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Prevalence, genetic characterization and virulence genes of sorbitol-fermenting Escherichia coli O157:H- and *E. coli* O157:H7 isolated from retail beef

Khalid Ibrahim Sallam [a,*], Mahmoud Ahmed Mohammed [a], Asmaa Mohammed Ahdy [a], Tomohiro Tamura [b]

[a] Department of Food Hygiene and Control, Faculty of Veterinary Medicine, Mansoura University, Mansoura 35516, Egypt
[b] Bio-production Research Institute, National Institute of Advanced Industrial Science and Technology (AIST), 2-17-2-1 Tsukisamu-Higashi, Toyohira-ku, Sapporo 062-8517, Japan

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ABSTRACT

Sorbitol-fermenting (SF) *Escherichia coli* O157:H- strains have emerged as important pathogens and have been associated with a higher incidence of progression to hemolytic–uremic syndrome (HUS) than non-sorbitol fermenting (NSF) *E. coli* O157:H7. The present study was carried out to determine the prevalence of SF *E. coli* O157:H- and NSF *E. coli* O157:H7 strains in retail beef products in Mansoura, Egypt. The contamination rates with $rfbE_{O157}$-positive *E. coli* O157 strains were 26.7% (8/30), 10% (3/30) and 3.7% (1/27) in ground beef, beef burger, and fresh beef samples, respectively with an overall mean of 13.8% (12/87) among all meat products tested. SF *E. coli* O157:H- were the most dominant among the isolated O157 strains. Of the fifteen O157 strains isolated, 11 (73.3%) were SF *E. coli* O157:H-, while the remaining 4 (26.7%) were NSF *E. coli* O157:H7. The 11 SF O157H- strains were genetically positive for *sfpA* gene. Restriction fragment length polymorphism (RFLP) analysis for *fliC* gene demonstrated a similar pattern for both SF and NSF O157 isolates. PCR assays verified the existence of *stx*1 gene in 7 (46.7%) and *stx*2 gene in 13 (86.7%) of the 15 O157 strains isolated. Unexpectedly, two of the 15 O157 strains isolated were negative for Shiga toxin genes. The *eae* gene was identified in all of the 15 O157 strains except in one NSF O157:H7 strain. EHEC-*hlyA* gene was detected in 14 (93.3%) of the 15 O157 isolates, nonetheless only 11 strains showed enterohemolytic phenotype on blood agar. A combination of the four virulence genes, *stx*1, *stx*2, *eae* and EHEC-*hlyA* were detected in 7 (46.7%) strains, while six (40%) strains were positive for *stx*2, *eae* and *hlyA* genes. This is the first record for isolation of *E. coli* O157: H- in Egypt as well as in the African continent.

1. Introduction

Enterohemorrhagic *Escherichia coli* (EHEC), particularly *E. coli* O157:H7 and O157:NM (nonmotile; referred to herein as *E. coli* O157:H-), has become a major pathogen associated with food-borne illness worldwide. *E. coli* O157:H7 was first recognized as a pathogen when an outbreak of unusual gastrointestinal illness traced to contaminated hamburgers in 1982 in the United States (Riley et al., 1983). *E. coli* O157:H7 is usually identified by classical microbiological diagnostic procedures based on its inability to ferment sorbitol.

Non-sorbitol-fermenting (NSF) *E. coli* O157:H7 are the primary Shiga toxin (STx)-producing *E. coli* (STEC) strains and are recognized as important worldwide food-borne pathogens associated with human diseases, including diarrhea, hemorrhagic colitis (HC), and hemolytic–uremic syndrome (HUS). Sorbitol-fermenting (SF) STEC O157:H- strains, however, have emerged as important pathogens and have been associated with a higher incidence of progression to HUS than NSF STEC O157:H7 especially in continental Europe (Ammon et al., 1999; Karch and Bielaszewska, 2001; Rosser et al., 2008). Several genomic and phylogenetic studies demonstrated a high heterogenetity of Stx-harboring strains of the O157 serogroup (Bono et al., 2012; Jenke et al., 2012).

SF STEC O157:H- strains were first recognized causing HUS in children in Bavaria, Germany, in 1988 (Karch and Bielaszewska, 2001), and since then, these strains have been isolated as a cause of human diseases, including life-threatening HUS, from patients in different European countries including Germany (Ammon et al., 1999; Schmidt et al., 1999), the Czech Republic (Bielaszewska et al., 1998, 2000), Hungary (Karch and Bielaszewska, 2001), Finland (Eklund et al., 2006), Austria (Orth et al., 2006), Ireland (Garvey et al., 2006), Belgium (Buvens et al., 2009), and Norway (Haugum et al., 2012). Isolation of SF STEC O157:H- was not reported outside continental Europe until 2002, when it was isolated from patient clinically diagnosed with HUS in Australia (Bettelheim et al., 2002).

The pathogenicity of both SF STEC O157:H- and NSF STEC O157:H7 strains is associated with several virulence factors. The main factors contributing to their pathogenicity, including Shiga toxins 1 and 2 (encoded by *stx1* and *stx*2 genes), intimin (encoded by *eae* gene),

* Corresponding author. Tel.: +20 1000479670.
E-mail address: khsallam@hotmail.com (K.I. Sallam).

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and EHEC hemolysin (encoded by *Ehly* gene). Most of the isolates from both groups of STEC O157 pathogens possess *stx*2 and *eae* genes (Karch and Bielaszewska, 2001).

Failure to implement appropriate farm management practices and/or strict sanitary conditions during the production process, handling and marketing of meat usually facilitates transfer of *E. coli* O157 to the meat and its associated food products. Cattle's feces and hides are considered to be sources of enterohemorrhagic *E. coli* contamination of carcasses during slaughter and during removal of the hide or the gastrointestinal tract. Contaminated bovine carcasses during slaughter and butchering allow the organisms to be thoroughly mixed into beef when it is ground into beef burger.

Until recently, very few studies have been done in developing countries, including Egypt, about the prevalence of STEC O157 in food products. The aim of the present study was to determine the prevalence of both NSF *E. coli* O157:H7 and SF *E. coli* O157:H- in retail beef products in Mansoura, Egypt, and to investigate their potential to cause severe infection in humans by detecting some virulence genes including *stx*1, *stx*2, *eae* and EHEC-*hlyA* genes in their genome.

