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Molecular Characterization of *Escherichia coli* O157:H7 and non-O157 Shiga Toxin Producing *E. coli* from Retail Meat and Humans

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Abstract

A total of 550 meat samples (300 minced beef and 250 chicken meat) marketed in Zagazig City, Sharkia Governorate, Egypt, as well as 150 human stool samples were examined for Shiga toxin producing *E. coli*. Results revealed that the isolation rates of *E. coli* O157:H7 versus non- O157 were 1.7% versus 2.3% in minced beef, 0.8% versus 2% in chicken meat and 0.7% versus 2.7% in human stools. Other identified serotypes were including O111:H8 (25%), O26:H11 (20.8%), O55:H7 (16.7%) and O113:H21 (4.2%). Virulence associated genes were identified in *E. coli* serotypes, *stx*1 and *stx*2 were characterized in 16.7% and 62.5% of the isolates, while, *eae*A and *hly*A genes were identified in 50% and 70.8% of the examined serotypes, respectively. Genotyping of *E. coli* O157:H7 serotype from different sources using Enterobacterial repetitive intergenic consensus polymerase chain reaction (ERIC-PCR) fingerprinting revealed heterogenicity of the isolates, however, human and minced beef isolates were grouped in the same cluster indicating potential transmission of infection from contaminated beef to human consumers. In conclusion, ERIC-PCR is a highly discriminatory, reliable and cost-effective tool for tracing sources of infection with bacteria. Public health education and application of strict hygienic measures during slaughtering, transportation and preparation of meat are essential to minimize the risk of contamination and transmission of infection to consumers.

Keywords: STEC, Poultry meat, Beef, Shiga toxin, *eaeA* gene, *hly* gene

Introduction

Escherichia coli is a normal intestinal microflora in humans and animals, however, certain serotypes can result in intestinal and extra-intestinal infections with manifestations ranging from mild diarrhea to severe hemorrhagic colitis (HC) and hemolytic uraemic syndrome (HUS) [1]. The pathogenicity of some *E. coli* serotypes is attributed to the acquisition of certain genes that enable them to produce different diseases [2]. According to their virulence factors, pathogenic *E. coli* have been classified to several pathotypes including Shiga toxin-producing *E. coli* (STEC) [1]. Nearly 82% of STEC strains of animal origin are related to those isolated from humans, which in turn indicate the zoonotic potential of such strains [3]. *E. coli* O157:H7 has been widely known to be directly linked with foodborne infections, however, outbreaks due to non-O157 *E. coli* have increased recently [4]. Other serogroups including *E. coli* O26, O91, O103, O111, O118, O113and O145 have been reported as STEC [5-7]. Many STEC harbor shiga toxin 1 and shiga toxin 2 genes (*stx*1and *stx*2), such genes are responsible for the damage of endothelial and tubular cells resulting in acute renal failure [8]. Moreover, the presence of *eae*A gene (*E. coli* attachment and effacement) encoding the intimin protein is essential for pathogenicity of *E. coli* resulting in a characteristic histopathological feature known as "attaching and effacing" which is associated

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with an increased risk of bloody diarrhea [9]. Other virulence factors such as enterohemolysin encoded by *hly*A gene causing enterocyte and leukocyte lysis have been reported [10]. Molecular typing is useful to determine the genetic relationship of pathogens isolated from different sources. Enterobacterial repetitive intergenic consensus polymerase chain reaction (ERIC PCR) is a simple, cost effective tool with high discriminatory power for genotyping of different bacterial pathogens [11-13]. In order to investigate the occurrence of STEC in Zagazig City, Egypt, minced beef and chicken meat purchased from food outlets and human samples from outpatient clinics at Sharkia Governorate were examined. Characterization of virulence associated genes and genotyping of the isolates from different sources were also carried out.

Material and Methods

Sampling

A total of 300 fresh minced beef and 250 fresh chicken breast meat samples obtained from retail outlets and 150 stool swabs diarrheic (n=48) and apparently healthy persons (n=102) attending the outpatient clinic of Al-Ahrar General Hospital, Sharkia Governorate were examined.

