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Multidrug-resistant and clonal dispersion of enterotoxigenic Escherichia coli from ready-to-eat meat products in Duhok province, Iraq

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Abstract

This research evaluated the effluent proportion of *E. coli* and ETEC in RTE meat products, characterized the isolated strains' clonal relatedness, and determined their antibiotic resistance. 130 RTE products were gathered from various restaurants and street fast food vendors in Duhok and Zakho Province. The Isolates of *E. coli* identified by culture methods were confirmed as ETEC by multiplex PCR of the identified virulence genes. ERIC-PCR was applied to establish the clonal relationships between strains. The disk diffusion method performed the susceptibility of antibiotics on the isolated ETEC. Out of 130 examined samples, 39 (30%) isolates of *E. coli* and 16 (12.3%) ETEC were detected. Pan-fried burgers were revealed to be the most frequent contaminated sample type, with both *E. coli* and ETEC 50% and 23.3%, respectively (P≤0.05). A high clonal dispersion (12 genotypes) was observed among the isolated ETEC strains. A strong genetic linkage was discovered between a few isolates retrieved from the same sample type and within the strains from the same geographic source area. A high antibiotic resistance rate was observed with total resistance to Amoxicillin/clavulanate, Clarithromycin, Doxycycline, Erythromycin, and Clindamycin. Isolates from burger samples showed a higher resistance rate when compared with the other sample types (P≤0.05). Multi-drug resistance was noticed in all ETEC isolates. RTE meat products sold in our area have a high rate of clonally heterogeneous carrying multi-drug resistant ETEC and may constitute a significant public health risk.

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Introduction

Due to cheapness and good taste, our area's most popular food products are ready-to-eat (RTE) foods such as sandwiches with red and chicken meat, burger, and kebab. The sanitation and health of these foods have become a significant public health problem (1). Because RTE foods are edible without extra preparation, the risk of foodborne illnesses is considerable if they are handled poorly. This is primarily due to a potential vehicle of microbial foodborne bacteria (2,3). The most common indicator of food and its product's hygienic quality is the existence of *E. coli* (4,5). ETEC strains are characterized by heat-labile (LT) and/or heat-stable (ST) enterotoxins which are plasmid-encoded (6,7). In developing nations, enterotoxigenic *E. coli* is a known causal agent of diarrhea in travelers and children (8,9). Various genotyping methods such as pulsed-field gel electrophoresis (PFGE) (10,11), enterobacterial repeated intergenic consensus PCR (ERIC-PCR) (12,13) and multilocus sequence typing (MLST) (14,15) have been used to differentiate strains of pathogenic *E. coli* genetically. When comparing all of these assay systems, the ERIC-PCR can give accurate, simple, and fast results, making it a useful tool for achieving the best outcomes for epidemiological data

analysis (13). The situations favorable for the acceleration of the selection, spread, and determination of drug-resistant bacteria are the misuse and overuse of antimicrobial agents in humans and animals for therapeutic and/or prophylaxis purposes (16,17). Moreover, with rising international travel and trade of meat animals and products, drug-resistant bacteria emerging in any country could quickly become a global issue (18,19).

Due to the importance of ETEC as the primary causal agent of traveler's diarrhea with an easy transmission with food in addition to their high drug resistance pattern, this study aimed to find out the exact effluent level of *E. coli*, particularly ETEC in RTE food samples collected from Duhok and Zakho districts, to find out genetic diversity and clonal relatedness of the isolated strains and to determine the resistant profile against different antibiotics that most commonly used for therapeutic purposes in animal and human being.

Materials and methods

Ethical approve

This study was approved by pathology and microbiology departments council at the college of Veterinary medicine/University of Duhok with registration number 3/10th April 2022.

Sample collection

130 RTE foods samples were collected randomly from diverse restaurants and street fast food around the Duhok and Zakho districts**.** The samples included 30 red meat sandwiches dry-heats, 40 chicken meat (shawarma), 30 pan-fried burger sandwiches, and 30 charcoal-fired kababs (sandwiches). All samples were collected from 82 open restaurants and street fast food areas. For each sample, the whole sandwich was taken (each sample unit was at least 400 g) and put into separate sterile plastic bags to prevent cross-contamination, and immediately transported to the microbiological laboratory (College of Veterinary Medicine /Duhok University, Duhok city, Iraq) in an ice box and processed within 1-2 hr.