2. Materials and methods

2.1. Collection of samples

Eighty seven beef meat samples, including 27 fresh beef, 30 ground beef, and 30 beef burger samples were purchased on 5 occasions, during the period of December 2010 through September 2011, from different supermarkets and butchers' shops at Mansoura city, Egypt. Fresh beef samples are fresh meat taken from cattle carcasses slaughtered and dressed at municipal abattoir then transported to butchers' shops, wherein they are sold, while ground beef and beef burger samples are meat products usually manufactured from imported frozen beef and sold in a deep frozen state under different market names. Each individual sample was aseptically packed into a polyethylene bag then transferred to the laboratory of Food Hygiene and Control Department, Faculty of Veterinary Medicine, Mansoura University, wherein the conventional bacteriological analyses were done.

2.2. Isolation and identification of E. coli O157 strains

Twenty five grams from each meat sample were blended with 225 ml of sterile modified tryptone soya broth (Oxoid, CM0989) containing vancomycin (40 μg/ml) followed by incubation at 37 °C for 18 h. Enriched culture was plated onto sorbitol MacConkey agar (Oxoid, CM0813) supplemented with cefixime and potassium tellurite (Oxoid, SR0172E). The plates were incubated at 37 °C for 24 h and examined for typical *E. coli* O157:H7 colonies (colorless, circular and entire edge colonies with brown centers), in addition to the pink colonies of sorbitol-fermenting strains. Ten colorless colonies of presumptive *E. coli* O157:H7 (non-sorbitol-fermenting; NSF) and 10 pink colonies (sorbitol-fermenting; SF) were picked and sub-cultured onto nutrient agar (Oxoid, CM0003s) slopes for identification.

A total of 980 colonies from both SF (pink) and NSF (colorless) strains were subjected to various biochemical tests intended for conventional identification of *E. coli* included indole, methyl red, Voges-Proskauer, and citrate (IMViC). *E. coli* O157 positive strains were further tested for sorbitol fermentation, glucuronidase activity, and enterohemolysin production. D-Sorbitol fermentation was examined in the tube test, while the β-glucuronidase activity was tested in 4-methylumbelliferyl-p-D-glucuronide containing Fluorocult BRILA broth (Merck, Darmstadt, Germany). Detection of the enterohemolytic phenotype was performed on blood agar plates containing 5% of washed sheep erythrocytes. Appearance of a narrow turbid zone of hemolysis within 18–24 h incubation at 37 °C was regarded as a positive result.

2.3. Serological identification of isolated E. coli strains

Serological characterizations of *E. coli* O157 isolates were performed according to the manufacturer's directions at the Center of Food Analysis, Faculty of Veterinary Medicine, Benha University, Egypt. *E. coli* O157 latex test kit (Oxoid, DR0620M) was used for the identification of *E. coli* O157, while *E. coli* H Antiserum H7 (Difco Laboratories, Detroit, Michigan, USA) was used for the identification of *E. coli* O157:H7.

2.4. Molecular characterization of E. coli O157

All of the one hundred isolates that were conventionally identified as *E. coli* strains were tested for the presence of $rfbE_{O157}$. Positive strains for $rfbE_{O157}$ were further analyzed for the detection of *sfpA* gene, besides some selected virulence genes including *eae*, *stx*1, *stx*2 and EHEC-*hlyA*. Primer sets for PCR amplification of the target genes, as well as the condition adopted for their amplification are listed in Table 1. The oligonucleotide primers were synthesized by Hokkaido System Science Co. Ltd. (Hokkaido, Sapporo, Japan). The reference strains used for PCR were provided by Prof. Trout Tobe (Department of Microbiology and Immunology, Graduate School of Medicine, Osaka University, Osaka, Japan). The reference strains were *E. coli* O157:H7 Sakai (positive for $rfbE_{O157}$, *eae*, *stx1*, *stx2* and EHEC-*hlyA* genes) and *E. coli* K12DH5α (a nonpathogenic negative control strain) that does not possess any virulence gene.

Genomic DNA of *E. coli* strains was isolated by the method reported by Choo et al. (2007) and used as a template for PCR assays. PCR for detection of the target genes was operated with the use of GeneAmp PCR system 2700 thermal cycler (Applied Biosystems, Foster City, CA, USA). The optimized protocol was carried out with a 10-μl mixture contained 0.8 μl *E. coli* DNA template, 1 μl each of forward and reverse primers (3.2 pmol each), 2 μl dNTPs (2 mM), 5 μl of 2 × PCR Buffer for KOD FX, and 0.2 μl KOD FX DNA Polymerase. After an initial denaturation at 94 °C for 2 min, 35 cycles were performed under the conditions specified in Table 1 for the various tested genes, followed by a final extension at 68 °C for 5 min. PCR products of each reaction mixture were separated by subjecting 4 μl aliquots to agarose (1.5%) gel electrophoresis for 30 min at 100 V followed by a 25-min staining in ethidium bromide solution. The separated PCR products were visualized and photographed under UV light.

2.5. fliC PCR-RFLP analysis

The *fliC* PCR-restriction fragment length polymorphism (RFLP) was performed to test whether or not the H- strains really belong to the H7 clone like typical NSF O157. PCR amplification of the *fliC* gene was carried out with the use of a specific primer pairs at the cycling conditions specified in Table 1. The amplified *fliC* gene was digested with *RsaI* (New England Biolabs, Inc., USA) for 2 h at 37 °C. Restricted fragments were separated on a 2% agarose gel and visualized under UV light after staining with ethidium bromide.

3. Results and discussion

3.1. Prevalence of E. coli O157 in beef products

Of the 980 colonies isolated from the examined meat products, only 100 colonies were biochemically identified as *E. coli*. These 100 strains were tested by PCR for the presence of $rfbE_{O157}$ gene, the specific gene for genetic identification of *E. coli* O157, and the results indicated that $rfbE_{O157}$ gene was present in 15 strains (Fig. 1A). Among the 87 beef product samples tested in the present study, 12 (13.8%) samples, were positive for *E. coli* O157 strains. Fresh beef from butchers' shops was the lowest contaminated product with *E. coli* O157. Of the 27 fresh beef samples tested, only one (3.7%)

Table 1
Oligonucleotide primers and PCR cycling conditions adopted for amplification of the various target genes in isolated O157 *E. coli*.

