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ORIGINAL ARTICLE

Research and molecular characteristic of Shiga toxin-producing *Escherichia coli* isolated from sheep carcasses

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Significance and Impact of the Study: PCR screening revealed the significant presence of the genetic markers of Shiga toxin-producing *Escherichia coli* (STEC) (*stx*$^+$/*stx*$^+$*eae*$^+$) on the surfaces of sheep carcasses. *Citrobacter braakii* (*stx*$_1$$^+$ *eae*$^+$) was isolated for the first time in this study. The risk of foodborne diseases due to STEC must be taken into account in Algeria. To prevent the emergence of epidemic outbreaks among children and older by people, preventive measures should be taken.

Keywords
multiplex PCR, sheep carcasses, slaughterhouse, STEC, virulence-factor encoding genes.

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Abstract

Domestic ruminants are regarded as the major reservoir of Shiga toxin-producing *Escherichia coli* (STEC) closely related to human infection. A total of 363 ovine carcasses were swabbed in an Algiers city slaughterhouse for research on STEC. First of all, screening of the STECs was carried out by a multiplex PCR searching for the genes coding for the virulence factors *stx*$_1$, *stx*$_2$ and *eae*. This step was followed by STEC isolation and serotyping. The presence of *stx*$^+$/*stx*$^+$*eae*$^+$ genes was shown in 116 sheep carcasses (31·95%). From the 116 positive samples, 20 bacterial strains (17·24%) were isolated. Nineteen strains belonged to the species *E. coli* (STEC), and 1 belonged to *Citrobacter braakii* (*eae*$^+$ *stx*$_1$$^+$). During this study, the presence of potentially pathogenic STEC for humans on the surface of sheep carcasses was confirmed. Corrective measures should be considered at the slaughterhouse level to avoid outbreaks of STEC in Algeria.

Introduction

Shiga toxin-producing *Escherichia coli* (STEC) are emerging bacteria that cause epidemic foodborne disease outbreaks; these outbreaks are characterized by a variety of clinical manifestations, ranging from simple bloody diarrhoea to haemorrhagic colitis (HC) in 80% of cases (Beutin *et al.* 2004). HC may progress to haemolytic uremic syndrome (HUS) and/or thrombotic thrombocytopenic purpura, which causes neurological disorders (Tarr 2009). These complications can ultimately lead to chronic renal failure (Ake *et al.* 2005) or death of the affected individuals in 3–5% of cases (AFSSA 2003). Children and elderly people are the first victims of HUS due to the deficiency of their immune systems. The first isolated strain causing HUS belongs to the O157:H7 serotype (Riley *et al.* 1983); however, there are other non-O157 *E. coli* responsible for worldwide epidemic outbreaks, such as O26, O45, O103, O104, O111, O119, O121, O145, O113, O118, O55, O91 and O128 (Johnson *et al.* 2006).

The pathogenicity of STEC is determined by the production of virulence factors, mainly intimin, Shiga toxins and enterohaemolysin (Momtaz *et al.* 2013). Intimin is responsible for the bacteria's intimate adhesion to intestinal cells, causing the appearance of attachment lesions and erasure of the microvilli of the brush border of enterocytes and diarrhoea (Joly and Reynaud 2003). Intimin is encoded by the *eae* virulence gene, carried by the bacterial chromosome (Schmidt *et al.* 1994). Shiga toxin types 1 and 2 (Stx1 and Stx2) are responsible for the death of intestinal, vascular, renal and brain cells (O'Loughlin and Robins-Browne 2001) and are the origin of HUS and PTT. They are encoded respectively, by the virulence genes *stx*$_1$ and *stx*$_2$ carried by prophages located on the

chromosome (Allison 2007). STEC strains isolated from patients with diarrhoea and/or HUS are referred to as EHEC (enterohaemorrhagic *E. coli*) (Riley *et al.* 1983).

