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The molecular identification of diarrheagenic *Escherichia coli* (DEC) isolated from meat and meat products

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Abstract

The present study aims to diagnose diarrheagenic *E. coli* in meat and meat products by the conventional polymerase chain reaction (PCR) technique using the *uidA* gene to confirm the existence of the bacterial isolates as *E. coli*. The multiplex PCR technique is adopted to detect the virulence genes of these bacteria using two groups of primers for detecting the gene (stx1, *stx2*, *aggR*, *esth*, *eae*, *invE*, *daaC*, *estp*, *elt*, and *bfpA*). In this study, these primers are applied to a total of 100 *E. coli* strains isolated from 782 samples of meat and meat products (fresh, minced, burger, pastirma, and chicken) from February to November 2020. The results of the present study show that all *E. coli* isolates are positive to have the uidA gene (147 bp). The study also detects 95/782 (12.15%) pathogenic species related to virulence genes by using multiplex PCR. The highest percentage of pathotype is ETEC 46.32% and the lowest percentage is the DAEC type 1.05%. In addition, the other pathotypes are 20.05, 14.74, 6.32, 6.32, and 5.26% of STEC, EHEC, aEPEC, EAEC, and EIEC respectively. The high rate of contamination with DEC reported in this study is associated with the poor hygiene conditions of slaughtering and meat storage in shops and markets resulting in health risks to consumers.

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Introduction

Escherichia coli is one of the most important types of the *Enterobacteriaceae* family that is naturally endemic to the alimentary canal of humans and animals (1). The opportunistic bacteria of *E. coli* can infect a body whenever an opportunity exists, causing many diseases such as diarrhea, meningitis, septicemia, and bacteremia (2,3). Meat and meat products are important food items for humans because they are rich in animal proteins, fats, minerals, and vitamins. Recently, the increasing meat consumption may be a source of the danger threatening human health. Meat is a suitable environment for the growth of many different types of pathogenic and non-pathogenic germs that are transmitted from meat to humans such as *E. coli* which can generate a large and diverse group of diarrheal bacteria called Diarrheagenic *E. coli* (DEC) (4-6). The pathogenicity of *E. coli* is closely related to many types of virulence factors and their pathological features. The pathological types are classified into six groups: Enteropathogenic *E. coli* (EPEC), Enterotoxigenic *E. coli* (ETEC), Enterohemorrhagic *E. coli*. or Shiga-toxigenic *E. coli* (STEC), Enteroaggregative *E. coli* (EAEC), Enteroinvasive *E. coli* (EIEC), and diffusely adhering *Escherichia coli* (DAEC) (7). It is not possible to use cultures or biochemical tests for distinguishing the symbiotic and pathological strains of these bacteria in a laboratory because of the large number of pathological types and virulence factors. Therefore, the polymerase chain reaction (PCR) is one of the most important techniques to detect virulence genes (8). Additionally, the multiplex Polymerase Chain Reaction (mPCR) technique is used to detect the presence or absence of several genes encoding

major virulence factors for the diagnosis of DEC isolated bacteria, particularly when dealing with large samples (9).

The current study aims to identify *E. coli* in meat and meat products using conventional molecular methods, detect the species-specific uidA gene of *E. coli*, and identify the different types of genes that encode the virulence factors of *E. coli* by using the mPCR technique.

Materials and methods

Sampling

A total of 100 *E. coli* strains were isolated previously from 782 samples of meat and meat products including fresh meat (beef, sheep, buffalo, and goats), minced meat, burgers (local and imported), pastirma, and chicken meat (local and imported) collected from different butchers' shops and restaurants at Mosul city from 2/3/2020 to 5/11/2020. The samples were transmitted directly to the laboratory of Microbiology, Department of Biology, College of Education for Pure Sciences, and College of Veterinary Medicine, University of Mosul, Iraq for preparation, isolation, and molecular detection of *E. coli* bacteria (10).

DNA extraction and amplification

The pure *E. coli* colonies were selected and added to 200 µl of sterile distilled water in a 1.5 ml Eppendorf tube. After that, they were mixed with a vortex mixer device. The cells were lysed for at least 15 seconds and the DNA was extracted using the laboratory kit prepared by (Jena) Bioscience (11).