Gene	Oligonucleotide primer sequences and their positions in the CDS of the gene[a]	Genbank accession number	PCR cycling (35 cycles) conditions	Size of amplified DNA (bp)	Reference
$rfbE_{O157}$	F: 5′-gcggaacaaaaccatgtgca-3′ (127–146) R: 5′-actggccttgtttcgatgag-3′ (907–926)	S83460	98 °C for 10 s 56 °C for 30 s 68 °C for 60 s	800	This study
sfpA	F: 5′-gccagccaaggccaagggattatt-3′ (61–84) R: 5′-gttagcaacagcagtgaagtctcc-3′ (481–504)	AF401292	98 °C for 10 s 57 °C for 30 s 68 °C for 45 s	444	This study
fliC	[b]F: 5′-*cggat*ggcacaagtcattaatacc-3′ (1–21) R: 5′-ttaaccctgcagcagagacagaa-3′ (1736–1758)	AM228905	98 °C for 10 s 58 °C for 30 s 68 °C for 90 s	1758	This study
*stx*1	F: 5′-tcctggtacaactgcggttac-3′ (366–386) R: 5′-acgcactcttccatctaccg −3 (851–870)	AB048231	98 °C for 10 s 58 °C for 30 s 68 °C for 45 s	505	This study
*stx*2	F: 5′-ctggcgttaatggagttcagtgg-3′ (484–506) F: 5′-cctgtcgccagttatctgaca −3′ (844–864)	EF079674	98 °C for 10 s 59 °C for 30 s 68 °C for 45 s	381	This study
eae	F: 5′-ctgaacggcgattacgcgaa-3′ (544–563) R: 5′-ccagacgatacgatccag-3′ (1444–1461)	–	98 °C for 10 s 57 °C for 30 s 68 °C for 60 s	917	Reid et al., 1999
EHEC-*hlyA*	F: 5′-cagcatcatcaagcgtacgtt-3 (68–88) R: 5′-gcttgctgatacagcagaaag-3′ (703–723)	X79839	98 °C for 10 s 57 °C for 30 s 68 °C for 45 s	656	This study

F: Forward; R: Reverse.
[a] Positions of the primer sequences calculated from the initiation codon of the open reading frame (ORF) of the gene.
[b] The first 3 nucleotides (in italics) are extra nucleotide that were added to get a melting temperature for the forward primer comparable to that of the reverse primer for proper amplification of *fliC* gene.

sample was positive for O157. This prevalence for *E. coli* O157 in our fresh beef is consistent with other reports for fresh retail beef cuts from China (Zhou et al., 2002), Palestine (Adwan and Adwan, 2004) and Ireland (Carney et al., 2006). Much higher contamination rate of 36% (9/25) for *E. coli* O157 was reported in retail fresh beef in Malaysia (Radu et al., 1998), while relatively higher contamination rates of 8%, 8.2%, and 9.1% were verified in fresh beef from Ethiopia (Hiko et al., 2008), Iran (Rahimi et al., 2012), and India (Dutta et al., 2000), respectively. On the contrary, lower prevalence of 1.1% was reported for *E. coli* O157 in retail beef cuts from UK (Chapman et al., 2000). Also, lower occurrences of 1.02%, and 1.4% were identified in beef carcasses from Belgium (Tutenel et al., 2003) and UK (Chapman et al., 2001),

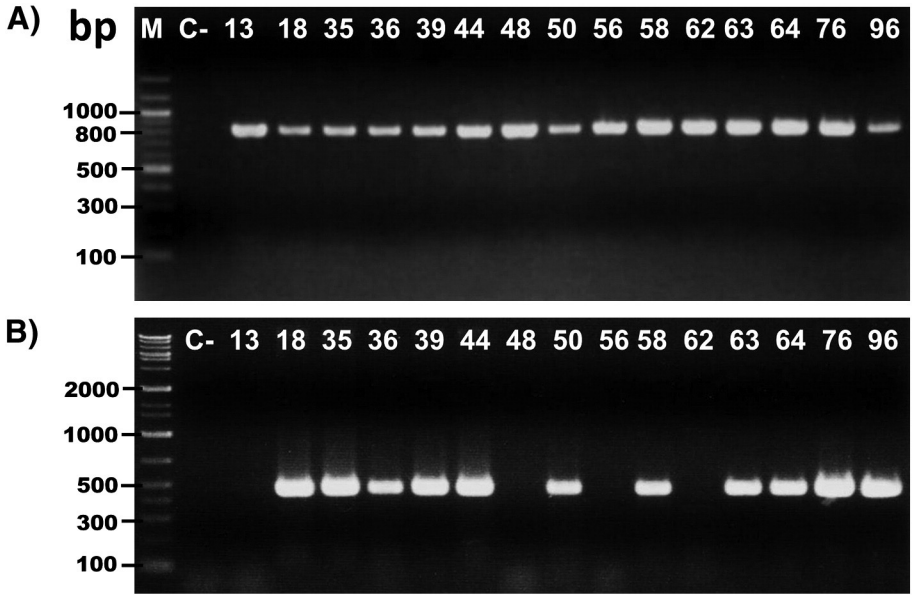

Fig. 1. Agarose gel electrophoresis for PCR products of $rfbE_{O157}$ and *sfpA* genes in *E. coli* O157 isolates. Four microliters from the amplified DNA at the expected molecular size of 800 bp for $rfbE_{O157}$ gene (A) and 444 bp for *sfpA* gene (B) were separated by electrophoresis on 1.5% agarose gel and visualized under UV light. M: DNA marker used as a reference for fragment size; Lane C-: *E. coli* K12 DH5α as a negative control strain; Lanes with the key numbers from 13 to 96 represented the positive strains for target genes.

Table 2
Phenotypes, serotypes and virulent genes in molecularly identified NSF *E. coli* O157:H7 and SF *E. coli* O157:H- strains.