Isolation and identification of STEC

Meat samples (25 g) homogenized with 225 mL modified tryptone soya broth base (Oxoid, CM0989) containing vancomycin (40 µg/mL) as well as stool swabs inoculated into tubes containing the same media were incubated at 37°C for 18 h. Enriched cultures were plated onto Sorbitol MacConkey agar (Oxoid, CM0813) containing cefixime and potassium tellurite (Oxoid SR0172E) Twenty-five grams from meat samples were homogenized with 225mL modified tryptone soya broth base (Oxoid, CM0989) containing vancomycin (40 µg/mL) and then incubated at 37°C for 18 h. Enriched cultures were plated onto Sorbitol MacConkey agar (Oxoid, CM0813) containing cefixime and potassium tellurite (Oxoid SR0172E). The plates were then incubated at 37°C for 24 h. Presumptive colorless (suspected *E. coli* O157:H7) and/or dark pink colonies (suspected non-O157 strains) were purified and sub-cultured for confirmation. The colonies were confirmed using biochemical teste as previously described [14]. *E. coli* isolates were then sero-grouped using rapid diagnostic *E. coli* antisera sets [15] (DIFCO Laboratories, Detroit Michigan 48232-7058, USA). Serotyping was carried out at Bacteriology Department, Faculty of Veterinary Medicine, Benha University.

Table 1: Prevalence of STEC serotypes meat and human samples (n: number of samples)

Sample type	*E. coli*O157:H7	Non-O157	Total
Minced beef (n=300)	5 (1.7%)	7 (2.3%)	12 (4%)
Chicken meat (n=250)	2 (0.8%)	5 (2%)	7 (2.8%7)
Human stool (n=150)	1 (0.7%)	4 (2.7%)	5 (3.3%)
Diarrheic (n=48)	1 (2.1%)	1 (2.1%)	2 (4.2%)
Apparently healthy (n=102)	0	3 (2.9%)	3 (2.9%)

Molecular characterization

DNA extraction

Genomic DNA was extracted from the serologically typed STEC isolates using Bacterial DNA extraction kit (Spin-column) (BioTeke Corporation Shanghai, China, Catalogue # DP2001) according to the manufacturer's guidelines. The PCR was carried out at the PCR Unit, Virology Department, Faculty of Veterinary Medicine, Zagazig University.

Molecular identification of virulence associated genes

The serologically identified STEC strains were subjected to PCR for the amplification of*stx*1, *stx*2, *hly*A and *eae*A genes. The reaction was performed using oligonucleotide primers sets specific for each gene (AlphaDNA, Montreal, Quebec, Canada). Primers for the amplification of the aforementioned genes are Stx1F- 5'-AAA TCG CCA TTC GTT GAC TAC TTCT-3' and Stx1R- 5'-TGC CAT TCT GGC AAC TCG CGA TGC A-3' producing 366 bp of *stx*1 gene [16], Stx2F – 5'-CGA

TCG TCA CTC ACT GGT TTC ATC A-3' and Stx2R- 5'-GGA TAT TCT CCC CAC TCT GAC ACC-3' producing 282 bp of *stx*2 gene [16], EAE1- 5'-TGC GGC ACA ACA GGC GGC GA-3' and EAE2- 5'-CGG TCG CCG CAC CAG GAT TC-3' amplifying 629 bp of *eae*A gene [17], finally, Hly F- 5'-CAA TGC AGA TGC AGA TAC CG-3' and Hly R- 5'-CAG AGA TGT CGT TGC AGC AG-3' producing 432 bp of *hly*A gene [18].

The reactions were performed in 20 µL reaction volume using readymade 2x power *Taq* PCR master mix (BioTeke Corporation, Shanghai, China) in Primus Thermal Cycler (MWG-Biotech Thermal Cycler, Ebersberg, Germany). Amplification products were resolved in 1.5% (w/v) agarose gels along with 100 bp molecular weight ladder (BioTeke Corporation, Shanghai, China). The gels were run in 1xTBE, 5 µM ethidium bromide for at least 45 minutes at 100 volts and then visualized under ultraviolet light of a transilluminator (Spectroline, Westbury, NY, USA).

