stx*1	1/6	16.7%
*Stx*2	0/6	0%
Rfb	0/6	0%

Table 4: The NCBI GenBank accession numbers for the *E. coli* sequences in Basturma

Accession numbers of the *uidA* gene	Bacteria
PP332803	*E. coli*
PP332804	*E. coli*
PP332805	*E. coli*
PP332806	*E. coli*
PP332807	*E. coli*

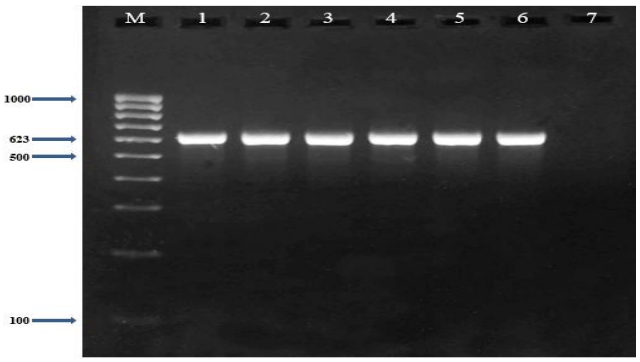

Figure 1: The molecular weight of the *uidA* gene with a molecular weight of 623 bp based on Agarose Gel Electrophoresis (2%). The amplification of DNA appears as a ladder-like pattern. Lanes M are DNA Marker 100 bp ladder (Biozym Diagnostic), Lane 1 is the positive control, Lanes 2 – 6 represent positive isolates, and Lane 7 is the negative control.

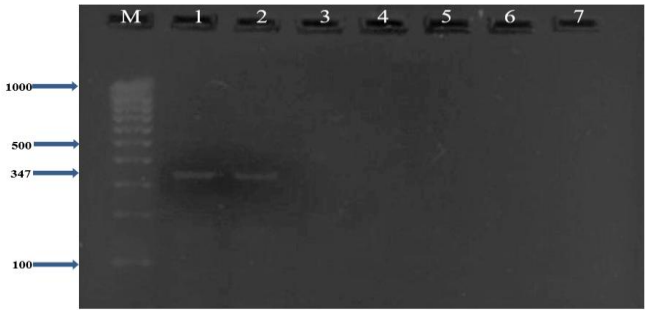

Figure 2: The molecular weight of the Stx1 gene with a molecular weight of 347 bp based on Agarose Gel Electrophoresis (2%). The amplification of DNA appears as a ladder-like pattern. Lanes M are DNA Marker 100 bp ladder (Biozym Diagnostic), Lane 1 is the positive control, Lanes 2 – 6 represent positive isolates, and Lane 7 is the negative control.

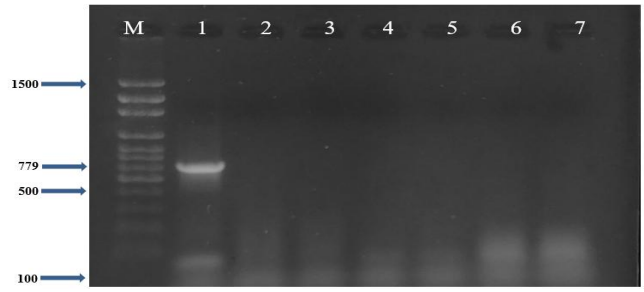

Figure 3: The molecular weight of the Stx2 gene with a molecular weight of 779 bp based on Agarose Gel Electrophoresis (2%). The amplification of DNA appears as a ladder-like pattern. Lanes M are DNA Marker 100 bp ladder (Biozym Diagnostic), Lane 1 is the positive control, Lanes 2 – 6 represent negative isolates, and Lane 7 is the negative control.

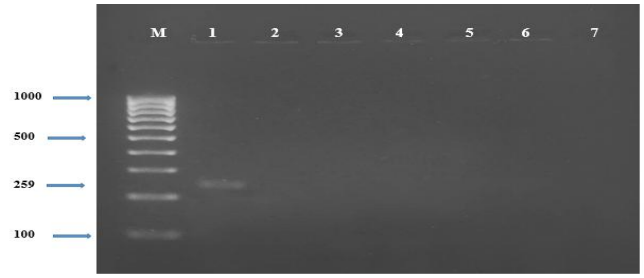

Figure 4: The perfect amplicon of the *rfb* gene with molecular weight 259 bp. The molecular weight of the *Rfb* gene was 259 bp based on Agarose Gel Electrophoresis (2%). The amplification of DNA appears as a ladder-like pattern. Lanes M are DNA Marker 100 bp ladder (Biozym Diagnostic), Lane 1 is the positive control, Lanes 2 - 6 represent negative isolates, and Lane 7 is the negative control.