Ruminants, particularly cattle (Blanco *et al.* 2003a; Chahed *et al.* 2006), sheep and goats (Beutin *et al.* 1993; Vettorato *et al.* 2003), are the main reservoirs of STEC.

The main source of human infection is ingestion of undercooked meat; but as statistics grows, consumption of other foodstuffs and/or water, direct contact with animals and human-to-human transmission through the faecal-oral route have on be of epidemics (Caprioli *et al.* 2005; Bertholet-Thomas *et al.* 2011).

In Algeria, mutton is highly valued, and sheep farming accounts of nearly 80% (27 million heads) of the total national population of herd animals and also represents the country's largest animal resource (Djaout *et al.* 2017). This production ranks Algeria sixth in terms of sheep farming worldwide.

The lack of data on the carriage of STEC by ovine carcasses in Algeria, as well as the possible presence of STEC in derived foods, could constitute a serious danger for consumers. This concern led us to look for and characterize STEC in samples taken from the surface of sheep carcasses produced in a slaughterhouse in Algiers.

Results and discussion

Screening result

The number of positive PCRs for the stx^+/stx^+ eae^+ genes was one hundred and sixteen (116), or 31·95% of total numbers samples (n = 363). This result is close to the findings, recorded by Zweifel and Stephan (2003) in Switzerland (36·6%) but higher than those recorded by Bai *et al.* (2015) in China (20·6%) and Mazzette *et al.* (2012) in Italy (14·7%).

The results of the virulence gene screening by multiplex PCR are presented in Fig. 1.

The stx_1^+ gene was predominant with a rate of (20.66%). Our results are lower than those reported by Mazzette *et al.* (2012) (36·91%).The combination of the two genes (stx_1^+ stx_2^+) was found in 1·38% of the carcasses. This rate is lower than the result (5·7%) obtained by Mazzette *et al.* (2012).

The presence of the *eae* gene in combination with the *stx* genes was detected in 8·26% of the total samples which is less than the rate (17·94%) obtained by Momtaz *et al.* (2013). The onset of HC and HUS is closely associated with the types of STECs that carry the *eae* gene encoding intimin (Blanco *et al.* 2004).

These studies show that genetic markers of STEC could be isolated in all the countries where STEC was screened for and that sheep represent major reservoirs of these pathogens, as do cattle and ruminants in general (Vettorato *et al.* 2003). The presence of STEC on carcasses is indicative of poor hygiene practices, for which corrective measures need to be considered.

Thirty-six samples out of 363 (9·92%) contained only the *eae* gene. Mazzette *et al.* (2012) were able to detect this gene at a rate of 18·9% in sheep carcasses, whereas Maluta *et al.* (2014) reported an isolation rate of 11·11%. The *eae* gene is present in all EPEC (enteropathogenic *E. coli*) (Trabulsi *et al.* 2002). To identify EPEC, it is necessary first to detect the presence of *eae* gene (Mora *et al.* 2016).

EPEC and STEC (stx^+ eae^+) belong to the attaching and effacing *E. coli* groups, due to their ability to cause the same 'Attachment/effacement' lesions in enterocytes (Blanco *et al.* 2006). The detection of virulence genes by PCR in this study revealed the eventual presence of two different types of pathogens, namely, STEC and EPEC.

Isolation and characterization of isolated strains

Isolation of the bacterial strains that were identified as STEC-positive samples from PCR, was performed on