Meat products	Isolates number	Serotyping	Colony on SMAC	Sorbitol test	β-glucuronidase activity	Enterohemolytic phenotype	*rfbE*$_{O157}$ gene	*sfpA* gene	*eae* gene	*Stx*1 gene	*stx*2 gene	*hlyA* gene
Fresh beef	96	O157:H-	Pink	+	+	+	+	+	+	+	+	+
Ground beef	39	O157:H-	Pink	+	+	+	+	+	+	+	+	+
	44	O157:H-	Pink	+	+	+	+	+	+	+	+	+
	48	O157:H7	Colorless	−	−	−	+	−	+	+	+	+
	50	O157:H-	Pink	+	+	+	+	+	+	+	+	+
	56	O157:H7	Colorless	−	−	−	+	−	+	−	+	+
	58	O157:H-	Pink	+	+	+	+	+	+	+	+	+
	62[a]	O157:H7	Colorless	−	−	−	+	−	−	−	−	−
	63[a]	O157:H-	Pink	+	+	+	+	+	+	−	−	+
	64[a]	O157:H-	Pink	+	+	+	+	+	+	−	+	+
	76	O157:H-	Pink	+	+	−	+	+	+	−	+	+
Beef burger	13	O157:H7	Colorless	−	−	+	+	−	+	+	+	+
	18	O157:H-	Pink	+	+	+	+	+	+	−	+	+
	35[b]	O157:H-	Pink	+	+	+	+	+	+	−	+	+
	36[b]	O157:H-	Pink	+	+	+	+	+	+	−	+	+

[a] Isolates were originated from a single ground beef samples.
[b] Isolates were originated from a single beef burger sample.

respectively. This difference could be referred to the difference in the hygienic measures and sanitation levels implemented during slaughter, handling and preparation of retail beef meat cuts.

Unlike fresh beef, which showed low prevalence for *E. coli* O157, a higher contamination rate of 26.7% (8/30) was detected in the ground beef analyzed. In a previous study from Egypt, Abdul-Raouf et al. (1996) detected O157 *E. coli* in ground beef at a lower occurrence of 6% (3/50). On the contrary to our high prevalence, many researchers from different countries could not isolate O157 strains from ground beef. Tarr et al. (1999) in Seattle, USA could not find O157 in any of the 1400 ground beef samples analyzed. Likewise, Fantelli and Stephan (2001) in Switzerland, and Barlow et al. (2006) in Australia could not identify O157 *E. coli* in any of the 400 and 285 minced beef samples analyzed, respectively. Other worldwide studies, however, revealed very low contamination rates of about 1% or less for O157 *E. coli* in ground beef. For instance, 0.12% (4/3450) in France (Vernozy-Rozand et al., 2002), 0.18% (1/549) in Belgium (Tutenel et al., 2003), 0.43% (9/931) in Italy (Conedera et al., 2004), 1.1% (13/1120) in UK (Chapman et al., 2000), 1.1% (6/571) in the Netherlands (Heuvelink et al., 1999), 1% (8/785) in Spain (Mora et al., 2007), and 1% (1/100) in Turkey (Cadirci et al., 2010).

Of the 30 beef burger samples analyzed in the present study, 3 (10%) samples were contaminated with *E. coli* O157. Similar finding was reported in Argentina (Chinen et al., 2009), where *E. coli* O157 was isolated from 10.1% (9/89) of the examined uncooked beef burger samples. Lower occurrences of *E. coli* O157, however, were reported in UK by Chapman et al. (2000), who detected O157 in 1.2% (13/1120) of the beef burger tested, and also in Italy by Stampi et al. (2004), who identified O157 in 3.3% (1/30) of the examined hamburger. Likewise, Cadirci et al. (2010) isolated O157 from 4% (4/100) of the meat balls tested in Turkey.

The higher contamination rate of *E. coli* O157 strains in ground beef and beef burger samples in comparison to fresh retail beef cuts in this study may be attributed to the fact that these two products were manufactured from frozen meat which imported from Europe where SF *E. coli* O157 is rather common (Bielaszewska et al., 2000; Rosser et al., 2008; Alpers et al., 2009). Additionally, it is possible that the contamination of ground beef or beef burgers occurred during their production process. It is also suspected that mixing of meat from several cows, when only one carcass is fecally contaminated with *E. coli* O157 may result in contamination of the whole batch of minced meat.

Few studies are published about the occurrence of SF *E. coli* O157:H- strains in food. Such strains are missed by diagnostic procedures recommended for the detection of NSF *E. coli* O157:H7, and their significance in many parts of the world might thus be underestimated (Karch and Bielaszewska, 2001). It is evident from our results (Table 2) that SF *E. coli* O157:H- were the most dominant among the isolated O157 strains. Of the 15 O157 strains isolated, 11 were SF *E. coli* O157:H-, while the remaining 4 were NSF *E. coli* O157:H7. Three of the O157:H7 strains were isolated from 3 ground beef samples, while the fourth was isolated from a single beef burger sample. Among the eleven SF *E. coli* O157:H- identified, seven were isolated from 6 ground beef samples, three from 2 beef burger samples and one strain from a single fresh beef sample. All SF *E. coli* O157:H- were positive for sorbitol fermentation as well as for glucuronidase activity

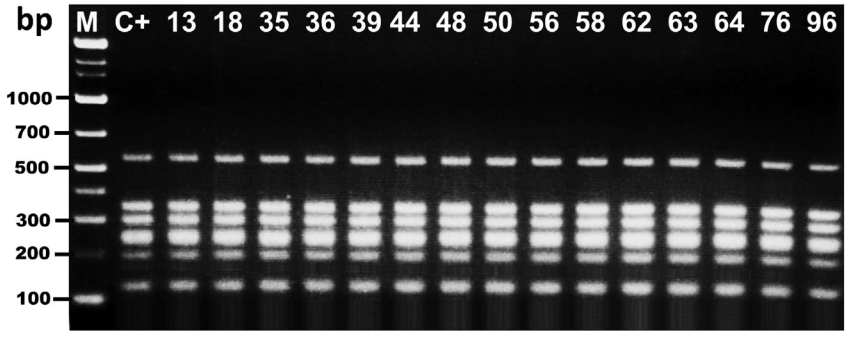

Fig. 2. Agarose gel electrophoresis showing RFLP pattern for *fliC* gene of SF O157:H- and NSF *E. coli* O157:H7 strains. *fliC* gene was PCR-amplified followed by restriction with *Rsa*I. Restricted fragments were separated by electrophoresis on 2% agarose gel. M: DNA marker (Gene Ladder Wide 1); Lane C+: *E. coli* O157:H7 Sakai (EHEC) as a reference strain; Lanes with the key numbers 13, 48, 56 and 62 represented NSF O157:H7 strains. The other 11 lanes represented SF O157:H- strains.

(Table 2). Similar to our finding concerning the dominance of SF O157:H- over NSF O157:H7, Rahimi et al. (2012) identified 13 SF O157:H- strains among the 14 O157 *E. coli* isolated from fresh meat of different ruminant species in Iran. Likewise, Cadirci et al. (2010) isolated 5 SF *E. coli* O157:H- strains from ground beef and meat ball samples from Turkey, while they could not isolate NSF O157:H7 from any sample.