Sample processing and *E. coli* identification

The obtained samples were completely blended upon arrival at the laboratory, and 10 g from each sample was added to 90 ml of buffered peptone water (BPW) (Lab M, UK) and incubated at 37°C for 18-24 hr (pre-enrichment), followed by 1 ml of BPW incorporated to 9 ml of MacConkey broth (Lab M, UK) and incubated as revealed previously (selective enrichment). The selective enrichment is then quarter-streaked onto the brilliance *E. coli*/coliform agar (Oxoid, UK) and incubated as described earlier. One or two deep purple colonies were cultured onto MacConkey agar (Lab M, UK) and incubated like before to show colonies with pink colorations (capable of lactose fermentation).

DNA extraction

According to Taha and Yassin (20), the thermal method of DNA extraction was used to extract the total DNA. Four to five pure MacConkey subculture (stock culture) colonies with similar morphology were mixed entirely with 500 µL of sterile double distilled water. The solution was brought to boil for 10 minutes at 94°C, then held on ice for at least 15 minutes before centrifugation. According to Taha *et al.* (21), a nanodrop (Thermo Scientific, USA) was used to test the pureness and concentration of the sample DNA extract. A total of 350 µL of supernatant was maintained at -20°C until it was utilized as a template DNA for molecular assay.

PCR verification of *E. coli* isolates

According to Taha and Yassin (20), the recognized *E. coli* colonies by cultural assay were subjected to monoplex PCR amplification of the *uidA* gene using primer pairs (F-AAAACGGCAAGAAAAAGCAG and R-ACGCGTGGTTACAGTCTTGCG), to encodes the enzyme B-glucuronidase, which is found in all *E. coli* species. The PCR amplification setting was performed in a PCR system 9700 GeneAmp (applied bio-system, USA) with 5-minute pre-PCR heating at 95°C, followed by 35 cycles (each cycle was repeated for 1 minute at 94°C, 58°C, and 72°C respectively), then, one cycle was run for 5-minutes at 72°C. The amplicons were checked by agarose gel prepared by dissolving 1 gm of agarose in 1× TAE buffer and then stained by red safe DNA staining solution (GeNetBio, Korea).

PCR detection of ETEC

All the final verified *E. coli* isolates were exposed to multiplex PCR (mPCR) to identify genetic markers with the ETEC pathotype using a group of primer pairs including *elt* (F- AACGTTCCGGAGGTCTTATG and R-CAACCTTGTGGTGCATGATG), *esth* (F-TTCACCTTTCGCTCAGGATG and *R*-ATAGCACCCGGTACAAGCAG) and *estp* (F-ACTGAATCACTTGACTCTTCA and R-TCACAGCAGTAAAATGTGTTGT) described by Fujioka and coworkers (22). The PCR mixtures were prepared in 25 µl total volume, which contained 12.5 µl of Hot-start Premix (Genet Bio, Korea), 3 µl primers mixture for each analogous gene (*elt, esth,* and *estp*), 2 µl of DNA from the sample (100 ng/µl), and sterile double distilled water up to 25 µl. The PCR program and the electrophoresis pattern were run according to conditions utilized by Taha and Yassin (20). A 50 bp DNA ladder (Genedirex, Taiwan) was used as a standard molecular marker to determine the PCR product sizes by gel electrophoresis in 2% agarose gel. The particular policy was applied for identifying ETEC by mPCR, as when the presence of *elt* and/or *st* variants (*estp* or *esth*) genes amplicon bands in agarose gel, the isolates were classified as ETEC.