Genotyping of E. coli O157:H7 isolates

Two primer sets with the sequences ERIC-DG111-F 5'-ATG TAA GCT CCT GGG GAT TCA C-3'and ERIC-DG112-R 5'-AAG TAA GTG ACT GGG GTG AGC G-3' were used to amplify repetitive sequences contained in the chromosomal DNA of *E. coli*O157:H7 isolates using single amplification profile [19]. Depending on the presence or absence of each band, a binary code was used to transform ERIC-PCR fingerprinting data. Un weighted pair group method with arithmetic average (UPGMA) and sequential hierarchical and nested clustering routine were used to generate a dendrogram using SPSS, Inc. version 22 (IBM Corp. 2013, Armonk, NY). The Simpson's index of diversity (*D*) was used to measure the discriminatory power of ERIC-PCR [20]. *D* value of more than 0.9 indicates good differentiation.

Results

Table (1) showed that *E. coli* O157:H7 was isolated from 1.7%, 0.8% and 0.7% of minced beef, chicken meat and human stools, respectively. out of the examined minced beef and chicken meat samples, 1.7% and 0.8% were positive for *E. coli* O157:H7, respectively, while, 0.7% of the human stool samples were positive.

However, 2.3%, 2% and 2.7% of minced beef, chicken meat and human stool samples were respectively positive for non-O157 STEC.

Serotypes of the identified non-O157 STEC strains were distributed in the examined samples as shown in Table (2). The most prevalent serotype was O111:H8 (25%), followed by, O26:H11 (20.8%), O55:H7 (16.7%) and O113:H21 (4.2%).

Virulence associated genes were detected in the examined isolates using PCR (Figure 1). Table (3) shows the distribution of the investigated genes in each serotype. STEC strains with *stx*2 gene were more frequent than that carrying stx1 gene (62.5% versus 16.7%, respectively). The *eae*A and *hly*A genes, were identified in 12 (50%) and 17 (70.8%), respectively.

Table 2: Distribution of *E. coli* serotypes in meat and human samples

Serotype	Source			Total
	Minced beef	Chicken	Humans	
O157:H7	5	2	1	8 (33.3%)
O26:H11	2	1	2	5 (20.8%)
O111:H8	3	2	1	6 (25%)
O113:H21	1	0	0	1 (4.2%)
O55:H7	1	2	1	4 (16.7%)
Total	12	7	5	24

The banding patterns obtained by ERIC-PCR revealed multiple DNA fragments ranging in size between 148 and 2900 bp (Figure 2). Eight profiles were produced and they were referred to as E1 to E8. Simpson's index of diversity calculation revealed a high

discriminatory index of ERIC-PCR (D = 1). The dendrogram analysis of eight *E. coli* O157:H7 isolates of minced beef, chicken meat and human origins showed three clusters, cluster I contained 2 isolates of beef and chicken meat origin, cluster II showed 2 isolates of beef meat and human origin, while, cluster III showed 2 isolates of beef and chicken meat origin. In addition, two single isolates of beef meat origin were identified.

Discussion

Cattle are considered the main reservoir of STEC that are carried in the gastrointestinal tract without apparent symptoms [21]. Contamination of the carcass usually occurs during slaughtering, dressing and evisceration [22]. Consumption of ground beef poses more risk than intact muscles because it can be contaminated during the grinding operation [23].

E. coli O157:H7 was isolated from 1.7% of minced beef meat in the present study. Nearly similar isolation rate (1%) was reported in Belgium [5], while, higher percentages of 3.2%, 6.2%, 7.6% and 26.7% were obtained in UK [24], Serbia [22], Turkey [25] and Egypt [26], respectively. The variation in the prevalence of *E. coli* O157:H7 in these samples could be attributed to the differences in the study areas and hygienic measures during handling and mincing of meat. In contrary, Cadirci, Siriken [27] reported that *E. coli* O157:H7 was not isolated from ground beef samples in Turkey and only 0.1% of minced beef samples in France were contaminated with the pathogen [28].

Globally, the prevalence of non-O157 STEC in beef is more than the prevalence of O157, the range of isolation rates of non-O157 from beef samples at outlets was 3-62.5% [7]. In the present study, 2.3% of minced beef samples were contaminated with different serotypes of STEC other than *E. coli* O157:H7. Higher isolation rates of 16.7% in Egypt [29], 12% in New Zealand [30], 12% in Spain [31] and 36.4% in Canada [32] have previously been reported.