The species-specific conventional polymerase chain reaction (PCR) technique was used to confirm the *E. coli* isolates with the application of the universal primers of uidA (Table 1). The master mix was prepared for all the PCR using the Gen Net Bio kit by calculating the required volumes of reaction components for each sample. The additives were mixed well and distributed in a volume of 18 µl into small PCR tubes in a 0.2ml volume for the PCR procedure. After that, the extracted DNA from the samples in a 2 µl volume was added separately to the tube of each sample and the total volume in each tube was 20 µl. All PCR tubes were placed in the T100TM thermocycler (Bio-Rad, USA) and the PCR program was used at 94 °C for 10 min as an initial denaturation. 35 cycles (94° C for 45 sec, 58 °C for 45 sec, and 72 °C for 1 min) were applied. The final elongation was 72 °C for 10 min. The tubes were removed from the apparatus and placed in the refrigerator at 4-8°C until the electrophoresis was performed to detect the products of the DNA amplification process. For further analysis of DEC using the PCR technique according to Fujioka & Coworkers (12), two groups of primer mixtures were prepared for the DEC classification in meat samples (Table 1).

Table 1: The Sequences of the Primers used in the study

Genes	Path type	Primer Sequence (5' to 3')	Size (bp)	Primer Conc. (pmol/µl)	Reference
uidA	*E. coli*	AAAACGGCAAGAAAAAGCAG ACGCGTGGTTACAGTCTTGCG	147	10	(13)
Stx1	STEC. EHEC	AGTTAATGTGGTGGCGAAGG CACCAGACAATGTAACCGC	347	5	(12)
Stx2	STEC. EHEC	TTCGGTATCCTATTCCCGG CGTCATCGTATACACAGGAG	592	4	(12)
Eae	EHEC.tEPEC.aEpEC	CCCGAATTCGGCACAAGCATAAGC CCCGGATCCGTCTCGCCAGTATTCG	881	5	(14)
bfpA	tEPEC	AATGGTGCTTGCGCTTGCTGC GCCGCTTTATCCAACCTGGTA	324	5	(15)
aggR	EAEC	GTATACACAAAAGAAGGAAGC ACAGAATCGTCAGCATCAGC	254	4	(14)
Elt	ETEC	AACGTTCCGGAGGTCTTATG CAACCTTGTGGTGCATGATG	511	3	(12)
Esth	ETEC	TTCACCTTTCCCTCAGGATG ATAGCACCCGGTACAAGCAG	172	4	(12)
Estp	ETEC	ACTGAATCACTTGACTCTTCA TCACAGCAGTAAAATGTGTTCT	120	10	(12)
invE	EIEC	GCAGGAGCATCTTGAAG GAAAGGCACGAGTGACTTTC	208	10	(12)
daaC	DAEC	CACTGTGGGCTCCGCGCAAGC CGGTGAGGTTCACTGTGTAT	418	10	(16)

uidA: the gene encoding the enzyme B-glucuronidase, Stx: Shiga-like toxins, eae: Intimin, bfpA: bundle-forming filaments, aggR: virulence genes that stimulate transcription in EAEC, elt: heat-labile toxins, esth and estp protein: heat-stable toxin, invE: invasive toxins, daaC: From the adhesion family.

The mixture groups were organized in such a way that each group had the closest initial annealing temperature and the farthest PCR product volumes. The first group was added to the reaction mixture to detect the genes (stx1, stx2, aggR, esth, and eae). The second set of primers was used to detect the invE, daaC, estp, elt, and bfpA genes for both sets of primers. The total volume of the PCR reaction mixture was 50 µl consisting of 25 µl of Hot Start Premix and 5 µl of primer mixture for each similar gene. 4 µl of DNA (100 ng/µL) of the sample and nuclease-free water were added up to 50 µL. The PCR preparation program was adjusted to preheat for 5 minutes at 95°C, followed by a 35 cycle with 1 minute at 94°C, 1 minute at 55°C, and 1 minute at 72°C, post-reaction, and sequential polymerization for 10 minutes at 72°C. The PCR products were migrated by the electrophoresis at 85 V for 40 minutes in a 2% agarose gel prepared from Tris-acetate-EDTA (TAE x1) solution, then stained by the Ethidium bromide to be photographed with a digital camera.