ERIC PCR of diversity analysis

To establish strain diversity and similarity, ERIC primers (ERIC1: 5'-ATGTAAGCTCCTGGGGATTCAC-3' and ERIC2: 5'-AAGTAAGTGACTGGGGTGAGCG-3') designated by Versalovic *et al.* (23) were amplified in all ETEC isolated strains using the ERIC PCR reaction. The PCR mixture and program conditions were applied according to Taha (24). As mentioned above, the amplicons were identified through agarose gel electrophoresis in the PCR verification of *E. coli* isolates section. A 100-bp DNA ladder (Genedirex, Taiwan) was applied for amplicons size verification. For ERIC data processing, an image was taken.

ERIC PCR data analysis

The existence or disappearance of amplified DNA by ERIC-PCR was first visually checked, and then the dendrogram was generated via GelJ computer software Version 2.0 (25). Unweighted Pair Group Method with Arithmetic Mean (UPGMA) analysis and Dice similarity coefficient was utilized to cluster the ETEC strains. When the similarity coefficient was equivalent to or over 90%, isolates were categorized as having the same genetic profile (26). Depending on the sample types and geographic area, all the isolated ETEC strains were genotyped.

Susceptibility testing for antibiotics

All confirmed strains of ETEC were subjected to susceptibility tests by disc diffusion assay on Mueller-Hinton agar (HiMedia, India). Sixteen antibiotic discs were utilized, including amoxicillin/clavulanate, meropenem, clarithromycin, doxycycline, norfloxacin, levofloxacin, streptomycin, aztreonam, cefotaxime, erythromycin, imipenem, clindamycin, ciprofloxacin, trimethoprim-sulfamethoxazole, ceftazidime, and gentamicin. All selected antibiotics represent the main classes of antibacterial drugs used in human and veterinary medicine. Using the inhibition zone breakpoints specified by the clinical and laboratory standards institute, all the ETEC strains were classified as susceptible or resistant (27). Multiple antimicrobial resistance was identified as any isolate resistant to three or more antibiotics.

Statistical analysis

All data were analyzed by one-way analysis of variance (ANOVA). Specific differences between the groups were determined using Duncan multiple range test by using SPSS version 20. The accepted level of significance was P≤0.05.

Results

Distribution of *E. coli* and ETEC

Figure 1 will show the gel banding patterns of a positive sample with both *E. coli* (*uidA* gene) and ETEC (*elt* and *estp* genes). The incidence rates of both *E. coli* and ETEC in the tested RTE foods are shown in table 1 and figure 2. Out of 130 RTE food samples, only 39 (30%) were found to be positive for *E. coli* in both cities (Duhok and Zakho), including 3 (10%) dry-heats cooked red meat, 7 (17.5%) dry-heats cooked shawarma, 15 (50%) pan fried burger, and 14 (46.6%) Kebabs sandwiches. All types of food items were found to be contaminated with *E. coli*. Pan-fried burgers and Kebabs sandwiches were the most commonly *E. coli* contaminated RTE food (P≤0.05). On the other hand, ETEC was isolated from 16 (12.3%) of the 130 tested RTE food samples, including 7 (23.3%) from the pan-fried burger, 5 (12.5%) from dry-heats cooked shawarma, 3 (10%) from Kebabs and only 1 (3.3%) isolate from dry-heats cooked red meat. At the same time, the most common meat product contaminated with the ETEC was the Pan-fried burger (P≤0.05).

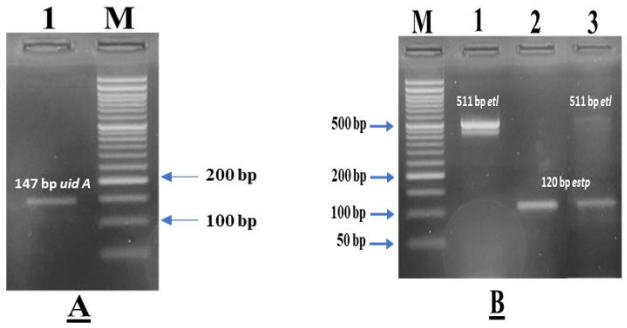

Figure 1: (A) Verifying *Escherichia coli* isolates using monoplex PCR. Lane M: 50 bp DNA ladder (Genedirex, Taiwan), lane 1: Amplified *uidA* gene (147 bp) from samples. (B) Identification of positive pathogenicity-related genes from ETEC isolates using triplex PCR. Lane M: 50 bp DNA ladder, Lane 1: 511 bp *elt* gene, lane 2: 120 bp *estp* gene, lane 3: positive sample for both *elt* and *estp* genes.

