Isolation and characterization of *Escherichia coli* O91:H21 in a sample obtained from cattle

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**INTRODUCTION**

Shiga toxin-producing *E. coli* (STEC) has been identified as the causative agent of foodborne diarrhea since 1982. STEC strains are characterized by the production of one or more types of cytotoxins which cause tissue damage in humans and animals. On the basis of their pathogenicity in humans, a subset of STEC strains are classified in the category of Enterohemorrhagic *E. coli* (EHEC). Symptoms of EHEC infection in humans range from abdominal pain and watery diarrhea and hemorrhagic colitis to hemolytic uremic syndrome (Paton and Paton, 1998; Riley et al., 1983; Verweyen et al., 2000). The natural reservoirs of STEC are domestic animals and wild ruminants, which eliminate the bacteria in their stool, disseminating them in the environment (Caprioli et al., 2005). STEC-infected animals usually do not show signs of infection and can be included in the food production chain. As a result, animal products such as meat or milk, pose a risk of contamination with STEC from such animals (Mellmann et al., 2008). Consumption of STEC contaminated food has been identified as the most important route for human infection with these pathogens (Caprioli et al., 2005; Mellmann et al., 2008).

Typical EHEC strains isolated from patients have, besides the encoding cytotoxins genes mentioned above, the eae gene that encodes the adhesin intimin (Bettelheim, 2007; Bielaszewska et al., 2006). However, a subgroup of EHEC strains associated with disease in humans lacks the eae gene, thus denominated atypical EHEC (Bettelheim, 2007; Karmali et al., 2003; Pradel et al., 2008; Pulz et al., 2003).
Table 1. Oligonucleotides used in the PCR reactions.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Primer sequence (5'-3')</th>
<th>Size bp</th>
<th>Reference source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stx1a</td>
<td>GAAGAGTCCGTGGGATTACG</td>
<td>130</td>
<td>Pollard et al., 1990</td>
</tr>
<tr>
<td>Stx1b</td>
<td>AGCGATGCAGCTATTAATAA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stx2a</td>
<td>TTACCCACACCCACCCGGGCA</td>
<td>346</td>
<td>Pollard et al., 1990</td>
</tr>
<tr>
<td>Stx2b</td>
<td>GCTCTGGATGCATCTCTGGT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>eae a</td>
<td>TGAGCGGCTGGCATGATCATAC</td>
<td>240</td>
<td>Pass et al., 2000</td>
</tr>
<tr>
<td>eae b</td>
<td>TCGATCCCATGCACGAGAAGG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>astA a</td>
<td>GCCATCACA GTA TAT CCG</td>
<td>108</td>
<td>Ruttler et al., 2006; Schmidt et al., 1995</td>
</tr>
<tr>
<td>astA b</td>
<td>GCG AGT GAC GGC TTT GTA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HlyA1</td>
<td>GGTGCAGCAGAAAAAGTGTAG</td>
<td>1551</td>
<td>Schmidt et al., 1995</td>
</tr>
<tr>
<td>HlyA4</td>
<td>TCTCGCCTGATAGTGTGGTA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SAAD F</td>
<td>CGTGATGAACAGGCTATTGC</td>
<td>119</td>
<td>Paton and Paton, 2002</td>
</tr>
<tr>
<td>SAAD R</td>
<td>ATGGACATGCCTGTGGCAAC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AggR F</td>
<td>AGACGCCTAAAGGATGCC</td>
<td>430</td>
<td>Ruttler et al., 2006</td>
</tr>
<tr>
<td>AggR R</td>
<td>GAGTTATCAAGCAGACGATGC</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Whereas EHEC O157:H7 was known to be the predominant serotype associated with most of the documented EHEC-related outbreaks worldwide, previous epidemiological studies imply that non-O157 EHEC infection can become problematic for public health (Caprioli et al., 2005). The non-O157 EHEC strains associated with human disease include the serotypes O8:H-; O26:H11, O91:H21, O103:H2, O111:H-, O113:H21 and O128:H2 (Padola et al., 2002).

