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Isolation and characterization of non-O157 Shiga toxin-producing *Escherichia coli* (STEC) isolated from retail ground beef in Santiago, Chile

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ABSTRACT

Shiga toxin-producing *Escherichia coli* (STEC) is one of the main cause of foodborne disease worldwide, but isolation rates or characteristics of this bacteria from ground beef in Chile are unknown. The present study aimed to isolate and characterize non-O157 STEC from ground beef sold at retail in the city of Santiago, Chile. We analyzed 430 ground beef samples for the presence of STEC, and isolated the microorganism in 10% of samples (43/430). We obtained 56 isolates from the 43 positive samples; 55 of these (98.2%) fermented sorbitol. Most isolates (98.2%; 55/56) showed β-glucoronidase activity, and only six (10.7%; 6/56) were resistant to tellurite. Among the virulence factors studied (stx_1, stx_2, *eae*, and *hlyA*), stx_2 was the only virulence factor in 41% of the isolates (23/56), whereas 10.7% (6/56) of isolates carried a combination of three virulence factors (*stx1* + *stx2* + *hlyA*). None of the isolates carried the gene *eae*. Finally, isolates were neither serogroups O157 nor "big six". In conclusion, ground beef sold in Santiago, Chile is contaminated with STEC; however, further studies are required for understanding their virulence potential.

1. Introduction

Shigatoxin-producing *E. coli* (STEC) is one of the most important foodborne pathogens in the world; the bacterium can cause large outbreaks and severe diseases such as hemolytic uremic syndrome (HUS) and can even cause death (Scallan et al., 2011; Majowicz et al., 2014; WHO, 2015). Bovines are regarded as one of the main reservoirs of the pathogen (Bettelheim, 2007); beef and beef products have been frequently associated with STEC outbreaks (EFSA, 2013; Robertson et al., 2016). STEC O157:H7 has been traditionally linked to human illnesses (EFSA, 2013; Robertson et al., 2016), but over 400 STEC serotypes have been associated with human disease in the world (Blanco et al., 2004; EFSA, 2013). Serogroups O26, O45, O103, O111, O121 and O145—known as the *big six*— are the within most prevalent non-O157 STEC causing disease in the United States (Hoang Minh et al., 2015; USDA, 2012) and in other countries (EFSA, 2013).

STEC strains can carry several virulence factors that are linked to their ability to cause disease. STEC main virulence factors are Shigatoxins Stx1 and Stx2 (encoded by stx_1 and stx_2 genes) and their variants, which interfere with protein synthesis and cause intestinal cell death (Johannes and Römer, 2010). The protein *intimin* (encoded by gene *eae*) is described in highly virulent isolates; it is involved in the close contact between the bacteria and the intestinal cell and the effacing lesions on intestinal mucosal cells (McWilliams and Torres, 2014). HlyA (plasmid gene EHEC-*hlyA*) is an exotoxin that lyses erythrocytes and other cells, promoting iron acquisition for bacterial nutrition (Lorenz et al., 2013). These virulence factors are considered among the main ones involved in STEC pathogenicity.

STEC have been detected and isolated from beef and beef products around the world: In China, STEC was detected in 48% of ground beef and isolated in 9.9% of the samples (Bai et al., 2015; Li et al., 2016); In the United States, non-O157 STEC has been isolated from 5.2% to 7.3% of ground beef samples (Bosilevac and Koohmaraie, 2011; Ju et al., 2012). In Argentina, 25% of beef cuts and 40.7% of minced beef samples were positive for non-O157 STEC at screening (Etcheverría et al., 2010), and non-O157 STEC were

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isolated from 14% of raw ground beef samples (Brusa et al., 2012). In Chile, STEC has been isolated from beef and pork cattle at a slaughterhouse (Borie et al., 1997) and from zoo animals (Marchant et al., 2016). However, the isolation rate of STEC from beef has not been reported. We hypothesized that ground beef is a vehicle for non-O157 STEC in Chile. To test this hypothesis we investigated the presence of STEC in 430 ground beef samples obtained across Santiago, capital of Chile, and determined the presence of the main virulence genes (*stx*$_1$, *stx*$_2$, *eae*, *hlyA*) in the isolates. To characterize the isolates better, we also studied phenotypic characteristics such as sorbitol fermentation, β-glucoronidase activity, resistance to tellurite and production of hemolysin. Finally, we analyzed whether the isolates belonged to some of the most frequent disease-causing serogroups in the world (O157 and big six).