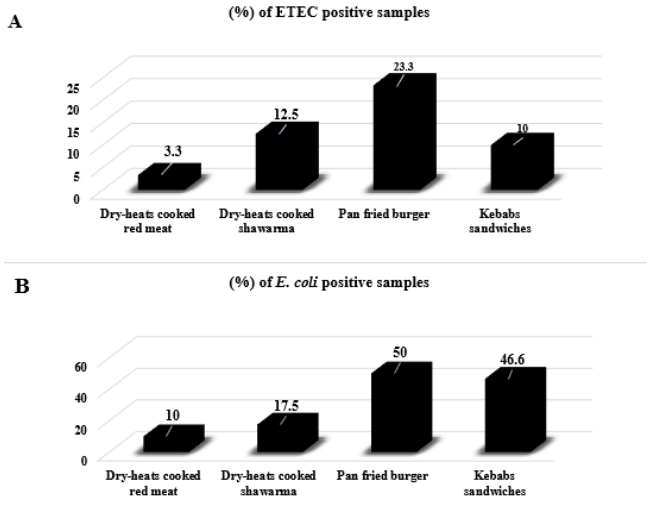

Figure 2: Percentages of the incidence rate of both *E. coli* and ETEC positive RTE samples (A and B, respectively).

Table 1: The incidence rate of *E. coli* and ETEC in the tested RTE meat products in Duhok and Zakho cities

Sample type	Tested (n)	*E. coli* +ve n(%)	ETEC +ve n(%)
Dry-heats cooked red meat	30	3 (10)	1 (3.3)
Dry-heats cooked shawarma	40	7 (17.5)	5 (12.5)
Pan fried burger	30	15 (50) *	7 (23.3) **
Kebabs sandwiches	30	14 (46.6) *	3 (10)
Total	130	39 (30)	16 (12.3)

* Indicates that *E. coli* was significantly seen in both Pan-fried burgers and Kebabs sandwiches at P≤0.05. ** Indicates that the ETEC was significantly seen in Pan-fried burgers at P≤0.05.

ERIC PCR diversity assay

Based on the number and size of ERIC sequence variances and depending on the ERIC-PCR fingerprinting analysis in each isolate, the outcomes showed that ETEC isolates similarity was between 54-100 %. The strains were divided into 12 genotypes based on a similarity limit of 90% (1 to12), in which the most prevalent clone was within genotype 1. Genotype 1 was the largest group containing 3 strains. After that, 2 strains were clustered within genotypes 11 and 12. In contrast, each genotype (2 to 10) comprises only one strain (Figures 3 and Table 2). Interestingly, the result of ERIC-PCR showed that all strains recovered from Zakho district have a distinct genetic profile from those strains isolated from Duhok city (The band profile of isolates from Zakho showed a cluster diversity to all representatives of Duhok isolates) (Figures 3).

Regarding the sample type, certain strains obtained from the same sample type as well as the strains from the same geographic area shared a high degree of genetic homology, including strain numbers 14, 15, and 16, 1 and 2 (Table 2 and Figure 3).

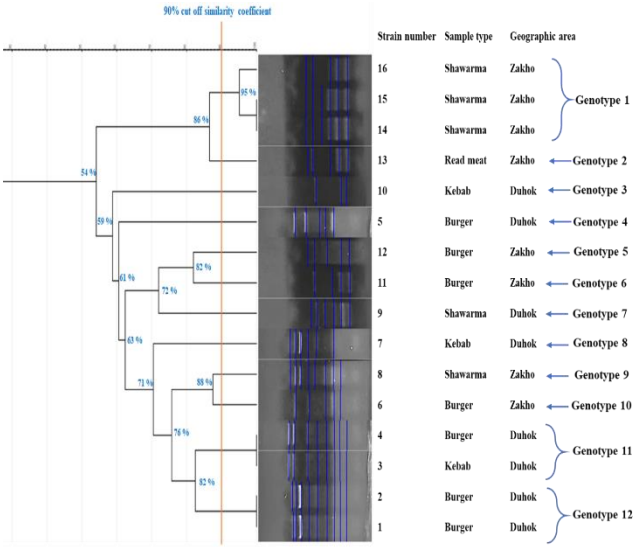

Figure 3: A dendrogram obtained from ERIC-PCR shows the banding pattern of 16 ETEC strains obtained from RTE meat products from Duhok and Zakho districts.