Statistical analysis

The differences in the distribution rates of DEC pathotypes among the different meat samples were calculated by the Chi-square test by using the SPSS statistical program at $P<0.05$.

Results

As far as the molecular assays and polymerase chain reaction are concerned, the DNA was extracted from the *E. coli* isolates from the different types of samples. The concentration of DNA extracted from the bacterial isolates ranged from 265 to 345 ng/µl and the DNA purity ranged from1.75 from 1.98. Figure 1 shows the genome packages of DNA extracted from the bacterial isolates of E. coli on the agarose gel. The results of the present study show that all positive *E. coli* isolates possessed the *uidA* gene and bands with a molecular weight of 147 bp (Figure 2). The mPCR technique was adopted to investigate the presence or absence of the virulence genes encoding important virulence factors of *E. coli* using the species-specific primers that target each virulence gene and to ensure the relevance of the isolates to these bacteria (DEC). The results of these primers show a difference in the number of amplification bands and their molecular weights according to the adopted primer (Figures 3).

The current study detects pathogenic species related to virulence genes in 95 (12.15%) samples (Table 2). The study shows that the highest percentage of these pathotypes are in minced meat from restaurants and butcher shops, 40% and 46.7%, respectively. In addition, meat products show a significant difference at 0.05 from all other types of meat and meat products. No significant difference between burger products and beef products is reported, whereas a significant difference between these products and the rest types in the study is noted. The results also show that there are no significant differences between other types of meat such as sheep, buffalo, goat, pastirma products, and the two types of local and imported chicken meat. The percentage of diarrheagenic *E. coli* (DEC) is shared by the rest of meat and meat products. Pastirma and imported chicken meat show the lowest percentage 6%.

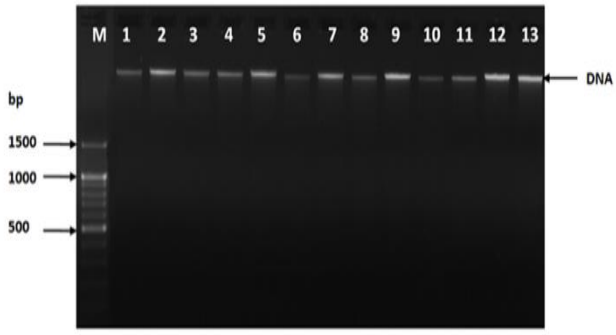

Figure 1: Agarose gel electrophoresis of DNA extracted from E. coli isolates. Lane M: Ladder. DNA 100 bp and Lane 1-13 represents extracted DNA.

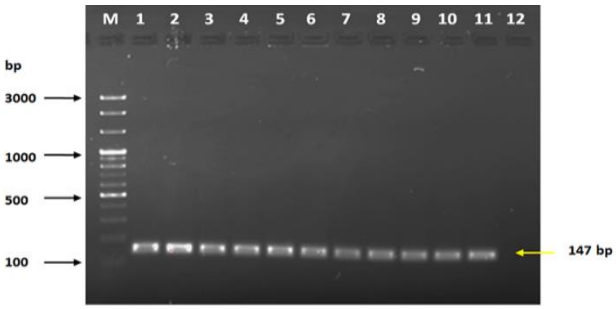

Figure 2: Agarose gel electrophoresis of PCR products for the detection of the uidA gene in *E. coli* isolates. Lane M: Ladder DNA 100 bp, Lane 1-11 represents samples of 147 base pairs, Lane 12 represents the negative control.

Figure 4 shows that the highest percentage of pathotype 46.32% is ETEC and that the lowest percentage of pathotype 1.05% in this study is due to DAEC. On the other hand, the percentages of the other pathotypes are 20.05, 14.74, 6.32, 6.32, and 5.26% for STEC, EHEC, aEPEC, EAEC, and EIEC respectively. The study shows that there is a significant difference between the ETEC pathotype and all the other pathotypes. In addition, the STEC pathotype shows a significant difference from the other pathotypes, noting that EAEC, EIEC, DAEC, and aEPEC pathotypes do not have any significant difference between them. Table 2 shows that there are significant differences between the types of meat and the meat products within the same pathotypes that cause diarrhea. On the contrary, there is no significant difference between meat and meat products within the same pathotype

in some types, as in the case of EAEC and AIEC pathotypes. As for the EHEC type which is considered important in food poisoning, the percentages are very low 14.74% in the fresh meat of cows, sheep, buffaloes, goats, minced meat of both types, and local and imported burgers. This pathological type is not detected in pastirma and chicken in this study.