Some evidence suggests that SF O157:H- infection more frequently progresses to HUS than that by NSF O157:H7 (Alpers et al., 2009; Rosser et al., 2008). Outbreaks caused by SF O157 strains are dominated by children with HUS who require more sessions of hemodialysis and have a higher risk of dying than do HUS patients infected with *E. coli* O157:H7 (Ammon et al., 1999; Alpers et al., 2009). SF O157 strains were reported to show more potent adherence to colonic epithelial cells and may, therefore, cause a more severe inflammatory host response, resulting in a higher risk for HUS (Rosser et al., 2008). The existence of SF *E. coli* O157:H- in beef products originated from cattle in the present study indicated that beef is a vehicle for infection by O157:H-, and substantiated the previous studies, which identified cattle as a reservoir for such dangerous pathogen (Bielaszewska et al., 2000; Lee and Choi, 2006; Orth et al., 2006).

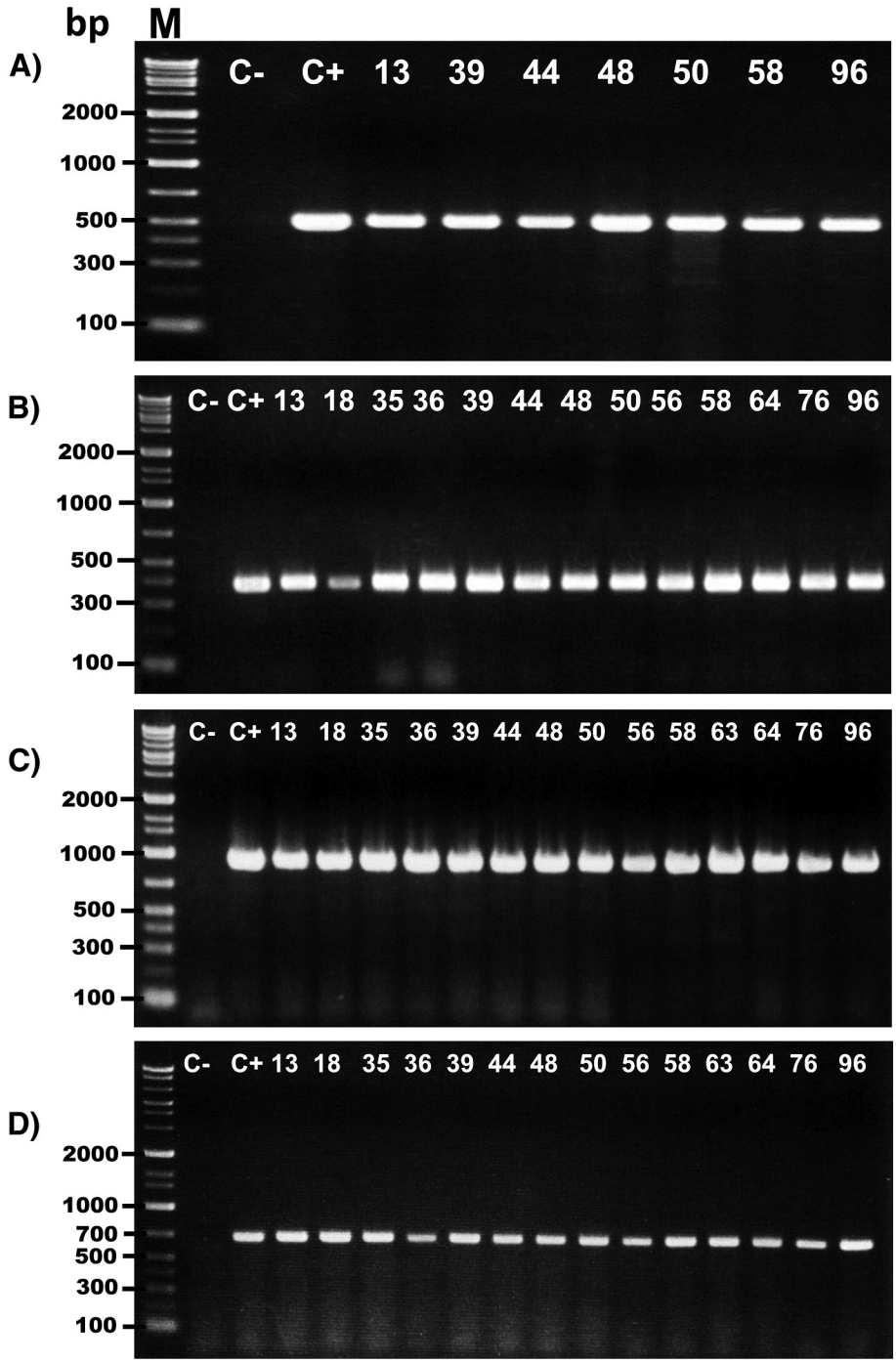

Fig. 3. Agarose gel electrophoresis for PCR-amplified virulence genes detected among the 15 *E. coli* O157 isolates. Amplified bands of the expected molecular sizes of 505 bp for *stx*1 gene (A), 381 bp for *stx*2 gene (B), 917 bp for *eae* gene (C), and 656 bp for EHEC-*hlyA* gene (D) were separated by electrophoresis on 1.5% agarose gel and visualized under UV light. M: DNA marker (Gene Ladder Wide 1); Lane C-: *E. coli* K12 DH5α as a negative control strain; Lane C+: *E. coli* O157:H7 Sakai as a positive reference strain; Lanes with the key numbers from 13 to 96 are the positive strains for target genes.

3.2. Genetic characterization of NSF E. coli O157:H7 and SF E. coli O157:H-

The *sfpA* gene is a unique marker for SF *E. coli* O157:H- that is not found in NSF O157:H7, other STEC strains, or other species of *Enterobacteriaceae* (Brunder et al., 2001; Friedrich et al., 2004). In the present study, all of the eleven SF O157:H- strains tested harbored *sfpA* gene, while this gene was absent in the four NSF O157:H7 (Fig. 1B).

Restriction fragment length polymorphism (RFLP) was developed by Fields et al. (1997) in order to characterize the *fliC* gene, which encodes the flagellin protein subunit of H antigens. Our RFLP analysis for *fliC* gene revealed an identical pattern for both SF and NSF O157 isolates (Fig. 2), which verified the presence of the H7-encoding *fliC* gene in all SF O157:H- strains. Similar findings were reported for SF O157:H- strains isolated from clinical samples (Schmidt et al., 1999; Bielaszewska et al., 2000).