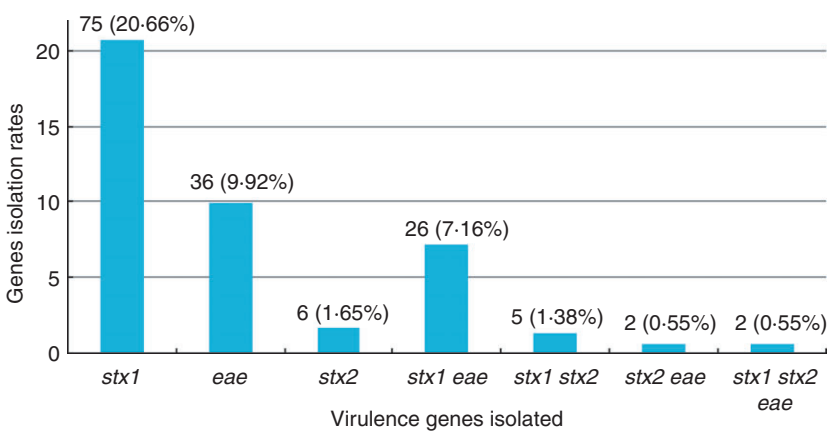

Figure 1 Results of the multiplex PCR screening for the genes *eae*, *stx1* and *stx2* in sheep carcasses (n = 363).

CHROMagar, which was chosen for its good sensitivity for several serogroups, such as O26 (90%), O111 (100%), O121 (100%), O145 (100%) and O157 (84·9%), with a specificity of 98·9% (Jari *et al.* 2012).

From the 116 positive PCRs for the stx^+/stx^+eae^+ genes, twenty strains (17·24%) were isolated. Nineteen (16·37%) belonged to the *E. coli* species (STEC). One strain (0·86%) belonged to *Citrobacter braakii* (eae^+ $stx_1{}^+$) (purple colonies with irregular edges and opaque centre, Fig 2).

The low level of isolated bacteria can be explained by the fact that many factors can influence the isolation rate of STEC in a sample, that is the presence of other bacterial species (stx^+) other than *E. coli*, such as *Shigella dysenteriae* type 1 and *Shigella flexneri* (Herold *et al.* 2004). The presence of competitive bacteria such as *Pseudomonas* spp., *Proteus* spp. and *Klebsiella* spp. may also mask a STEC outbreak (Fukushima and Seki 2004) and the viability of the present STECs. Verstraete *et al.* (2014) reported that no STEC strain could be isolated from 15 samples that were PCR-positive for the *eae* and *stx* genes.

Five strains (26·3%) of STEC, including three $stx_1{}^+eae^+$, one $stx_1{}^+$ and one $stx_2{}^+$, agglutinated with serum O26. Fourteen strains (73·7%) gave a negative result against the different sera tested.

The results of the characterization of the isolated strains are presented in Table 1.

STEC O26 was also isolated from ovine faeces, information according to by Blanco *et al.* (2003b). STEC O26 is an emerging pathogen found globally (EFSA 2011, 2012). In Europe, 127 O26 strains have been isolated from patients with HUS (Bielaszewska *et al.* 2013). These strains have been the cause of epidemic outbreaks in 66% of the cases in the United States (Luna-Gierke *et al.* 2014).

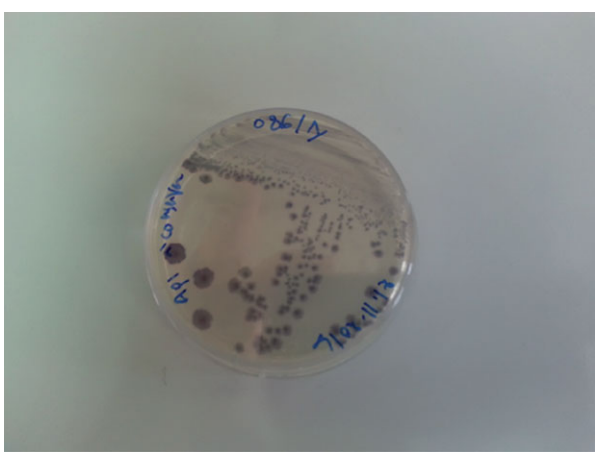

Figure 2 Appearance of *Citrobacter braakii* colonies on CHROMagarTM Shiga toxin-producing *Escherichia coli* (purple colonies with irregular edges and opaque center: *C. braakii*).