Table 3: Distribution of virulence genes (*stx1, stx2, eaeA, hly*) in the identified *E. coli* O157:H7 andnon-O157 isolates

Serotype	Sources	Virulence associated genes			
		***Stx*1**	***Stx*2**	***eae*A**	***hly*A**
O157:H7	Minced beef	+	+	+	+
O157:H7	Minced beef	-	+	-	+
O157:H7	Minced beef	-	+	-	+
O157:H7	Minced beef	+	+	+	-
O157:H7	Minced beef	+	+	-	+
O157:H7	Chicken	-	+	+	+
O157:H7	Chicken	-	+	+	+
O157:H7	Humans	+	+	+	+
O26:H11	Minced beef	-	-	-	-
O26:H11	Minced beef	-	-	-	-
O26:H11	Chicken	-	-	-	-
O26:H11	Humans	-	-	+	+
O26:H11	Humans	-	+	-	+
O111:H8	Minced beef	-	+	-	+
O111:H8	Minced beef	-	+	-	-
O111:H8	Minced beef	-	+	+	-
O111:H8	Chicken	-	-	+	-
O111:H8	Chicken	-	+	-	+
O111:H8	Humans	-	-	-	+
O113:H21	Minced beef	-	-	+	+
O55:H7	Minced beef	-	-	+	+
O55:H7	Chicken	-	+	+	+
O55:H7	Chicken	-	+	+	+
O55:H7	Humans	-	-	-	+
Total		4 (16.7%)	15 (62.5%)	12 (50%)	17 (70.8%)

The role of chicken meat as a potential source of *E. coli* O157:H7 has been previously reported [33-35]. The isolation rate of *E. coli* O157:H7 from chicken meat samples during the current study (0.8%) was lower than the prevalence of 4% [36], 5% [37] and 1.3% [38] reported also in Egypt. Studies in other countries have also shown different isolation rates, for example1.1% in Turkey [33], 3% in China [39], 7.6% in Argentina [40] and 7.4% in Iran [35].

Non-O157:H7 STEC of serotypes O111:H8, O26:H11 and O55:H7 were identified in 2% of chicken meat samples in the current study. In Iran, 34.6% of chicken meat samples were contaminated with STEC of different serogroups including O111 (1.2%), O26 (11%) [35]. In another study in Korea, *E. coli* other than O157 were identified in 4.6% of chicken meat samples [41].

In the current study, *E. coli* O157:H7 was isolated from 0.7% of human stool samples. This is similar to studies carried out in South Africa [42] and Canada where prevalence was reported to be 0.8% and 2.3%, respectively. However, higher isolation rate (7.5%) was reported in a different study in South Africa [43]. The isolation of non-O157 strains from human stools (2.7%) in the present study was comparable to 2.5% reported in Canada [44] and USA, where 4.2% of stool sample were positive [45].

