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Prevalence and Characterization of Virulence Genes Toxin-producing *Escherichia coli* Enterohemorragic 0157:H7 Strain Isolated from Frozen Imported Bovine Meat in Algeria

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The presence of *E. coli enterohemorragic* O157:H7 was inspected in 756 samples of frozen bovine meat imported from different countries, Argentina, Australia, Brazil, Ireland and Uruguay, using the microbiological standard method of culture and serotyping technique. The pathogen strain of *E. coli* was detected in five samples (0.66 %). All the isolates were tested using the method of KIRBY and BAUER according to the nccls standards to view resistance to antibiotics and was considered sensitive to the sixteen antimicrobial agents tested. All isolates were further characterized by serotyping and molecular methodology. The PCR technique showed that all the strains carried the *stx2* and the *ehxA* genes 1 isolate carried *stx1* genes, 1 both *stx1* and *stx*2. Intimin (*eae* c) virulence genes were detected in 4 isolates. The interaction with lactic acid bacteria revealed that all *E. coli* O157:H7 isolates were inhibited.

Key words: *Escherichia coli* O157:H7, *stx1, stx2, eae c, ehxA*, PCR, frozen bovine meat, Algeria.

Escherichia coli O157:H7 (*E. coli*) is an important enterohemorrhagic food-borne pathogen which causes hemorrhagic colitis with symptoms such as diarrhoea, hemolytic uremic syndrome, and thrombotic thrombocytopenic purpurea (Lin *et al.*, 2010). *Escherichia coli* enterohaemorrhagic (EHEC ; also variously referred to as Verocytotoxin producing *E. coli*, VTEC and Shiga toxin producing *E. coli*, STEC) are an important group of food-borne pathogens that cause infections to humans in many parts of the world (Nataro *et al.*, 1998 ; Armstrong *et al.*, 1996). *E. coli* O157:H7 is the most important serotype causes simple diarrhea to the more complicated hemorrhagic colitis (HC) and hemolytic uremic syndrome (HUS) (Browning *et al.*, 1990). Cattle, especially the young ones, have been implicated as a principal source for *E. coli* O157:H7 (Ateba and Mbewe, 2013). Most infections caused by *E. coli* O157: H7 result from the consumption of contaminated and undercooked ground beef and unpasteurized fruit juices. Dairy products, cheese from raw milk (Allerberger *et al.*, 2001, Ghanbarpour and Oswald, 2010).

Virulence factors include hemolysins and Shiga toxins (Stx) (Brooks *et al.*, 1998 ; Murray *et al*, 1999). Members of family include *Stx1* and *Stx2* toxins encoded by *stx1* and *stx2* genes, respectively. Shiga toxins are the major virulence factors of EHEC

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which have cytotoxicity effects for human and animal eukaryotic cells and cause dangerous details like HUS and TTP (Saif *et al.*, 2003) other virulence factor *eae* c and *ehxA* have been described (Pilpott and Ebel, 2003 Ghanbarpour and Oswald, 2010). In Algeria, no data were available until now on the fate and characteristics of STEC isolated from frozen bovine meat. The objective of the present study was to isolate *E. coli* O157:H7 from frozen bovine meat samples by conventional culture method and to confirm it by a serogroup-specific and PCR assay.

MATERIALS AND METHODS

Sample and bacterial isolates

Seven hundred and fifty six samples of imported frozen bovine meat were analyzed. After received by the laboratory, enrichment cultures for each sample were carried out by combining 25 g of each sample with 225 ml of buffered peptone water supplemented into a stomacher bag, homogenized for at least 2 min and incubated at 37°C for 16–18 h. After incubation, the isolates belonging to *E. coli* were cultured on Sorbitol Mac Conkey medium added with MUG supplement (Biokar diagnostics) for detecting non sorbitol and MUG negative variants. This test required the identification guides to the pathogenic EHEC strains. The strains metabolizing sorbitol are excluded, however the rest of the strains are tested for their immunological confirmation by the method of Farmer and Davis. Three *Lactobacillus* strains and one strain of *Leuconostoc* were used in this study. *Lactobacillus* strains were obtained from laboratory of applied microbiology, biology department, Oran university (Barka and Kihal, 2010; Ngwa *et al*., 2013).

Biochemical and serological tests

All the identified nonsorbitol fermenting colonies from the SMAC Agar were biochemically tested for confirmation by the API 20E, and subjected to slide agglutination with the the *E. coli* Latex kit O157:H7 (« Statens Serum Institute », Copenhague, Danemark) and the agglutinating colonies were further processed for definite confirmation (Farmer and Davis, 1985).