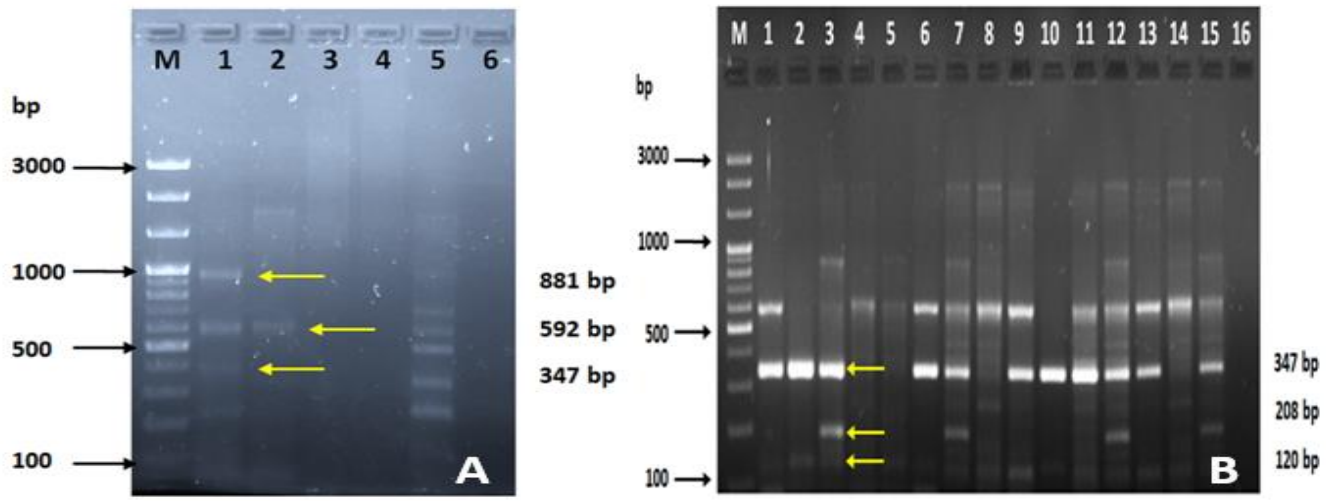

Figure 3: A: Agarose gel electrophoresis of PCR products for the detection of virulence genes. Lane M: Ladder DNA 100 bp, and Lane 1 represents a positive sample of genes are = 881 bp, stx2 = 592 bp, and stx1 = 347 bp., Lane 6 represents the negative control. B: PCR products for the detection of virulence genes in *E. coli* isolates. Lane 3 represents a positive sample for estp = 120 bp, invE = 208 bp, and STX1 = 347 bp. Lane 16 represents the negative control.

Table 2: Distribution of Diarrheagenic *E. coli* (DEC) pathotypes in the studied samples of meat and meat products