Several virulence factors have been associated with the pathogenicity of both NSF *E. coli* O157:H7 and SF *E. coli* O157:H- strains. These factors include production of at least one of two Shiga toxins (*stx*1 and/or *stx*2), intimin (*eae*), and enterohemolysin (EHEC-*hly*A). In the present study, we investigated the existence of these four virulence genes in the isolated *E. coli* O157 strains. PCR analysis verified the presence of *stx*1 gene, which appeared at the expected molecular size of 505 bp, in seven (46.7%) of the 15 O157 strains tested (Fig. 3A). Detection of *stx*1 gene in SF *E. coli* O157:H- is very rare. Brandal et al. (2012) published the first report about the existence of *stx*1 in SF O157 in clinical samples from a patient with bloody diarrhea, while Rahimi et al. (2012) detected *stx*1 in one of the 13 SF *E. coli* O157:H- isolated from raw meat from ruminant species in Iran. Interestingly, our results verified the presence of *stx*1 in five (45.5%) of the 11 SF *E. coli* O157:H- isolated (Table 2). On the other hand, two of the 4 NSF *Evcoli* O157:H7 strains isolated harbored *stx*1 (Table 2).

PCR verified the presence of *stx*2 at the expected molecular size of 381 bp in 13 (86.7%) of the 15 O157 strains isolated (Fig. 3B). The 2 strains that lacked *stx*2 were from a single ground beef sample (Table 2). The strains carrying *stx*2 are potentially more virulent than those carrying *stx*1 or even strains carrying both *stx*1 and *stx*2 (Thorpe et al., 2002). Epidemiological studies, together with in vivo and in vitro experiments, have indicated that Stx2 is the most important virulence factor associated with severe human disease and that Stx2-producing strains are more frequently related to HUS than those producing Stx1. Indeed, Stx2 is reported to be 1000 times more cytotoxic than Stx1 towards human renal microvascular endothelial cells (Louise and Obrig, 1995). Unexpectedly, two out of the 15 O157 strains tested were negative for Shiga toxin genes. One of these 2 strains was SF O157: H-, while the other was NSF O157: H7. Both of these two strains were derived from a single ground beef sample (Table 2). In a previous study, Schmidt et al. (1999) isolated one NSF *E. coli* O157:H7 strain and five SF *E. coli* O157:H- strains that lacked *stx* genes from patients with diarrhea and HUS. Similarly, in a recent study on meat products in Turkey, Cadirci et al. (2010) isolated two *stx*-negative *E. coli* O157 strains.

Besides the *stx* genes, human pathogenic O157 strains often carry the *eae* gene, encoding the adherence factor intimin, which is required for intimate attachment to the host intestinal mucosa. PCR analysis indicated that *eae* gene was present at the expected molecular size of 917 bp (Fig. 3C) in 14 of the 15 O157 strains tested. Indeed, *eae* gene is found almost in all *E. coli* O157 strains tested in different studies. Similar to our findings, Cagney et al. (2004) detected *eae* gene in 41 of the 43 O157 isolates from minced beef and beef burger in Ireland. Also, Chapman et al. (2000) found *eae* gene in all of the 72 O157 strains isolated from raw beef and lamb products from UK, while Chinen et al. (2009) identified *eae* gene in 24 STEC O157 that were isolated from beef and chicken in Argentina. In this study, only one O157 strain (isolate number 62) that was isolated from a ground beef sample was negative for *eae* gene. The finding of *eae*-negative *E. coli* O157:H7 strain is unusual, but has been reported previously. An *eae*-negative O157:H7 strain was isolated from cattle in Europe (Stephan et al., 2009). Also, 12 *eae*-negative O157 strains were isolated from meat of ruminant species in Iran (Rahimi et al., 2012).

EHEC-*hlyA* is another chief cause of *E. coli* virulence. In the present study, 14 (93.3%) of the 15 O157 isolates were genetically positive for EHEC-*hlyA* gene (Fig. 3D), nonetheless their enterohemolytic phenotype on blood agar was observed only in 11 (73.3%) strains (Table 2).

Our results indicated that seven (46.7%) of the 15 *E. coli* O157 strains were positive for the four virulence genes, *stx*1, *stx*2, *eae* and EHEC-*hlyA*, while six (40%) strains were positive for *stx*2, *eae* and EHEC-*hlyA*. A single (6.7%) strain, however, was positive for both *eae* and *hylA* genes. On the other hand, another single strain of O157:H7 type did not contain any of the virulence genes tested (Table 2).

The present study concluded that the retail beef products, especially ground beef marketed in Mansoura, Egypt are considerably contaminated with SF *E. coli* O157:H- and NSF *E. coli* O157:H7. Such microbial contaminants harbor one or more of the virulence genes, which have potential to cause severe infection in humans.

References

Abdul-Raouf, U.M., Ammar, M.S., Beuchat, L.R., 1996. Isolation of *Escherichia coli* O157:H7 from some Egyptian foods. International Journal of Food Microbiology 29, 423–426.

Adwan, G.M., Adwan, K.M., 2004. Isolation of Shiga toxigenic *Escherichia coli* from raw beef in Palestine. International Journal of Food Microbiology 97, 81–84.

Alpers, K., Werber, D., Frank, C., Koch, J., Friedrich, A.W., Karch, H., An Der Heiden, M., Prager, R., Fruth, A., Bielaszewska, M., Morlock, G., Heissenhuber, A., Diedler, A., Gerber, A., Ammon, A., 2009. Sorbitol-fermenting enterohaemorrhagic *Escherichia coli* O157:H causes another outbreak of haemolytic uraemic syndrome in children. Epidemiology and Infection 137, 389–395.

Ammon, A., Petersen, L.R., Karch, H., 1999. A large outbreak of hemolytic uremic syndrome caused by an unusual sorbitol-fermenting strain of *Escherichia coli* O157:H. The Journal of Infectious Diseases 179, 1274–1277.

Barlow, R.S., Gobius, K.S., Desmarchelier, P.M., 2006. Shiga toxin-producing *Escherichia coli* in ground beef and lamb cuts: results of a one-year study. International Journal of Food Microbiology 111, 1–5.

Bettelheim, K.A., Whipp, M., Djordjevic, S.P., Ramachandran, V., 2002. First isolation outside Europe of sorbitol-fermenting verocytotoxigenic *Escherichia coli* (STEC) belonging to O group O157. Journal of Medical Microbiology 51, 713–714.