Antibiotic susceptibility test

The isolated strains were tested for susceptibility to 16 antibiotics using the standard Kirby–Bauer method (Bauer *et al.*, 1966) and the following antibiotics discs were used: ampicillin (10 µg), chloramphenicol (30 µg), gentamycin (10 µg), colistin 10 µg , flumequin (30 µg) , enrofloxacin (5 µg), nitrofurantoin (300 µg), cephalotin (30 µg), sulfamethoxazole-trimethoprim (1.25/23.75 µg), nalidixic acid (30 µg), norfloxacin (10 µg), ofloxacin (5 µg), kanamycin (30 µg), neomycin (30 µg), ceftiofur (30µg) and tetracycline (30 µg). Isolates were classed as sensitive or resistant to each antibiotic according to the Clinical and Laboratories Standards Institute (formally NCCLS) guidelines (NCCLS, 2003). *E. coli* strain ATCC 25922 was used for quality control.

Interaction with lactic acid bacteria

The research of the effect of bacteriocins produced by lactic acid bacteria is carried out by the method of the double layer (Barefoot and Klaenhammer, 1983). Strains of *E. coli* O157: H7 inoculated on BHIB medium liquid are incubated 18 hours at 37 °C; while the lactic acid bacteria are inoculated in MRS spot on solid buffered in order to eliminate the effect of lactic acid (pH 5.4) then they were incubated 24 h at 30 °C. After incubation of the *E. coli* O157: H7 were inoculated into BHIB semi solid Petri dishes are flooded with MRS strains to study and let incubated for 24 h at 37 °C.

Extraction of DNA

The extraction of DNA was realized by the technique of phenol-chloroform. The bacteria are spread initially on MacConkey sorbitol, and then a clone is grown in 5 ml of LB medium. 1 ml of each culture was centrifuged 2500 g x 10 min, then the pellet was recovered and treated with lysis buffer (20% SDS, 10 mM Tris pH 7.5, 1M EDTA) and proteinase K at 10mg/ml. After heating at 56 °C for 2 hours, add the phenol-chloroform-isopropanol and follow the protocol of Sambrook *et al*. (1989). The DNA is recovered in 50 µl of sterile water.

PCR amplification

The virulence genes of *E. coli* O157: H7 isolates were identified by polymerase chain reaction. *E.coli* ECL6611 were used as the positive control, *E. coli* ECL3463 and *E. coli* 29 as the negative control. The PCR reaction was performed in a 50 µl amplification mixture consisting of 5 µl 10 × PCR buffer (500 mM KCl, 200 mM Tris-HCl), 8 µl dNTPs (10 mM), 1.5 µl $MgCl_2$ (50 mM), 2.5 µl of each primer (10 µM), 0.5 µl of Taq DNA polymerase

(5 Unit/µl) and 1 µl of template (Padola *et al*., 2004; Rubén Gordillo *et al*., 2011). The primers used in this PCR are presented in the following table:

Electrophoresis of PCR products

PCR products were separated by electrophoresis on 1, 5 % (w/v) agarose gel (Invitrogen) at 100 V for 40 min in Tris acetate buffer. The gels were stained in ethidium bromide, illuminated by UV-transilluminator and documented by a gel documentation apparatus. Each gel contained a 1 kb DNA molecular weight marker (Fermentas, USA).

RESULTS

Isolation and identification of *E. coli* O157:H7

From 756 samples of meat analyzed; 3 *Escherichia coli* sorbitol negative were isolated and identified from Uruguay samples, 1 from Brazil and 1 from Argentina (Table 2). Then the strains are identified by the API 20E system that enables us to confirm the sorbitol negative test and especially to have the chemotype of these strains. One strain has chemotype 5144112, in fact we find that this strain in addition to sorbitol, it does not ferment sucrose, melibiose and amygdalin. While the four other strains have the same chemotype 5144172.

Interaction with lactic acid bacteria

All strains were inhibited in contact with lactic acid bacteria used. Fig. 1 shows the zones of inhibition

Antibiotic susceptibility test

Antibiotic susceptibility profiles indicated that only one resistance strain to tetracycline was observed, the strain number 25uru.