2. Materials and methods

2.1. Sampling

We obtained 430 ground beef samples from grocery stores and butcher shops across the city of Santiago, Chile. Samples were taken biweekly from March to December 2016. The city was divided into 4 main areas (north, west, south and east), and a similar number of samples were taken in each area and from each type of store (Table 1). Samples were transported below 8 °C to the Microbiology and Probiotics Laboratory, University of Chile, and processed the same day.

2.2. Sample processing and screening

Ground beef samples were enriched as previously described (Ju et al., 2012). Briefly, 25 g of ground meat were manually homogenized with 225 ml of modified TSB [30 g TSB (DIFCO) + 1.5 g bile salts N°3 (DIFCO) + 1.5 g K_2HPO_4 (Merck, Germany) for 1 L] in a sterile BagFilter® P bag (Interscience, France). Samples were incubated at 42 °C for 20–22 h. After incubation, 3 loops of the enriched sample were streaked on McConkey agar and incubated at 37 °C for 24 h.

After incubation, DNA was extracted from both the enrichment broth and the McConkey agar plates of each sample, using the InstaGene ™ Matrix (Bio-Rad, Carlsbad, CA) following manufacturer's instructions; two templates for each sample were used to screen for the presence of STEC through a multiple PCR reaction targeting the *stx*$_1$ and *stx*$_2$ genes (Toro et al., 2013). PCR were performed in 25 μl reaction mixture containing 2.5 μl DNA template, 12.5 μl GoTaq® Green Master mix 2X (Promega, Wisconsin), and 0.5 μl (final concentration of 0.2 μM) of each oligonucleotide (IDT, Coralville, IA) (Table 2). The PCR protocol included an initial denaturation step at 95 °C for 5 min, followed by 30 cycles of denaturation (95 °C for 30 s), annealing (56 °C for 30 s) and extension (72 °C for 40 s), with a final extension step at 72 °C for 10 min. PCR products were resolved in 2% (wt/vol) agarose gels on 0.5% TAE buffer at 100 mV for 30 min.

2.3. STEC identification and isolation

When a template tested positive for one or both *stx* genes, 30 individual colonies were examined for the presence of *stx* genes. Positive colonies for the Shigatoxin genes were then confirmed as *E. coli* by a PCR previously described (Chen and Griffiths, 1998) with some modifications. Briefly, the PCR reaction contained 10 μL of GoTaq® Green Master mix 2X (Promega), 0.5 μL (final concentration of 0.3 μM) of each oligonucleotide (IDT; Table 2), 1 μL DNA template and molecular grade water for a final reaction volume of 17 μL. The PCR program included initial denaturation at 94 °C for 5 min, 25 cycles of denaturation (94 °C for 30 s), annealing (58 °C for 30 s) and extension (72 °C for 40 s). The final extension step was at 72 °C for 5 min. STEC isolates (isolates that tested positive for one or both *stx* genes and for *E. coli* by PCR) were stored in 20% glycerol at −80 °C for further analysis.

2.4. Virulence profiling and molecular serogrouping

Each STEC isolate was later characterized for the presence of virulence genes *eae* and *hlyA* by PCR as previously described (Fratamico and Strobaugh, 1998; Xia et al., 2010) (Table 2). DNA was extracted for each individual isolate as described above.

Additionally, we performed a multiplex PCR reaction to determine whether the isolates were of serogroups O26, O45, O103, O111, 0121, 0145, or 157, as described by Toro et al. (Toro et al., 2013) (Table 2). If a band was present, the DNA was tested for each serogroup in individual PCR reactions using the same primers.

DNA from strain ATCC350150 was used as positive control for genes *eae, hlyA* and for serogroup O157. Positive controls for the remaining serogroups were DNA obtained from strains 88–353 (O26), A9619-C2 (O45), B27828/95 (O103), P1338 (O111), SJ18 (O121), and CVM9777 (O145) which were provided by the Laboratory of Food Safety, University of Maryland, College Park, United States.