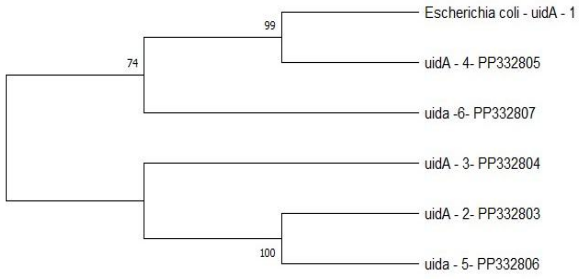

Figure 5: Phylogenetic tree of the partial sequence of the *uidA* gene of the local sequences of the *E. coli* reported in the NCBI GenBank. The numbers at the branches indicate bootstrap supports (500 replications).

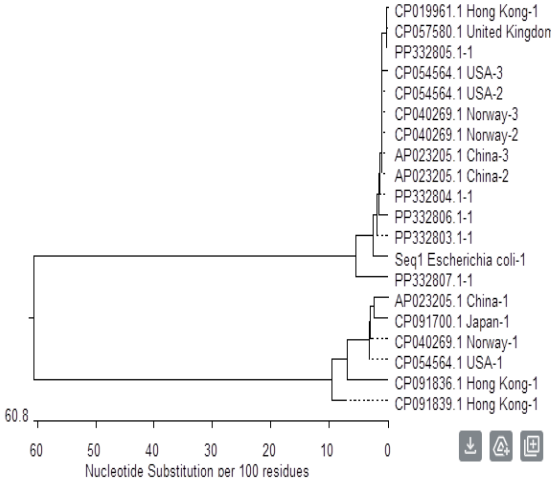

Figure 6: Clustering analysis of the *uidA* gene sequence of *E. coli* and other *uidA* sequences of *E. coli* isolates retrieved from NCBI GenBank.

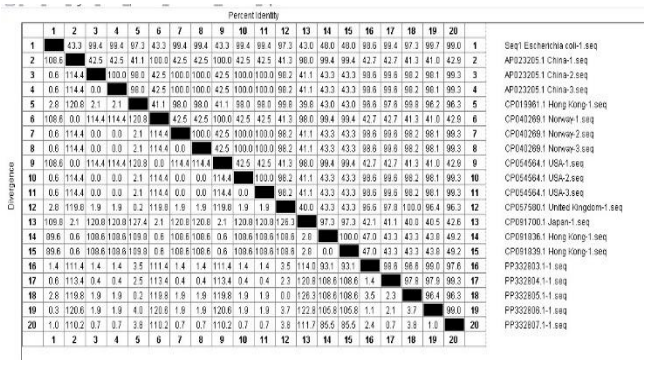

Table 7: *uidA* gene sequence similarity and divergence of each pair for *E. coli* calculated by DNASTAR. A percentage divergence is computed by contrasting sequence pairs with the tree that MegAlign DNASTAR has generated. Sequences are directly compared using percent similarity, which ignores evolutionary relationships.

Discussion

Food is a medium via which many pathogenic bacteria can spread; *E. coli* is a major foodborne microorganism that causes food poisoning around the globe (21). Numerous investigations have found that meat and its products had greater levels of *E. coli* contamination (22). Butcher shops are a hub for *E. coli* contamination of meat and its products via worker hands and utensils, according to a prior study conducted in Iraq (23). Basturma, an Iraqi meat product, is regarded as one of the most famous foods eaten during the winter season in various areas of Iraq, especially in Mosul (Nineveh governorate). In this investigation, none of the isolates was *E. coli* O:157, H7, and the percentage of *E. coli* in Basturma was 13.4% due to exposure of the meat to several stages of contamination started from farm passed through the slaughterhouse ended in the supermarket (24). Different cultures or geographical features of various systems for feeding, variances when preparing meat, and changes in the techniques employed in microbiological testing could all contribute to varying contamination rates of *E. coli* in meats and its products (25). When preparing Basturma, adding spicy garlic and salt to the meat lowers the pH of the flesh, which inhibits the formation and proliferation of bacteria in meat (26). During storage, the moisture content of Basturma dropped, which also caused an *E. coli* fall (27). The majority of people in underdeveloped nations choose to purchase inexpensive meats from unofficial marketplaces, which do not follow safety regulations or hygienic guidelines for storing meat (28,29).