Type	n sample	Total n (%)	n (%) of diarrheagenic *Escherichia coli* pathotypes						
			EHEC	ETEC	STEC	EAEC	EIEC	DAEC	a EPEC
Beef	128	18(14.06) bc	3(16.7) bc	7(38.9) c	5(27.8) ab	2(11.11) a	-	-	1(5.55) b
Sheep meat	128	8(6.25) c	2(25) ab	4(50) bc	1(12.5) c	-	-	(12.5)1	-
Buffalo meat	128	11(8.59) c	1(9.09) c	5(45.5) c	2(18.18) bc	1(9.09) a	1(9.09) a	-	1(9.09) b
Goat meat	128	10(7.81) c	2(20) ab	4(40) c	2(20) bc	1(10) a	1(10) a	-	-
Ground meat (shops)	30	14(46.7) a	1(7.14) c	7(50) bc	2(14.29) c	1(7.14) a	2(14.29) a	-	1(7.14) b
Ground meat (restaurants)	30	12(40) a	2(16.67) bc	5(41.79) c	3(25) ab	1(8.33) a	-	-	1(8.33) b
Imported beef Burger	30	5(16.7) bc	1(20) ab	3(60) ab	1(20) bc	-	-	-	-
Local beef burger	30	7(23.33) b	2(28.57) a	3(42.9) c	1(14.29) c	-	1(14.29) a	-	-
Pastirma	50	3(6) c	-	2(66.67) a	1(33.33) a	-	-	-	-
Imported chicken	50	3(6) c	-	2(66.67) a	-	-	-	-	1(33.33) a
Local chicken	50	4(8) c	-	2(50) bc	1(25) ab	-	-	-	1(25) a
Total number	782	95(12.15) c	14(14.74) cd	44(46.32) a	19(20.05) bc	6(6.32) de	5(5.26) e	1(1.05) e	6(6.32) de

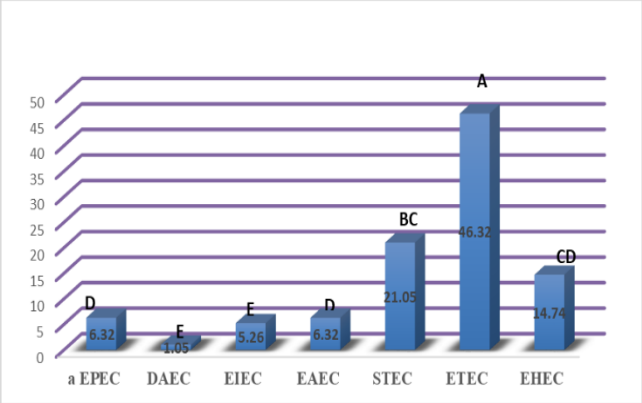

Figure 4: the distribution of pathological patterns of Diarrheagenic *E. coli* (DEC) in meat and meat products and the significant differences between them at the level of significance (P<0.05).

Discussion

The contamination with *E. coli* depends on the type of animal, breeding conditions, and slaughtering. Therefore, meat products consumed as human food need special attention and care as they play a major role in the transmission of many common diseases and food poisoning to humans (17). The high level of *E. coli* contamination in beef and buffalo meat is since the meat of these two cattle types is the main reservoirs for *E. coli* and is higher than the rest of the other cattle types because of their hooves and internal entrails. Meat may also be contaminated by the hands of the butchers, the walls, and floors of the slaughterhouses (18). The phenotypic characteristics of *E. coli* on a selective medium agree with the previous studies (10,19).

Ramires-Martinez and colleagues (13) indicate that when the primers of the uidA gene that encodes the enzyme (B-glucuronidase) common in all *E. coli* species in the PCR technique are used, a band with a molecular weight of 147 base pairs is given in all isolates and is positive for this gene.

The multiplexed polymerase chain reactions (mPCR) are performed to investigate the presence or absence of the virulence genes that encode the important virulence factors for the diagnosis of (DEC) by using specific primers to target each primer of a specific sequence of one of these genes and to ensure the subordination of these isolates in this study to these bacteria (DEC). The results of this study show that the primers differ in the number of amplification bands and their molecular weights according to the primer used. The difference between primers results is due to the presence or absence of complete sites for that initiator with similar sequences present in the genome of each isolate, and the absence of replication products for some isolates indicates the absence of binding sites for this primer in the genome of the bacterial isolate (12).

In other studies, the percentage of pathological types that cause diarrhea in Korea is 1.3% (20), and in Mexico is 1% (21), while the results of our study are in agreement with a positive sample 11.6% for the pathogenic *E. coli* types related to virulence genes and causing diarrhea in Dohuk governorate (22).