E. coli O91: H21 is a common serotype among EHEC eae negative subset. The strains belonging to this serotype have been classified within the seropathotype "C" to cause disease in humans, although with low incidence and low association with outbreaks, are known to be highly virulent (Bielaszewska et al., 2006; Bugarel et al., 2010; Kim et al., 2013; Kruger et al., 2006; Mellmann et al., 2008; Mellmann et al., 2009). The strains within this serogroup appear to be transmitted predominantly by food, because food vehicles have been identified as the only risk factors for adults with sporadic STEC O91 infection in Germany; O91 is the second most frequently isolated STEC serogroup in routine food samples in that country, and O91 is the only major STEC serogroup with no association between incidence of human infection and cattle density. Despite frequent isolation of STEC O91 from humans, the clonal relatedness of the serotypes of this serogroup is poorly understood (Mellmann et al., 2009).

Although this serotype has been isolated previously in Argentina in cattle, and its strains have been phenotypically and genotypically described (Kruger et al., 2006), there are no reports of strains belonging to this serotype until now, from either animal or human samples in Mendoza. This work reports the finding of a bovine-derived E. coli O91:H21 strain in a slaughterhouse in the Directorate of Livestock in the province of Mendoza in 2007.

MATERIALS AND METHODS

This isolation carries identified pathogenicity factors that enhance its virulence. So far, there has not been reported illness in patients associated with this serotype in our city.

This strain was found in the gut content of a freshly slaughtered animal intended for consumption. Detection was performed by PCR, looking for strains carrying Stx2 genes, and then the isolation of the strain was carried out following the methodology described: 91 rectal swabs of 91 animals intended for slaughter and 108 plating samples from the carcasses of 50 of them were analyzed during a period of nine months in 2007.

The samples were taken according to the rules and regulations set forth by SENASA, the organization that controls agriculture and livestock farming in Argentina. This procedure was carried out during the anal enucleating of animals.

PCR was carried out as a screening technique, looking for strains carrying encoding Shiga toxin1 (Stx1), and Shiga toxin 2 (Stx2) genes (Paton and Paton, 1998). A total of 199 (91 rectal swabs and 108 carcass) samples were studied. The reactions were carried out in 50-μL reaction volumes containing 2 μL of bacterial lysate, 200 mmol of deoxynucleotide mix (dATP, dTTP, dCTP, and dGTP), 100 pmol of each primer, 5 μL of 10XPCR buffer with 1.5 mM MgCl₂, and 2.5 U of Taq DNA polymerase (InbioHighway).

After the screening by PCR, the positive broths were reseeded on plates, and then pools of 10 colonies were tested again until the colony carrying the gene was found. Then, the E. coli strain was identified by standard biochemical tests as oxidase negative, indole positive, Simon's citrate negative, urease negative, and hydrogen sulfide negative (MacFaddin, 2003). Once the strains were isolated and reconfirmed by PCR, their genotyping was conducted by identification of coding genes of these proteins: intimin (eae) (Pass et al., 2000), enterohemolysin (HlyA) (Schmidt et al., 1995), enteroaggregative thermoestable toxin EAST1 (astA) (Ruttler et al., 2006; Schmidt et al., 1995) STEC binding adhesin (Saa) (Paton and Paton, 2002) (Table 1). The detection of Stx1 and Stx2 genes was carried out in a mutiplex reaction, while the remaining reactions
were performed in a single reaction. The following strains were used as positive controls: E. coli EDL 933 (O157: H7, Stx1 +, Stx2 +, eae +, astA +), E. coli O157: H7 Hly +, (Strains provided by National Institute of Infectious Diseases *Dr. Carlos G. Malbrán*); E. coli saa + (strain provided by Dr. Paula Lucchesi, Faculty of Veterinary Medicine, National University of Centro, Tandil, Buenos Aires, Argentina).