Genetic methods for detecting *E. coli* O157: H7

In this study all strains possess the genes *stx2* and *ehxA*, one strain (number 27uru) does not possess the *eae c* gene but instead it is the only

Table 1. Oligonucleotide primers sequences used in PCR (*stx1a* and *stx2b* for shigatoxine, *ehxA* for *enterohemolysin* and *eae c* for intimin*)*

Gene Primer	Oligonucleotide	Sequence (5'-3')	Fragment size (bp)	Tm	Reference
stx1a	VT1-A	CGCTGAATGTCATTCGCTCTGC	302	71.2	Blanco *et al*. (2003)
	VT1-B	CGTGGTATAGCTACTGTCACC	58.5		
stx2b	VT2-A	CTTCGGTATCCTATTCCCGG	516	64.2	Blanco *et al.* (2003)
	VT2-B	CTGCTGTGACAGTGACAAAACGC	69.1		
ehxA	HlyA1	GGTGCAGCAGAAAAAGTTGTAG	1551	62.7	Schmidt *et al.* (1995)
	HlyA4	TCTCGCCTGATAGTGTTTGGTA	63.4		
eae c	EAE-1	GAGAATGAAATAGAAGTCGT	775	52.4	Blanco *et al*. (2003)
	EAE-2	GCGGTATCTTTCGCGTAATCGCC	72.6		

The amplifications were performed for:
stx1 at 95°C 30 s, 30 cycles of 95°C for 15 s; 52°C for 60s; 72°C for 90s.
stx2 at 95°C 30 s, 30 cycles of 95°C for 15 s; 55°C for 30 s; 72°C for 90s.
eae c at 95°C 30 s, 30 cycles of 95°C for 15 s; 45°C for 60 s; 72°C for 90s.
ehxA at 95°C 30 s, 30 cycles of 95°C for 15 s; 55°C for 60 s, 72°C for 90s.
Both positive and negative control reactions were included in each PCR amplification experiment.

Table 2. Number of *E. coli* 0157:H7 detected in imported frozen meat in Algeria

Country	Uruguay	Brazil	Argentina	Ireland	Australia	New Zealand	Total
Number of Samples examined	365	243	105	21	16	06	756
Number of positive samples (%)	3 (0.39)	1 (0.13)	1 (0.13)	0 (0.0)	0 (0.0)	0 (0.0)	5

The identification of the strains was followed by the procedure applied by Barka and Kihal (2010).

Table 3. Genes detected in the different strains *E. coli* O157: H7 isolated from frozen bovine meat in Algeria

Gene Strain of *E. coli*	*Stx1*	*Stx2*	*Stx1 and Stx2*	*eae c*	*ehxA*
25uru	-	+	-	+	+
26uru	-	+	-	+	+
27uru	+	+	+	-	+
28bra	-	+	-	+	+
30arg	-	+	-	+	+
E. coli ECL 6611	+	+	+	+	+
E. coli ECL 3463	-	-	-	-	-

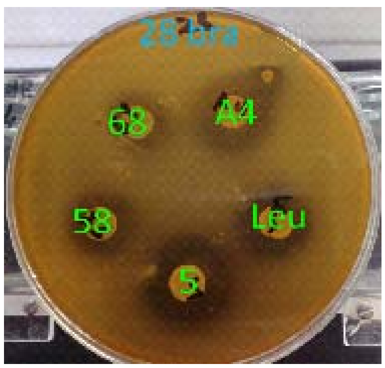

Fig. 1. Inhibition effect of lactic acid bacteria A4 : *Lactobacillus johnsonii*, 5 : *Lactobacillus plantarum*, 58: *Lactobacillus paracasei*, 68: *Lactobacillus plantarum*, leu : *Leuconostoc*, toward *E. coli* strain 28bra

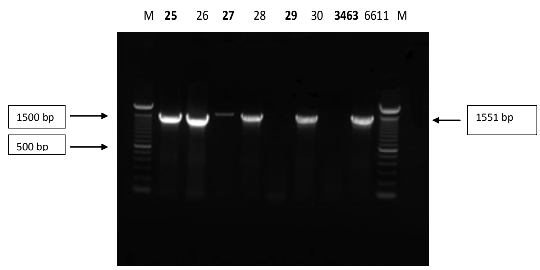

Fig. 2. Amplification products of *ehxA* gene by polymerase chain reaction (PCR). M (1kb), *E. coli* ECL6611 strain is the positive control. The negative control is the strain of *E. coli* ECL3463. The strains 25uru, 26uru, 27uru, 28bra and 30arg have the *ehxA* gene

strain that has the gene *stx1*. Table 3 summarizes the results of pcr and Figures 2 show the presence of bands corresponding to *ehxA* gene on the electrophoresis gel.

DISCUSSION

Enterohemorrhagic *Escherichia coli* (EHEC) O157:H7 infections cause serious public health problems worldwide. Approximately 93% of strains of *E. coli* of human origin were sorbitol positive in 24 hours and are ^{2}-glucuronidase positive, in contrast, *E. coli* O157: H7 does not ferment sorbitol and are β-glucuronidase negative. The absence of sorbitol fermentation, for example, justified the use of sorbitol MacConkey agar (SMAC), which has undergone several changes in the objective of increasing the selectivity against O157: H7. The biochemical methods are interesting and fundamental for the detection of *E. coli* O157: H7. Beutin *et al*. 2007).