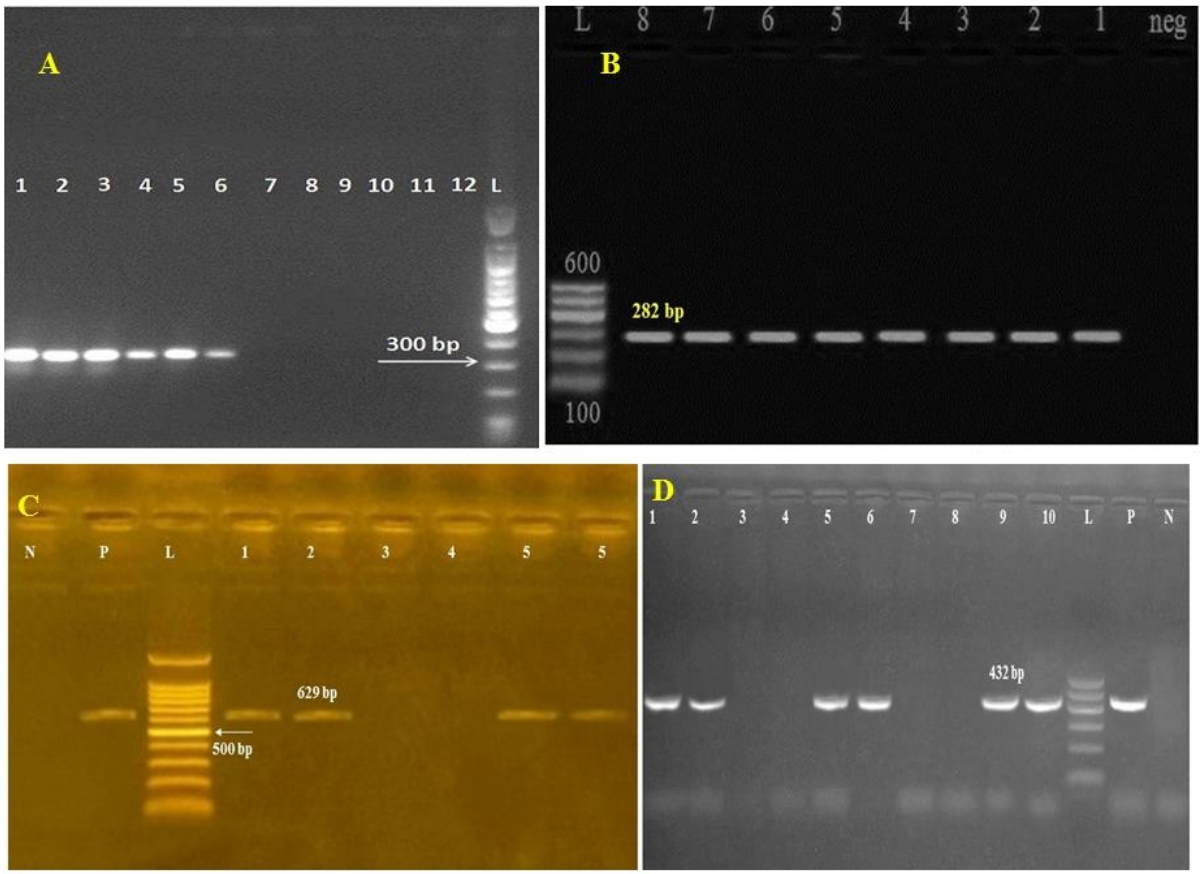

Figure 1: Sample of gel picture showing amplification of different virulence associated genes in *E. coli* isolates. A: stx1 gene amplified 366 bp product, B: *stx*2 gene amplified 282 bp product, C: *eae*A gene amplified 629 bp product, D: *hly*A gene amplified 432 bp product. L: 100 bp ladder, Numbers indicated positive and negative samples, P: positive controls kindly obtained from Microbiology Department, Faculty of Veterinary Medicine, Zagazig University. N: negative controls.

Serotyping revealed that O111:H8 was the most prevalent serogroup followed by O26:H11, O55:H7 and O113:H21. These serotypes are associated with human illness [46]. Another study in Egypt reported similar findings [29]. They reported that the distribution of the identified 18 isolates from beef samples was 7 (38.9%) O111:H8, 6 (33.3%) O26:H11, 2 (11.1%) O111: He and one (5.56%) for each of O55:H7, O126:H5, and O128:H2. In human stool samples, O157:H7, O26:H11, O111:H8 and O55:H7 were identified. In accordance, the most strains of EHEC isolated from humans in Japan are belonging to serotypes O157, O26, O111, and O128 [47]. *E. coli* O26:H11/He was found to be the most often serotype associated with HUS development in 40% of patients [48].

In the current study, four *E. coli* O157:H7 isolates harbored *stx*2 gene only (50%), while, the other 4 (50%) were positive for *stx*1/*stx*2 genes. However, 5 (62.5%) and 7 (87.5%) carried *eae*A and *hly*A genes, respectively. The present findings supported that most *E. coli* O157 isolates harbored *stx*2 [27]. *E. coli* O157:H7 isolates harboring all investigated genes were isolated from the case of diarrhea. This was consistent with other findings [42]. However, a study reported *E. coli* O157:H7 isolate from diarrheic patient without *stx* genes [49]. Thus, pathogenicity of *E. coli* O157:H7 depends on the production of different virulence factors in addition to physiological factors in the host [42].