Table 1 Characterization of isolated strains

Strains	Number of positive carcass sampled from which strains have been isolated (20/116)	Number of strains isolation	Serogroup	Pathotype
STEC	2	2	—	stx_1
STEC	2	2	O26	stx_1 *eae*
STEC	2	2	—	stx_2 *eae*
STEC	1	1	O26	stx_2
STEC	1	1	—	stx_2
STEC	3	3	—	stx_1 sx_2
STEC	3	3	—	stx_1*eae*
STEC	1	1	O26	stx_1 *eae*
STEC	1	1	O26	stx_1
STEC	3	3	—	stx_2
Citrobacter braakii	1	1	—	stx_1 *eae*

STEC, Shiga toxin-producing *Escherichia coli.*

The O26 serogroup bearing the pathotype eae^+ $stx_1{}^+$ highlighted in this study was isolated from a patient with HUS (Mora *et al.* 2012) and patients with diarrhoea in China according to the data by Bai *et al.* (2016).

This confirms that the presence of STEC O26 on the surfaces of ovine carcasses can be a potential risk for humans.

Some STECs (14/19) in this study belonged to other serogroups that possessed both the *stx* and *eae* genes. This genetic combination confers increased virulence to the bacteria, resulting in more severe clinical manifestations in infected individuals (Werber *et al.* 2003). Of these fourteen STEC strains, nine carried the stx_2 gene, either alone or in combination with other genes, namely, stx_1 or *eae*. The strains producing the Stx2 toxin are one-thousand-times more virulent (cytotoxic) than those producing the Stx1 toxin (Joly and Reynaud 2003).

Regardless of serogroups, the results show that STECs carrying virulence genes isolated in this study are considered potentially pathogenic. These STECs represent a clear threat if vulnerable populations were to consume this contaminated meat; such exposure would risk the development of HUS cases and even deaths, as was the case in Europe and the United States (Bielaszewska *et al.* 2013; Luna-Gierke *et al.* 2014). Consumption of mutton in the form of dried sausages has already caused 10 cases of HUS among children, including one fatal case in Norway. This food contained *E. coli* (O103, Stx2) (Schimmer *et al.* 2008).

Citrobacter braakii carrying the virulence genes $stx_1{}^+$ eae^+ was isolated in this study for the first time. The virulence gene *eae* had already been isolated from *Citrobacter rodentium* (Luperchio and Schauer 2001; Verstraete *et al.*

2014). The *stx* gene is present in *E. coli*, *Shigella*, *Enterobacter*, *Acinetobacter*, *Campylobacter* and *Citrobacter* (Moore *et al.* 1988).

The *eae* gene was initially carried by a particular pathogenicity island called Locus of Enterocyte Effacement (LEE), located on a plasmid similar to the plasmid R100 of *Shigella spp*. and pO157 of EHEC (Deng *et al.* 2001). Then, the LEE was eventually acquired by other pathogens through the horizontal transfer of these plasmids (Donnenberg and Whittam 2001). The *stx* genes are carried by mobile genetic elements, phages, whose genomes are inserted into the bacterial chromosome (Beutin *et al.* 1997). The transfer of phage genetic material to other *E. coli* by transduction has led to the emergence of new STEC clones (Quirós and Muniesa 2017) as well as the acquisition of these genes by other bacterial species, such as *Citrobacter freundii* and *Enterobacter cloacae* (Herold *et al.* 2004). This explains the presence of the genes *eae* and stx_1 in *C. braakii acquis*, which presumably acquired these genes by a transfer of mobile elements, namely, plasmids or bacteriophages, encoding the virulence factors from other bacterial species. *C. freundii*, a producer of Shiga toxin, was responsible for severe cases of gastroenteritis and HUS in a nursery in Germany (Tschape *et al.* 1995). As a result, *C. braakii* (eae^+ stx_1^+) may be considered potentially pathogenic for humans.