From 756 samples tested, five *E. coli* O157: H7 strains were isolated with a rate of 0.66%, the rate of *E. coli* O157: H7 varies from one country to another. The only known study on *E. coli* O157: H7 in Algeria was made in 2006 by Chahed after analyzing 230 samples of beef carcasses from slaughterhouse in the region of Algiers in which had isolated two *E. coli* O157: H7. In France Vernozy-Rozand *et al*., (2002) have detected a rate of 0.12% in the meat industry and 1.9% of carcasses (Rogers *et al*., 2001), in Switzerland no *E. coli* O157: H7 were detected from 400 samples (Fantelli and Stephan, 2001). 1% of the samples were positive for *E. coli* O157: H7 in Spain (Azucena Mora *et al*., 2007), 1% and 0.4% in England (Chapman *et al*., 2000), 1% in the Netherlands (Heuvelink, 1999) and 0.4% in Italy (Conedera *et al*. 2004; Stampi *et al*., 2004) and 0.3% in the Czech Republic (Lukásová *et al*., 2004).

Other studies have revealed much higher rates including one made in Argentina with 3.8% (Chinen *et al*., 2001), Peru 19% (Mora *et al*., 2007), and Jordan with 7.8% in carcasse (Osaili *et al*. 2013).

According to Nastasijevic *et al.*, (2009) the presence of *E. coli* O157: H7 is usually the result of contamination during the slaughter process. It was previously reported that this contamination is correlated with the presence of STEC in feces and hides (Elder *et al*., 2000).

Concerning the interaction with lactic bacteria some studies have shown inhibition of gram-negative bacteria by bacteriocins (Todorov *et al*., 2004).

Currently to prevent food contamination by pathogenic bacteria in the case of *E. coli* O157: H7, some authors recommend to use in addition to the barriers used in food safety which are temperature, water activity (Aw), pH, redox potential (Eh), vacuum packaging, modified atmosphere, high hydrostatic pressure (HHP), UV and competitive flora (LAB producing antimicrobial compounds), the combined use of bacteriocins produced by the plant competitive with storage methods listed above to create a series of obstacles during the manufacturing process to reduce food spoilage. Indeed, these different barriers positively influence the activity of many bacteriocins by increasing the permeability of cell membranes of target bacteria (Chawla *et al*., 2004, Ananou *et al*., 2009).

Antibiotic susceptibility shows result different of other studies around the world or a fairly high rate of resistance was observed. Recent studies have revealed an increasing trend of *E. coli* O157: H7 against antibiotics (Magwira *et al*., 2005; Solomakos *et al*., 2009).

For example, in 2005, about 35% of *E. coli* O157: H7 isolated from meat and meat products in Gabone, Botswana, were resistant to cephalothin, sulfatriad, colistin sulfate and tetracycline (Magwira *et al*., 2005). In Korea one study found a rate of 71% resistance to at least one antibiotic and is the highest rate was reported today (Ju-Yeon You, *et al*., 2006). An American study has shown a resistance rate of 20% to tetracycline and 14% to sulfonamides (Schroeder *et al*., 2002).

In our study, we found that *stx*2 was the predominant gene over *stx*1 and all the strains possesses the *ehxA*. It can be concluded that all strains carry one or more genes in pathogenicity. These results are similar to those obtained by Blanco *et al*. (1996 and 1997), 0% of cows, calves 0.6%, but less than obtained by (Oporto *et al*., 2008) in calves 6.7% and (Sánchez *et al*., 2010a) 7 strains of 268 samples analyzed.

Khan in 2002 in India found 44.5% of strains of *E. coli* O157: H7 bearing both the *stx1* and *stx2*. The genes *stx2* and *eaeA* are more frequently isolated than *stx1* in most studies in the United States, Japan and European countries (Lim *et al*., 2010, Brusa *et al.*, 2012). In a U.S.A study the gene *stx1* have been detected in any sample, while 22% of isolates carried the *stx2* gene (Osaili *et al*., 2012).

Some studies show that the presence of the stx2 gene was most of the time associated with the presence of the gene *eaeA* (Omisakin *et al*., 2003). Only some studies have shown the presence in the same strain genes *stx1*, *stx2* and *eaeA* (Guyon *et al*. 2001; Tutenel *et al*., 2003).

In our study the genes *stx2* and *eaeA* were detected in most strains analyzed. This is important because the presence of these genes is strongly associated with human disease (Sánchez *et al*., 2010b)

These results show that there may be contamination of frozen meat must done research of these pathogen bacteria before the placing on the market.

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