Table 2: Genotypic pattern of ETEC strains obtained from RTE meat products from Duhok and Zakho districts

Strain number	Sample type	Geographic area	Genotypic pattern
16	Shawarma	Zakho	
15	Shawarma	Zakho	Genotype 1
14	Shawarma	Zakho	
13	Red meat	Zakho	Genotype 2
10	Kebab	Duhok	Genotype 3
5	Burger	Duhok	Genotype 4
12	Burger	Zakho	Genotype 5
11	Burger	Zakho	Genotype 6
9	Shawarma	Duhok	Genotype 7
7	Kebab	Duhok	Genotype 8
8	Shawarma	Zakho	Genotype 9
6	Burger	Zakho	Genotype 10
4	Burger	Duhok	Genotype 11
3	Kebab	Duhok	
2	Burger	Duhok	Genotype 12
1	Burger	Duhok	

Antibiotic susceptibility test

All ETEC isolates 100% were multi-drug resistant (at least 3 different antibiotics are resistant) (Figure 4). All of them showed a total resistance 100% to each of Amoxicillin/clavulanate, Clarithromycin, Doxycycline, Erythromycin, and Clindamycin, followed by 75% resistance to Gentamicin, 35% to Streptomycin, 31.2% to Ciprofloxacin, 25% to each of Aztreonam and Cefotaxime, 12.5% to Ceftazidime and 6.2% to each of Norfloxacin and Trimethoprim-sulfamethoxazole. Alternatively, all isolates showed complete susceptibility to Meropenem, Levofloxacin, and Imipenem (Figure 4 and Table 3). Regarding the sample type, the resistance rate of ETEC isolates from a Pan-fried burger showed a higher resistance rate than the other sample types (Table 3).

Table 3: Antibiotic-resistant patterns of all ETEC isolates among various RTE meat products

Antimicrobial	Total ETEC (n=16) n(%)	Dry-heats cooked red meat (n= 1) n(%)	Dry-heats cooked shawarma (n= 5) n(%)	Pan fried burger (n= 7) n(%) *	Kebabs sandwiches (n= 3) n(%)
AMC	16 (100)	1 (100)	5 (100)	7 (100)	3 (100)
CLR	16 (100)	1 (100)	5 (100)	7 (100)	3 (100)
DOX	16 (100)	1 (100)	5 (100)	7 (100)	3 (100)
ERY	16 (100)	1 (100)	5 (100)	7 (100)	3 (100)
CLI	16 (100)	1 (100)	5 (100)	7 (100)	3 (100)
GEN	12 (75)	0	5 (100)	5 (71.4)	2 (66.6)
STR	6 (37.5)	0	3 (60)	2 (28.5)	1 (33.3)
CIP	5 (31.2)	0	2 (40)	2 (28.5)	1 (33.3)
ATM	4 (25)	0	2 (40)	2 (28.5)	0
CTX	4 (25)	0	2 (40)	1 (14.2)	1 (33.3)
CAZ	2 (12.5)	0	1 (20)	1 (14.2)	0
NOR	1 (6.2)	0	0	0	1 (33.3)
SXT	1 (6.2)	0	0	0	1 (33.3)

* Indicates that the resistance rate of ETEC isolates from pan-fried burgers was significantly higher than the isolates from the other sample types at P≤0.05.