Furthermore, this study showed that only one strain of *E. coli* has the *Stx1* gene (16.7%) and lacks the *Stx2* gene. Previous studies' findings showed that the *Stx1* gene was more commonly detected in *E. coli* than the *Stx2* gene (30). Numerous investigations found that the *Stx1* gene produced findings that were greater than our results; in Spain, the *E. coli* possessed the *Stx1* gene at 29% (31), and in Namibia, 40.68% (30). Simultaneously, the *Stx1* and *Stx2* genes failed to identify any *E. coli* isolated from Vietnamese meat and seafood (32). Furthermore, a close relationship between *E. coli* isolated from Ras Al-Jada and Bab Al-Tob areas was observed in the phylogenetic tree of the *uidA* gene sequences in *E. coli* isolated from Mosul city. This relationship could be explained by the animals being raised on the same farm and exposed to *E. coli* infection or by the animals contracting the infection during the processing of carcasses in the abattoir, such as cutting equipment and tools, an unclean atmosphere, and a decline in worker hygiene (33). Genomic sequencing analyses were carried out to verify the isolates' identities even more. The findings demonstrated a high degree of sequence similarity ranging from 96.3 to 99% between the sequence of this study and the sequence of *E. coli* isolated in the world. It has been established that these molecular targets are effective tools for *E. coli* genotyping (34-38). The study's high similarity findings supported the

identification of the samples as *E. coli* species. This thorough genetic research supported the finding that PCR was an effective method for characterizing *E. coli* molecules at the molecular level.

Conclusion

The primary food-borne pathogen responsible for producing the Shiga toxins is *E. coli,* which causes several problems in the digestion system and food poisoning in humans. Meat products can be contaminated by *E. coli*. The tools that butchers utilize (knives, hooks, tables, machinery, and employee hands) are crucial in the contamination and spread of *E. coli* to meats. Basturma is a type of meat product that may be contaminated with *E. coli* or other foodborne pathogens. Furthermore, this work provides a valuable molecular diagnostic tool to identify and characterize *E. coli* isolates, providing insight into the possible implications of this species in companion animals and facilitating larger-scale study.

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Conflict of interest

The authors of this manuscript have declared that no conflicts of interest arose during the writing or data analysis phases.

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التوصيف الجزيئي وشجرة التحليل التطوري لجراثيم الإشريكية القولونية المنتجة للسموم الشيكا من الباسطرمة المحلية في مدينة الموصل، العراق

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الخلاصة

تبرز أهمية جراثيم الإيشريكيا القولونية باعتبارها من الكائنات الحية الدقيقة التي تنتقل عن طريق الغذاء، وتمتلك جراثيم الإيشريكيا القولونية أنواعا مختلفة من عوامل الفوعة مثل *uidA* و *Stx1* و*Stx2* و *Rfb* التي يمكن أن تسبب التسمم الغذائي لدى البشر. تعتبر جراثيم الإيشريكيا القولونية من النوع و١٥٧: هـ ٧ من اهم الأنواع والأنماط المصلية في جراثيم الإيشريكيا القولونية المعروفة بقدرتها على إنتاج سم يشبه شيجا. وهو مصدر للأمراض، ويرتبط عادة بالأمراض المنقولة بالغذاء الناتجة عن تناول الأغذية المطبوخة والغير مطبوخة، مثل الأغذية ذات المصدر الحيواني والتي تم معاملتها بالحرارة الغير مثالية. تعد البسطرمة أحد من اهم المنتجات اللحوم التقليدية والغذائية التي يتم تصنيعها بغرض حفظ اللحوم في العراق. تم جمع خمسة وأربعين عينة من البسطرمة المنتجة محليا من المتاجر المحلية في مناطق مختلفة في مدينة الموصل. امتدت فترة الدراسة من نيسان ٢٠٢٣ إلى حزيران ٢٠٢٣. وظهرت نتائج دراستنا أن طرق الأحياء الدقيقة الكلاسيكية وطرق تفاعل السلسلة المتبلمرة تفاعل السلسلة المتبلمرة اتفقت في التعرف على الإيشريكيا القولونية في عينات البسطرمة المحلية بمعدل ٦ من ٤٥ (١٣,٤%). وقد وجد أن جميع عزلات الإيشريكيا القولونية تمتلك جين *uidA* النوعي ١٠٠%، بوزن جزيئي قدره ٦٢٣ كيلو دالتون. كشف تحليل تفاعل السلسلة المتبلمرة أيضا أن عزلة واحدة من أصل ٦ عزلات (١٦,٧%) تحمل جين *Stx1* بوزن جزيئي ٣٤٧ كيلو دالتون في حين لا تحتوي أي عزلة من جراثيم الإيشريكيا القولونية على جين *Stx2* و*rfb* . جميع العزلات المعزولة من البسطرمة لم تكن من جراثيم الإيشريكيا القولونية نوع و١٥٧: هـ ٧. وإشارة الدراسة الى وجود تشابه كبير بين جراثيم الإيشريكيا القولونية المعزولة من باب الطوب ورأس الجادة. علاوة على ذلك، أظهرت الدراسة الى وجود تشابه كبير بين جراثيم الإيشريكيا القولونية المعزولة في الدراسة مع الإيشريكيا القولونية المعزولة من مختلف دول العالم.

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