2.5. Biochemical characterization of STEC isolates

All isolates were characterized for the following biochemical features: a) Sorbitol fermentation test: individual isolates were inoculated on Sorbitol McConkey (SMAC) agar (BD, MD) and incubated at 37 °C. Results were recorded as positive or negative depending on the development of pink colored colonies after 24 h incubation (Miko et al., 2014); b) β-glucoronidase activity test: individual isolates were inoculated on TBX (Tryptone Bile X-glucuronide) chromogenic agar (Biomérieux, France) and incubated at 35 °C and 44 °C for 24 h. Cultures containing the enzyme developed a blue-green color on the agar after incubation (Verhaegen et al., 2015); c) Tellurite resistance was assessed by inoculating isolates on SMAC agar supplemented with 2.5 μg/ml tellurite (CT-SMAC). Plates were incubated at 37 °C for 24 h; cultures with the ability to grow in the media were defined as resistant to tellurite (Miko et al.,

Table 1
Positive samples for STEC at screening and isolation per area in Santiago, Chile.

Type of store	Grocery stores			Butcher shops			Total		
Area	Isolated (%)	Screening (%)	Total samples	Isolated (%)	Screening (%)	Total samples	Isolated (%)	Screening (%)	Total samples
North	7 (13.0)	16 (29.6)	54	8 (14.8)	27 (50)	54	15 (13.9)	43 (39.8)	108
West	3 (5.7)	18 (34.0)	53	5 (9.3)	32 (59)	54	8 (7.5)	50 (46.7)	107
South	4 (7.4)	22 (40.7)	54	5 (9.3)	38 (70.4)	54	9 (8.3)	60 (55.6)	108
East	6 (11.1)	22 (40.7)	54	5 (9.4)	37 (69.8)	53	11 (10.3)	59 (55.1)	107
Total	20 (9.3)	78 (36.3)	215	23 (10.7)	134 (62.3)	215	43 (10.0)	212 (49.3)	430

Statistical comparisons between type of store and area were made using the Pearson Chi-square. No significant differences were detected among isolation rates ($p < 0.05$). All tests were performed in SSPS v25.

Table 2
Primers and PCR conditions used in this study for detection of target genes.

Type of PCR	Target	Forward Primer (5′ to 3′)	Reverse Primer (5′ to 3′)	Annealing Temp (°C)	Amplicon size (bp)	Reference
Multiplex or Singleplex	*stx*$_1$	CAGTTAATGTGGTGGCGAAGG	CACCAGACAATGTAACCGCTG	56	348	(Paton and Paton, 1998; Toro et al., 2013)
	stx$_2$	ATCCTATTCCCGGGAGTTTACG	GCGTCATCGTATACACAGGAGC		584	
Singleplex	*E. coli uspA*	CCGATACGCTGCCAATCAGT	ACGCAGACCGTAGGCCAGAT	58	884	(Chen and Griffiths, 1998)
Singleplex	*eae*	ATTACCATCCACACAGACGGT	ACAGCGTGGTTGGATCAACCT	63	397	(Fratamico and Strobaugh, 1998)
Singleplex	*hlyA*	AGCCGGAACAGTTCTCTCAG	CCAGCATAACAGCCGATGT	60	526	(Xia et al., 2010)
Multiplex or Singleplex	O26 *wzx*	GTGTGTCTGGTTCGTATTTTTTATCTG	CCTTATATCCCAATATAGTACCCACCC	56	438	(Toro et al., 2013)
	O45 *wzx*	GGTCGATAACTGGTATGCAATATG	CTAGGCAGAAAGCTATCAACCAC		341	
	O103 *wzy*	TTATACAAATGGCGTGGATTGGAG	TGCAGACACATGAAAAGTTGATGC		385	
	O111 *wzx*	TTCGATGTTGCGAGGAATAATTC	GTGAGAGCCCACCAGTTAATTGAAG		362	
	O121 *wzy*	AGTGGGGAAGGGCGTTACTTATC	CAATGAGTGCAGGCAAAATGGAG		366	
	O145 *wzy*	CCTGTCTGTTGCTTCAGCCCTTT	CTGTGCGCGAACCACTGCTAAT		392	
	O157 *wzy*	TCGTTCTGAATTGGTGTTGCTCA	TCGTTCTGAATTGGTGTTGCTCA		278	

2014) and d) EHEC-Enterohemolysin and α-hemolysin production test: individual isolates were inoculated on washed sheep blood agar (AGRL) and on blood agar (AGRSL) and incubated at 37 °C as described by Beutin et al., 1989. Cultures were considered positive for EHEC-Enterohemolysin when hemolysis was present exclusively on AGRL plates after 18 h of incubation. Cultures were positive for α-hemolysin when hemolysis was observed on AGRL and/or AGRSL plates after 3 h of incubation, or when both plates presented hemolysis after 18 h of incubation (Beutin et al., 1989; Rivas et al., 2007; Lorenz et al., 2013).