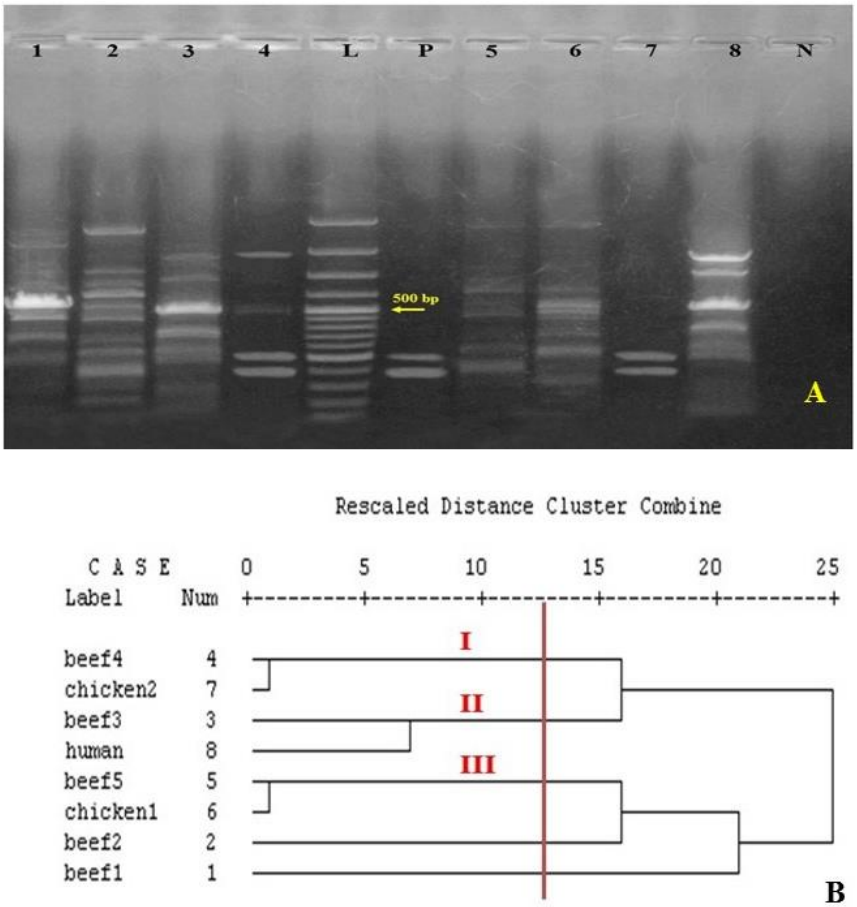

Figure 2: A: ERIC-PCR fingerprinting of 8 *E. coli* O157:H7 isolates of different origins in 1.5% agarose gel, L: 100 bp Molecular marker, N: Negative control. B: Dendrogram showing the relatedness of the isolates as determined by ERIC-PCR fingerprinting using SPSS computer software program.

Among non-O157 STEC isolates, 7 (43.8%), 6 (37.5%) and 10 (62.5%) harbored *stx*2, *eae*A and *hly*A genes, respectively. In Egypt, 9 (50%), 11 (61.1%), and 9 (50%) non-O157 STEC strains harbored*stx*1,*stx*2, and *eae*A genes, respectively [29].In France, STEC isolated from fresh beef showed that *stx*1, *stx*2 and both genes were identified in 39%, 50% and 11% of the isolates, while, none of them carried *eae*A gene [50].Another study in Korea, revealed that STEC from fresh beef carried *stx*2 (64%), both *stx*1 and *stx*2 (14%), both s*tx*2 and *eae*A (14%) and both *stx*1 and *eae*A (7%) [41]. While, in Germany, STEC isolated from meat harbored *stx*1, *stx*2 and *eae*A with the respective percentages of 40, 80.8, 5% [51].

Strains that carry only *stx*2 are more virulent than those carrying only *stx*1 or even both *stx*1 and *stx*2, and are more associated with cases of HUS in humans [29, 46, 52]. Production of Stx2 is mainly associated with HUS, moreover, this toxin is reported to be 1000 times more cytotoxic than Stx1 on human renal endothelial cells [53]. While, *eae*A gene was reported in STEC strains associated with severe human illnesses such as bloody diarrhea and HUS [46, 54, 55]. However, STEC strains lacking *eae*A gene have been reported to cause outbreaks [56].

The higher frequency of *stx*2 isolation in STEC isolates from beef was in accordance with other studies [23, 55, 57, 58]. However, in beef, *eae*A gene was reported in 100% of STEC O157:H7 and 19% of non-O157 isolates [31].In contrary, all STEC strains from meat products in Australia were negative for *eae*A gene [59].

The detection of *eaeA* gene in *E. coli* O157:H7 isolates in the current study was consistent with 54.7% reported by Ojo, Ajuwape [60] in Nigeria. The presence of this gene in STEC strains has been strongly correlated to their implication in severe illness resulting in HUS [60, 61]. However, some STEC strains that are *eaeA* negative also cause sporadic HUS cases [60, 62].