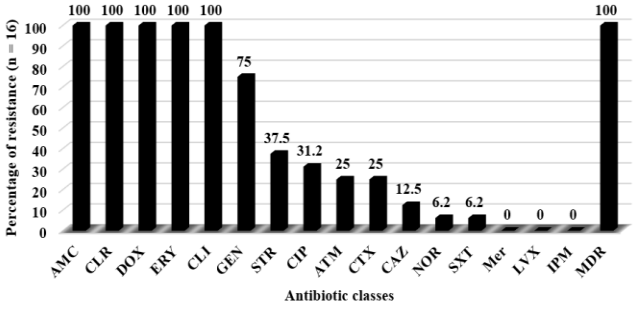

Figure 4: Antibiotic-resistant pattern of 16 ETEC strains isolated from RTE meat products. AMC: Amoxicillin/clavulanate, CLR: Clarithromycin, DOX: Doxycycline, ERY: Erythromycin, CLI: Clindamycin, GEN: Gentamicin, STR: Streptomycin, CIP: Ciprofloxacin, ATM: Aztreonam, CTX: Cefotaxime, CAZ: Ceftazidime, NOR: Norfloxacin, SXT: Trimethoprim-sulfamethoxazole, Mer: Meropenem, LVX: Levofloxacin, IPM: Imipenem, MDR: Multi-drug resistant.

Discussion

The focuses of this research were to establish the precise *E. coli* and ETEC contamination level of RTE meat products sold in our area, to find out the extent of genetic similarity between the isolated ETEC strains (to determine the source of contamination), and to investigate the fact that at which extend these ETEC strains were resistant to available antibiotics.

Escherichia coli's existence in food indicates unsanitary conditions and fecal contamination (28,29). This can arise at any point in food processing (30). Whereas RTE food products are normally consumed lacking any further dealing out, this raises the risk of infection associated with their consumption, and these risks are predominantly high if they are held hopelessly, resulting in foodborne illnesses (31,32). This study found a high threshold of both *E. coli* and ETEC contamination, indicating a poor hygienic standard in retail RTE foods sold in our area. RTE foods have been heated, which can kill all or most microbes found in these foods. As a result, the presence of microorganisms in such RTE food stuffs could be due to cross-contamination during processing and handling procedures, including storing, cutting, weighing, and packaging (2,33). To minimize the microbial load of RTE foods, reasonable personal hygiene procedures should be followed.

Furthermore, according to this study's results, the contamination rate of *E. coli* and ETEC in pan-fried burgers was relatively higher than elsewhere food products. This could be because burgers are typically made in thick balls, and the temperature from the grilling step cannot penetrate to inside, leaving it undercooked and raw product inside the burger's ball core without killing the pathogens (34). As a result, it is advised to confirm the cooking quality of the burger's core by providing an adequate temperature and a correct holding period.

A high clonal dispersion (12 genotypes) was observed in these isolated ETEC strains. These genetic dissimilarities could be because the contaminating bacteria have come from multiple sources and ecosystems (generally, the raw food products that this RTE food was prepared were imported to Iraq from various countries). Multiple ETEC strains could be transported through food processing facilities (from manufacturing till final processes step) (35). All of the factors mentioned above could influence the clonal dispersion of ETEC strains in this investigation, implying that diverse foods and their products could be used as a

vehicle for ETEC transmission. Furthermore, some isolates from the same geographic area and sample type had the same genetic profile (strain number 14, 15 with 16), (1 with 2), implying that a dominant ETEC clone is ubiquitous in our area and that there is only one contamination source in these strains (2,36).

Regarding antibiotic resistance characteristics, these ETEC isolates had an increased resistance rate and were all found to be multi-drug resistant. This alarming resistance could be attributed to the easy availability and haphazard usage of antibacterial agents in the veterinary field, as the need for the prophylactic or therapeutic use of sub-therapeutic dosages in animals consumed by humans (37) or perhaps due to the existence of elevated levels of various antibiotic residues in food products, with subsequent of resistance selections (38,39). Moreover, when compared to other types of samples, ETEC strains from pan-fried burgers had a greater resistance rate. This could be mainly attributable to the haphazard and widespread application of antibiotics as prophylactic or therapeutic against infectious diseases in animals from whom the meat products were obtained (36).

The existence of multidrug-resistant (MDR) ETEC in local RTE food is a public health concern because these RTE foods can behave as potential vectors for pathogenic *E. coli* carrying the multi-drug resistant trait, allowing these resistant bacteria or genes to spread to humans via food and establish themselves in the intestinal flora (40,41).