Bielaszewska, M., Schmidt, H., Karmali, M.A., Khakhria, R., Janda, J., Bláhová, K., Karch, H., 1998. Isolation and characterization of sorbitol fermenting Shiga toxin (verocytotoxin)-producing *Escherichia coli* O157:H- strains in the Czech Republic. Journal of Clinical Microbiology 36, 2135–2137.

Bielaszewska, M., Schmidt, H., Liesegang, A., Prager, R., Rabsch, W., Tschäpe, H., Cizek, A., Janda, J., Bláhová, K., Karch, H., 2000. Cattle can be a reservoir of sorbitol-fermenting Shiga toxin-producing *Escherichia coli* O157:H- strains and a source of human diseases. Journal of Clinical Microbiology 38, 3470–3473.

Bono, J.L., Smith, T.P., Keen, J.E., Harhay, G.P., McDaneld, T.G., Mandrell, R.E., Jung, W.K., Besser, T.E., Gerner-Smidt, P., Bielaszewska, M., Karch, H., Clawson, M.L., 2012. Phylogeny of Shiga toxin-producing *Escherichia coli* O157 isolated from cattle and clinically ill humans. Molecular Biology and Evolution 29, 2047–2062.

Brandal, L.T., Løbersli, I., Stavnes, T.L., Wester, A.L., Lindstedt, B.A., 2012. First report of the Shiga toxin 1 gene in sorbitol-fermenting *Escherichia coli* O157:H-. Journal of Clinical Microbiology 50, 1825–1826.

Brunder, W., Khan, A.S., Hacker, J., Karch, H., 2001. Novel type of fimbriae encoded by the large plasmid of sorbitol-fermenting enterohemorrhagic *Escherichia coli* O157:H-. Infection and Immunity 69, 4447–4457.

Buvens, G., Pierard, D., Hachimi-Idrissi, S., Lauwers, S., 2009. First sorbitol-fermenting Verocytotoxin-producing *Escherichia coli* O157: H- isolated in Belgium. Acta Clinica Belgica 64, 59–64.

Cadirci, O., Siriken, B., Inat, G., Kevenk, T.O., 2010. The prevalence of *Escherichia coli* O157 and O157:H7 in ground beef and raw meatball by immunomagnetic separation and the detection of virulence genes using multiplex PCR. Meat Science 84, 553–556.

Cagney, C., Crowley, H., Duffy, G., Sheridan, J.J., O'Brien, S., Carney, E., Anderson, W., McDowell, D.A., Blair, I.S., Bishop, R.H., 2004. Prevalence and numbers of *Escherichia coli* O157:H7 in minced beef and beef burgers from butcher shops and supermarkets in the Republic of Ireland. Food Microbiology 21, 203–212.

Carney, E., O'Brien, S.B., Sheridan, J.J., McDowell, D.A., Blair, I.S., Duffy, G., 2006. Prevalence and level of *Escherichia coli* O157 on beef trimmings, carcasses and boned head meat at a beef slaughter plant. Food Microbiology 23, 52–59.

Chapman, P.A., Siddons, C.A., Cerdan Malo, A.T., Harkin, M.A., 2000. A one year study of *Escherichia coli* O157 in raw beef and lamb products. Epidemiology and Infection 124, 207–213.

Chapman, P.A., Malo, A.T.C., Ellin, M., Ashton, R., Harkin, M.A., 2001. *Escherichia coli* O157 in cattle and sheep at slaughter, on beef and lamb carcasses and in raw beef and lamb products in South Yorkshire, UK. International Journal of Food Microbiology 64, 139–150.

Chinen, I., Epszteyn, S., Melamed, C.L., Aguerre, L., Martínez Espinosa, E., Motter, M.M., Baschkier, A., Manfredi, E., Miliwebsky, E., Rivas, M., 2009. Shiga toxin-producing *Escherichia coli* O157 in beef and chicken burgers, and chicken carcasses in Buenos Aires, Argentina. International Journal of Food Microbiology 132, 167–171.

Choo, E., Jang, S.S., Kim, K., Lee, K.G., Heu, S., Ryu, S., 2007. Prevalence and genetic diversity of *Bacillus cereus* in dried red pepper in Korea. Journal of Food Protection 70, 917–922.

Conedera, G., Dalvit, P., Martini, M., Galiero, G., Gramaglia, M., Goffredo, E., Loffredo, G., Morabito, S., Ottaviani, D., Paterlini, F., Pezzotti, G., Pisanu, M., Semprini, P., Caprioli, A., 2004. Verocytotoxin-producing *Escherichia coli* O157 in minced beef and dairy products in Italy. International Journal of Food Microbiology 96, 67–73.

Dutta, S., Deb, A., Chattopadhyay, U.K., Tsukamoto, T., 2000. Isolation of Shiga toxin-producing *Escherichia coli* including O157:H7 strains from dairy cattle and beef samples marketed in Calcutta, India. Journal of Medical Microbiology 49, 765–767.

Eklund, M., Bielaszewska, M., Nakari, U.M., Karch, H., Siitonen, A., 2006. Molecular and phenotypic profiling of sorbitolfermenting *Escherichia coli* O157:H- human isolates from Finland. Clinical Microbiology and Infection 12, 634–641.

Fantelli, K., Stephan, R., 2001. Prevalence and characteristics of shigatoxin-producing *Escherichia coli* and *Listeria monocytogenes* strains isolated from minced meat in Switzerland. International Journal of Food Microbiology 70, 63–69.

Fields, P.I., Blom, K., Hughes, H.J., Helsel, L.O., Feng, P., Swaminathan, B., 1997. Molecular characterization of the gene encoding H antigen in *Escherichia coli* and development of a PCR-restriction fragment length polymorphism test for identification of *E. coli* O157:H7 and O157:NM. Journal of Clinical Microbiology 35, 1066–1070.

Friedrich, A.W., Nierhoff, K.V., Bielaszewska, M., Mellmann, A., Karch, H., 2004. Phylogeny, clinical associations, and diagnostic utility of the pilin subunit gene (*sfpA*) of sorbitol-fermenting, enterohemorrhagic *Escherichia coli* O157:H-. Journal of Clinical Microbiology 42, 4697–4701.

Garvey, P., McKeown, P., Carroll, A., McNamara, E., 2006. Epidemiology of Verotoxigenic *E. coli* in Ireland, 2005. EPI-Insight 7, 2–3. http://www.ndsc.ie/hpsc/EPI-Insight/Volume72006/File,1936,en.pdf.