In this study, ETEC pathotype is the most common type that existed in approximately all positive samples that were examined, especially in imported chicken meat, pastirma, imported burger, minced meat, and beef. The results of the present study show lower percentages than those reported by Taha and Yassin (22). This does not agree with the study of Rugeles and coworkers (23) in which this pattern does not appear in the studied meat samples. Several studies, on the other hand, indicate that the ETEC pathotype is the most common type in fresh meat and meat products (24). Also, many studies indicate that the intestinal *E. coli* (ETEC) in ground meat and beef samples is the result of the contamination during the slaughtering by the fecal contamination of carcasses or the contamination in restaurants using a contaminated mincing machine or by the hands of workers. This indicates that meat, especially beef, and minced meat, is the main source of ETEC pathotype which is the main cause of traveler's diarrhea episodes (25).

This study shows a small percentage of diarrhea-causing type of DAEC, which is about 1.05% of the total isolates in the studied samples. The results of the study (18,19) reveal that a high contamination rate with DEC is associated with poor hygiene conditions during slaughtering, and poor meat storage in the shops, which may pose societal health risks to local people (26).

Conclusion

Cows and buffalos are considered the main reservoirs for *E. coli* more than the rest of the other cattle types. The meat produced from these animals needs more hygienic attention and care during slaughtering and treatment by workers to avoid any potential contamination. Also, the walls and floors of slaughterhouses must be carefully cleaned to maintain a healthy environment. All these factors play a major role in the transmission of many common diseases and cases of food poisoning. Because of the large number of pathotypes and virulence factors, culture or biochemical tests cannot distinguish between the pathological strains of these bacteria. Therefore, the multiplex Polymerase Chain Reaction (mPCR) technology is used to detect the presence or absence of several genes encoding major virulence factors for the diagnosis of DEC, particularly when dealing with large numbers of samples.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgments

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التحديد الجزيئي للإشيريكيا القولونية المسببة للإسهال والمعزولة من اللحوم ومنتجاتها

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الخلاصة

تهدف الدراسة الحالية الى تشخيص الايشيريكيا القولونية المسببة للإسهال في اللحوم ومنتجاتها بالتقنية التقليدية لتفاعل البلمرة المتسلسل باستخدام جين uidA لتأكيد عزلات بكتريا الايشيريكيا القولونية وكذلك استخدام مجموعتين من البادئات المتخصصة وهي *stx1* و*stx2* و *aggR* و*esth* و *eae* و*invE* و *daaC* و *estp*و *elt* و *bfpA* للكشف عن جينات الضراوة بتقنية تفاعل البلمرة المتسلسل المتعددة الى ١٠٠ عزلة من بكتريا الايشيريكيا القولونية التي تم أخذها من ٧٨٢ عينة من اللحوم ومنتجاتها والتي شملت اللحوم الطازجة واللحوم المفرومة والبركر والبسطرمة والدجاج للفترة من شباط إلى تشرين الثاني ٢٠٢٠. تظهر النتائج أن جميع عزلات الايشيريكيا القولونية كانت موجبة للجين uidAبحزم ذات وزن جزيئي ١٤٧ زوج قاعدي. وتكشف هذه الدراسة أيضًا عن ٩٥ (١٢,١٥٪) نوعا مرتبط بجينات الفوعة باستخدام تقنية تفاعل البلمرة المتسلسل المتعددة، حيث تبين الدراسة أن أعلى نسبة من النمط المرضي هي الايشيريكيا القولونية السامة للأمعاء بمعدل ٤٦,٣٢٪ وأن أقل نسبة من النمط المرضي في هذه الدراسة يرجع إلى النوع الايشيريكيا القولونية الملتصقة بشكل مستمر وبمعدل ١,٠٥٪ بينما كانت النسبة المئوية للأنماط المرضية الأخرى ٢٠,٠٥ ، ١٤,٧٤ و ٦,٣٢ و ٦,٣٢ و ٥,٢٦٪ لكل من الايشيريكيا القولونية المنتجة لسموم الشبيهة بالشايكا والإيشريكيا القولون النزفية للأمعاء والايشيريكيا القولونية الممرضة للأمعاء غير النموذجية والإيشريكيا القولونية المعوية التكتلية والإيشريكيا القولونية الغازيـــة للأمعاء على التوالي. ويقترن التلوث بالايشيريكيا القولونية المسببة للإسهال الذي شوهد في هذه الدراسة بالظروف غير الصحية أثناء عملية الذبح وسوء تخزين اللحوم في المحلات والأسواق، مما يشكل مخاطر صحية للمستهلكين.