Conclusion

Our findings reveal a high incidence rate of *E. coli* and ETEC in RTE food sold in our area, indicating deprived sanitary practices throughout product preparation and retail. High resistant rates to the commonly used antibiotics in both the veterinary field and in human beings are a dominant contributor to public health issues, as these resistant phenotypes could be transferred through the food chain to humans and may further complicate the therapeutic options of illnesses caused by not only ETEC strains by the other bacterial pathogens. Furthermore, most of the ETEC strains in this study exhibited a wide variety of genetic dispersion in meat products imported into our country. In addition, a dominant clone of ETEC strains is widespread in our area, as indicated by the identical genetic profile in the same geographic area and sample type. The study's findings indicate that ETEC contamination of RTE food poses a possible risk to consumers and travelers, emphasizing the importance of maintaining proper hygiene procedures during food preparation. These findings will aid Iraqi federal regulators in developing a supervisory framework for controlling ETEC and other foodborne pathogens to ensure the microbiological safety of RTE meat products.

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Conflicts of intertest

No conflicts are affecting the publication of this work.

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مقاومة المضادات الحيوية ومعرفة العلاقة النسيلية للإشريكيا القولونية المعوية السمية في الأطعمة الجاهزة للأكل في محافظة دهوك، العراق

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الخلاصة

تم إجراء هذا البحث لتقييم نسبة تواجد جراثيم الإشريكيا القولونية المعوية الســـــمية في عينات الأغذية الجاهزة للأكل مع وصف العلاقة

النسيلية لهذه السلالات المعزولة وتحديد مقاومتها للمضادات الحيوية. تم جمع ١٣٠ منتجا غذائيا جاهزا للأكل من عدة مطاعم ومن بائعي الوجبات السريعة في الشوارع في محافظة دهوك. تم تأكيد عزلات الإشريكية القولونية التي تم تحديدها بواسطة طريقة الزرع الجرثومي على انها إشريكيا قولونية معوية سمية بواسطة تفاعل البلمرة المتسلسل المتعدد لجينات الفوعة المحددة. كذلك تم تطبيق تفاعل البلمرة المتسلسل المتكرر بين الجينات المعوية باستخدام بادئات موافقة لإيجاد العلاقات النسيليّة بين السلالات كما تم إجراء اختبار حساسية المضادات الحيوية للعزلات الجرثومية باستخدام طريقة الانتشار القرصي. أظهرت النتائج عزل ٣٩ (٣٠٪) عزلة من الإشريكيا القولونية و ١٦ (١٢,٣٪) عزلة إشريكيا قولونية معوية سمية من اصل ١٣٠ عينة من الغذاء الجاهز للأكل، تم الكشف أن البركر المقلي هو العينة الملوثة الأكثر تكرارا بتواجد جرثومة الاشريكيا القولونية والإشريكيا القولونية المعوية السمية بنسبة ٥٠ % و ٢٣,٣٪ على التوالي. لوحظ وجود علاقة نسيلية تضمن ١٢ نمطا وراثيا بين سلالات الإشريكيا القولونية المعوية السمية وتم اكتشاف ارتباط وراثي بين بضع عزلات تعود لنفس نوع العينات من نفس المنطقة الجغرافية. لوحظ ارتفاع معدل المقاومة للمضادات الحيوية وخصوصا أموكسيسيلين / كلافولانات، كلاريثروميسين، دوكسيسيكلين، إريثروميسين، وكليندامايسين وأظهرت العزلات من عينات البركر نسبة مقاومة أعلى مقارنة بأنواع العزلات الأخرى واظهرت جميع عزلات الاشريكيا القولونية السمية مقاومة متعددة للمضادات الحيوية فضلا عن أن المنتجات الغذائية الجاهزة للأكل المباعة في دهوك وزاخو لديها معدلات عالية من العزلات غير المتجانسة نسيليا والحاملة لجينات مقاومة متعددة للمضادات الحيوية والتي قد تشكل خطرا كبيرا على الصحة العامة.