Haugum, K., Lindstedt, B.A., Løbersli, I., Kapperud, G., Brandal, L.T., 2012. Identification of the anti-terminator qO111:H- gene in Norwegian sorbitol-fermenting *Escherichia coli* O157:NM. FEMS Microbiology Letters 329, 102–110.

Heuvelink, A.E., Zwartkruis-Nahuis, J.T.M., Beumer, R.R., De Boer, E., 1999. Occurrence and survival of verocytotoxin-producing *Escherichia coli* O157 in meats obtained from retail outlets in the Netherlands. Journal of Food Protection 62, 1115–1122.

Hiko, A., Asrat, D., Zewde, G., 2008. Occurrence of *Escherichia coli* O157:H7 in retail raw meat products in Ethiopia. Journal of Infection in Developing Countries 2, 389–393.

Jenke, C., Leopold, S.R., Weniger, T., Rothgänger, J., Harmsen, D., Karch, H., Mellmann, A., 2012. Identification of intermediate in evolutionary model of enterohemorrhagic *Escherichia coli* O157. Emerging Infectious Diseases 18, 582–588.

Karch, H., Bielaszewska, M., 2001. Sorbitol-fermenting Shiga toxin producing *Escherichia coli* O157:H- strains: epidemiology, phenotypic and molecular characteristics, and microbiological diagnosis. Journal of Clinical Microbiology 39, 2043–2049.

Lee, J.H., Choi, S.J., 2006. Isolation and characteristics of sorbitol-fermenting *Escherichia coli* O157 strains from cattle. Microbes and Infection 8, 2021–2026.

Louise, C.B., Obrig, T.G., 1995. Specific interaction of *Escherichia coli* O157:H7-derived Shiga-like toxin II with human renal endothelial cells. The Journal of Infectious Diseases 172, 1397–1401.

Mora, A., Blanco, M., Blanco, J.E., Dahbi, G., López, C., Justel, P., Alonso, M.P., Echeita, A., Bernárdez, M.I., González, E.A., Blanco, J., 2007. Serotypes, virulence genes and intimin types of Shiga toxin (verocytotoxin)- producing *Escherichia coli* isolates from minced beef in Lugo (Spain) from 1995 through 2003. BMC Microbiology 7, 13.

Orth, D., Grif, K., Dierich, M.P., Würzner, R., 2006. Sorbitol-fermenting Shiga toxin-producing *Escherichia coli* O157: indications for an animal reservoir. Epidemiology and Infection 134, 719–723.

Radu, S., Mutalib, S.A., Rusul, G., Ahmad, Z., Morigaki, T., Asai, N., Kim, Y.B., Okuda, J., Nishibuchi, M., 1998. Detection of *Escherichia coli* O157:H7 in the beef marketed in Malaysia. Applied and Environmental Microbiology 64, 1153–1156.

Rahimi, E., Kazemeini, H.R., Salajegheh, M., 2012. *Escherichia coli* O157:H7/NM prevalence in raw beef, camel, sheep, goat, and water buffalo meat in Fars and Khuzestan provinces, Iran. Veterinary Research Forum 3, 13–17.

Reid, S.D., Betting, D.J., Whittam, T.S., 1999. Molecular detection and identification of intimin alleles in pathogenic *Escherichia coli* by multiplex PCR. Journal of Clinical Microbiology 37, 2719–2722.

Riley, L.W., Remis, R.S., Helgerson, S.D., McGee, H.B., Wells, J.G., Davis, B.R., Hebert, R.J., Olcott, E.S., Johnson, L.M., Hargrett, N.T., Blake, P.A., Cohen, M.L., 1983. Hemorrhagic colitis associated with a rare *Escherichia coli* serotype. The New England Journal of Medicine 308, 681–685.

Rosser, T., Dransfield, T., Allison, L., Hanson, M., Holden, N., Evans, J., Naylor, S., La Ragione, R., Low, J.C., Gally, D.L., 2008. Pathogenic potential of emergent sorbitol-fermenting *Escherichia coli* O157:NM. Infection and Immunity 76, 5598–5607.

Schmidt, H., Scheef, J., Huppertz, H.I., Frosch, M., Karch, H., 1999. *Escherichia coli* O157:H7 and O157:H- strains that do not produce Shiga toxin: phenotypic and genetic characterization of isolates associated with diarrhea and hemolytic–uremic syndrome. Journal of Clinical Microbiology 37, 3491–3496.

Stampi, S., Caprioli, A., De Luca, G., Quaglio, P., Sacchetti, R., Zanetti, F., 2004. Detection of *Escherichia coli* O157 in bovine meat products in northern Italy. International Journal of Food Microbiology 90, 257–262.

Stephan, R., Zhang, W., Bielaszewska, M., Mellmann, A., Karch, H., 2009. Phenotypic and genotypic traits of Shiga toxin-negative *E. coli* O157:H7/H(−) bovine and porcine strains. Foodborne Pathogens and Disease 6, 235–243.

Tarr, P.I., Tran, N.T., Wilson, R.A., 1999. *Escherichia coli* O157:H7 in retail ground beef in Seattle: results of a one-year prospective study. Journal of Food Protection 62, 133–139.

Thorpe, C.M., Ritchie, J.M., Acheson, D.W.K., 2002. Enterohemorrhagic and other Shiga toxin-producing *Escherichia coli*. In: Donnenberg, M.S. (Ed.), *Escherichia coli*: Virulence Mechanisms of a Versatile Pathogen. Academic Press, Boston, MA, pp. 119–154.

Tutenel, A.V., Pierard, D., Van Hoofa, J., Cornelis, M., De Zutter, L., 2003. Isolation and molecular characterization of *Escherichia coli* O157 isolated from cattle, pigs and chickens at slaughter. International Journal of Food Microbiology 84, 63–69.

Vernozy-Rozand, C., Ray-Gueniot, S., Ragot, C., Bavai, C., Mazuy, C., Montet, M.P., Bouvet, J., Richard, Y., 2002. Prevalence of *Escherichia coli* O157:H7 in industrial minced beef. Letters in Applied Microbiology 35, 7–11.

Zhou, Z., Nishikawa, Y., Zhu, P., Hong, S., Hase, A., Cheasty, T., Smith, H.R., Zheng, M., Haruki, K., 2002. Isolation and characterization of shiga toxin-producing *Escherichia coli* O157:H7 from beef, pork and cattle fecal samples in Changchun, China. Journal of Veterinary Medical Science 64, 1041–1044.