The reactions were performed in a thermocycler Eppendorf Mastercycler Personal, as previously described protocols (Pollard et al., 1990; Schmidt et al., 1995; Pass et al., 2000; Rüttler et al., 2006; Paton and Paton, 2002). For detection of sero-group, agglutination was conducted with monoclonal antisera anti-O and anti-H at the Veterinary Medicine School, Buenos Aires Central National University, at Tandil City. The strain being studied agglutinated with antisera anti O91:H21.

Vero cells have a high sensitivity to Shiga toxins and trial on cytotoxicity using this cell line is the "gold standard" technique as control strains were used; E. coli O157: H7 C984 (Stx1 +) and E. coli O157: H7 from 1271-84 (Stx2+) (Strains provided by National Institute of Infectious Diseases *Dr. Carlos G. Malbrán*). The cytotoxicity trial was performed as previously described (Karmali et al., 1985) on Vero cells (passage 8) cultured with Minimum Essential Medium (MEM) supplemented with 10% fetal bovine serum (FBS) and gentamicin (50 µg/mL). In order to achieve convergence, the required cell concentration was used.

RESULTS AND DISCUSSION

Twelve strains of E. coli were identified as having at least one virulence factor present in E. coli pathogenic. It was possible to recover only one strain O91: H21 from a sample of intestinal contents.

The strain confirmed as E. coli O91: H21 carried the genes encoding Stx2 toxin, the enterohemolysin (HlyA), and STEC binding adhesin (Saa).

Cytotoxic activity was estimated in E. coli O91:H21 Stx2+ strain, considering the reverse title of highest dilution provided a 50% destruction of the monolayer as a cytotoxic unit (DC50) (Basta et al., 1989; Karmali, 1989) Strain E. coli O91: H21 showed marked cytotoxic effect up to 1:64 dilutions.

It also turned out to be negative for the gene encoding the synthesis thermostable toxin enterotoxigenic E. coli (astA gene) that is present in some EHEC strains as well (Savarino et al., 1996).

It is known that most of the strains isolated from human origin, have common properties directly related to virulence, including hemolysin production, induction of injury "attaching and effacing" in intestinal epithelial cells and production of toxins Stx1 and Stx2. Therefore, to predict whether the isolates from animal sources are potentially pathogenic to humans, it is necessary to determine the presence and the virulence factor profile (Paton and Paton, 1998; Bielaszewska et al., 2006).

Moreover, recent “Enter-Net” data, a global consortium of enteric disease surveillance of 35 countries, showed that the number of cases of disease caused by non-O157 EHEC increased by 60.5% compared to 13% increase in cases involving EHEC O157 (Pihkala et al., 2012).

In other works consulted, similar results were obtained with respect to the phenotypic characteristics of strain E. coli O91: H21 in this study (Mellmann et al., 2008; Mellmann et al., 2009). This strain could be considered as atypical EHEC that encode the synthesis of Shiga Toxin 2, enterohemolysin STEC Binding adhesin but not for intimin.

Based on the fact that Shiga toxin type 2 (Stx2) is primarily responsible for renal failure in the HUS, extremely hygienic-sanitary controls are necessary for cattle products, especially beef, so they do not pose a risk to the population.

These data highlight the importance of studying these potentially pathogenic strains and their comprehensive characterization to be alert to the existence of virulent strains circulating in the population’s food chain. The information will also facilitate the comparison of the characteristics of the animal strains with those isolates obtained from patients with clinical symptoms, for the purpose of defining the potential pathogenic strains of this serotype isolated in the region.

The implementation of prevention and control strategies in public health impact are essential to reduce morbidity and mortality associated with HUS, as emphasizes the World Health Organization. Therefore, it is necessary to improve pathogen control measures through the agro-food chain to ensure food quality. Education programmes should be aimed to the community in general, warning about the risks of this agent, its ways of transmission as well as to implement prevention strategies.

Conflict of Interests

The author(s) have not declared any conflict of interests.

REFERENCES