Several studies have also reported inconsistent carriage percentages of *stx1, stx2* and *eae*A genes in *E. coli* O157:H7 strains. For instance, in Thailand, two *E. coli* O157:H7 isolates recovered from chicken meat were negative for Shiga like toxin production [63]. Whereas, in Iran, all 31 *E. coli* O157:H7 isolates from chicken meat were positive for *stx1* and *eaeA*, while negative for *stx2* [35].In a Czech study, only 40.9% of *E. coli* O157:H7 isolated from poultry meat samples were positive for *stx1, stx2* and *eaeA* [64].

ERIC-PCR has been used previously for genotyping of different bacteria including *E. coli* isolates of different origins in order to determine the genetic relationship between the isolates [11, 12, 65].

The present study revealed that *E. coli* O157:H7 isolates of beef, chicken and meat origin were of different genotypes indicating heterogeneity. However, the presence of human and beef meat isolate in the same cluster indicated the possibility of potential *E. coli* transmission from contaminated beef meat to human consumers. Another study on *E. coli* O157:H7 isolates reported that 94 isolates of pig, pork, cattle, beef, water and human origin in South Africa were arranged into eight clusters with similarities ranged from 72-91% between the isolates from animal feces and meat samples indicating the possibility of cross contamination [65]. They also concluded that ERIC-PCR genotyping is reliable in comparing *E. coli* O157:H7 isolates of different sources.

Conclusion

It could be concluded from the current study that STEC are prevalent in chicken and beef meat sold in the study area as well as in their consumers. These results substantiate the role of chicken and beef meat in transmitting these pathogens to human. The study recommended using of ERIC-PCR for genotyping of the isolates from different sources due to its reliability, cost effectiveness and high discriminatory power.

Conflict of interest

The authors declare no conflict of interest.

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المخلص العربي

التوصيف الجزيئي للايشيريشيا كولاي O157:H7 والايشيريشيا كولاي الغير O157 المنتجة لسموم الشيجا من اللحوم المسوقة ومن الإنسان

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تم جمع ٥٥٠ عينة من ا للحوم (٣٠٠ من لحم البقر المفروم و ٢٥٠ من لحم الدجاج) المسوقة في مدينة الزقازيق، فضلا عن ١٥٠ عينة من البراز البشري لفحصها بكتيريولوجياً لمعرفة مدى تواجد ميكروب الإيشيريشيا كولاي بها. أوضحت النتائج ان نسبة الإيشيريشيا كولاي O157: H7 و غير O157 كانت معزولة بنسبتي ١.٧٪ و ٢.٣٪ من لحم البقر المفروم، بينما تم تحديدها في ٠.٨٪ و ٢٪ من عينات لحم الدجاج. وبالإضافة إلى ذلك، تم عزلها من مسحات البراز البشري بمعدل ٠.٧٪ و ٢.٧٪ على التوالي. تم أيضا تحديد بعض الأنماط المصلية الأخري مثل: O111: H8 (٢٥%) ، O26: H11 (٢٠.٨%)، O55: H7 (١٦.٧%) وO113: H21 (٤.٢%) في العينات التي تم فحصها. وقد تم التعرف على الجينات المرتبطة بالضراوة في الأنماط المصلية للإيشيريشيا كولاي ومنها *stx*1 و *stx*2 في ١٦.٧٪ و ٦٢.٥٪ من العزلات، في حين تم تحديد جينات *eae*A و *hly*A في ٥٠٪ و ٧٠.٨٪ من العزلات على التوالي. تم استخدام بصمات ERIC-PCR للكشف عن الأنماط الجينية للإيشيريشيا كولاي O157: H7 من مصادر مختلفة ومع ذلك، تم تجميع العزلات البشرية ولحم البقر المفروم في نفس المجموعة مما يشير إلى إحتمال إنتقال العدوى من لحوم البقر الملوثة إلى المستهلكين. وأكدت هذه الدراسة ERIC-PCR أداة متميزة وفعالة لتعقب مصادر العدوى البكتيرية. ويعتبر التثقيف الصحي العام وتطبيق تدابير صحية صارمة أثناء ذبح اللحوم ونقلها وإعدادها أمرا ضروريا للحد من خطر التلوث ونقل العدوى إلى المستهلكين.