STECs, carrying genes coding for virulence factors, isolated from the surfaces of sheep carcasses produced at the slaughterhouse, confirm that sheep are major reservoirs of potentially pathogenic STEC for humans in Algeria. This study also revealed the emergence of a new bacterium bearing the virulence genes stx_1 *eae*. The presence of these pathogens on the surface of sheep carcasses reflects non-compliance with good hygiene practices during slaughter operations.

Materials and methods

Sampling

Sampling was carried out in a slaughterhouse located of Algiers. The animals slaughtered came from different livestock markets in the region. A total of 363 carcasses were collected over a period of 9 months, from September to May 2015, at the rate of 10 sheep carcasses per week and were randomly selected for STEC research. Sampling was conducted using a non-destructive method based on double (wet/dry) swabbing the carcass surfaces as described in ISO (2003). For each sheep carcass, four areas of 100 cm^2 each located in the posterior-external area of the thigh, flank, large breast and posterior aspect of the forelegs were swabbed. Eight swabs were used for each carcass, and one wet and one dry swab were applied to each area. The samples were sent to the laboratory in a cooler and stored at a temperature of +4°C for analysis within 24 h.

Research and characterization of STEC

The search for STEC consisted of screening the samples for the stx_1, stx_2 and *eae* genes with a multiplex PCR. The isolation of the bacteria from the PCR-positive samples was performed on CHROMagar STEC chromogenic medium (CHROMagar, Paris, France), followed by serotyping. The entire research methodology was carried out in the Enterobacteriaceae Laboratory of the Pasteur Institute of Algiers (IPA).

Molecular screening by multiplex PCR

The swabs of each carcass, together composing a sample, were subjected to the following steps: pre-enrichment in 100 ml of peptone-filled broth (EPT; Oxoid, Basingstoke, Hampshire, UK) for 6 h at 37°C, from which the extraction and amplification steps were carried out.

Extraction and amplification of DNA

Extraction and amplification of the DNA were carried out according to the protocol of China *et al.* (1996).

For the detection of each gene, 5 μl of DNA was transferred into a PCR tube to which we added 2 μl of $MgCl_2$ (25 mmol l^{-1}) (Invitrogen, Waltham, MA, USA), 2·5 μl of dNTP (2 mmol l^{-1}) (Invitrogen), 5 μl of buffer (pH 8·8) (Invitrogen), 5 μl of the primer pair in question (10 μmol l^{-1}) (China *et al.* 1996), 0·5 μl of Taq polymerase (5 U μl^{-1}) (Invitrogen) and 30 μl of sterile distilled water. The PCR tubes were placed in the thermocycler set for the following times: denaturation at 94°C for 5 min; followed by 30 denaturation cycles at 94°C for 30 s, hybridization at 50°C for 30 s and extension at 72°C for 30 s.

Isolation and serotyping of the STEC strains

One hundred (100) microlitres of the enrichment broths that showed positive after PCR amplification was seeded on CHROMagar STEC chromogenic medium and incubated at 37°C for 18 h. Isolated mauve colonies, presumed STEC positive, were characterized by serotyping and biochemical identification. Nine serogroups (O157, O91, O111, O103, O145, O26, O128, O121 and O45) most often implicated in worldwide epidemics were sought. Five mauve colonies were randomly taken from each plate and subjected to anti-O157 (Oxoid), anti-O45 and anti-O121 (Statens, Seruminstitut, Copenhagen S, Denmark) sera agglutination assays as well as the anti-latex agglutination tests for O91, O111, O103, O145, O26

and O128 (DrySpot Serocheck and Seroscreen for *E. coli*; Oxoid). The biochemical characterization was performed with the API 20E gallery (BioMérieux, Craponne, France).

Molecular characterization of isolated strains

After isolation of the bacterial strains, we performed a second, multiplex PCR done under the same conditions described above to determine the genotype of each isolated strain.

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Conflict of Interest

The authors have no conflicts of interest.

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