2.6. Statistical analysis

Statistical analysis comparing STEC isolation rates was performed using Pearson's Chi square test in SPSS for windows v25.0.

3. Results

In this study we isolated STEC strains from ground beef samples and then characterized the isolates for virulence factors, phenotypic characteristics and molecular serogroup for the main disease-causing STEC serotypes in the world (O157 and "*big six*").

3.1. Isolation rate of STEC from ground beef

We detected that 49% of samples tested positive to *stx* genes at screening, and differences between grocery stores (78/215; 36.6%) and butcher shops (134/215; 62.3%) were identified ($p < 0.05$) (Table 1). Positive samples were further processed to obtain STEC isolates, and we were able to isolate STEC from 43 (10%) of them. We did not detect a significant difference in isolation rates between grocery stores (20/215; 9.3%) and butcher shops (23/215; 10.7%) or among types of store by area ($p < 0.05$) (Table 1).

3.2. Virulence profiling and serogrouping of STEC isolates

We obtained 56 different isolates from 43 ground beef samples based on their virulence gene profile. The gene *stx*$_2$ was the most frequently detected shigatoxin gene; it was present in 60.7% (34/56) of isolates as the only *stx* gene. The combination *stx*$_1$ + *stx*$_2$ was found in 19.6% (11/56) of isolates (Fig. 1). None of the isolates tested positive for the *eae* gene, while 37.5% tested positive for *hlyA* (21/56).

Considering the presence/absence of the four tested virulence genes (*stx*$_1$, *stx*$_2$, *eae*, *hlyA*), we detected six different virulence profiles; most strains (41%; 23/56) carried the gene *stx*$_2$ as their only virulence factor, and less than 50% of the isolates were positive for more than one virulence gene. For instance, the combination *stx*$_1$+*stx*$_2$+*hlyA* was present in 10.7% (6/56) of the isolates (Table 3).

None of the 56 isolates studied tested positive for serogroups O157 or *big six* at the PCR.

3.3. Biochemical characteristics of STEC isolates

All but one of the 56 isolates obtained fermented sorbitol (98%),

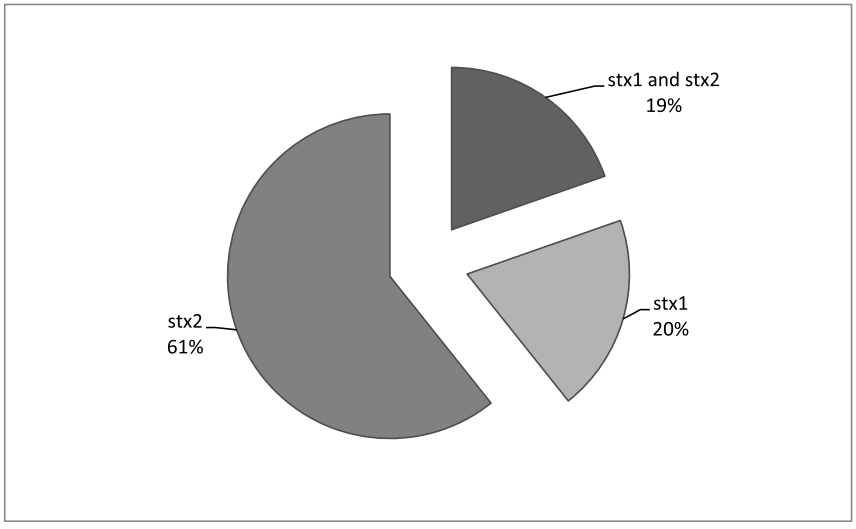

Fig. 1. Shiga toxin profiles of STEC isolates (n = 56). Percentage of the isolates displaying the different combinations of shigatoxin (*stx*) genes.

Table 3
Virulence profile of STEC isolates and their frequency.

Virulence profile	Number of isolates (%)
stx2	23 (41.1%)
stx2 + *hlyA*	11 (19.6%)
stx1	7 (12.5%)
stx1 + *stx2* + *hlyA*	6 (10.7%)
stx1 + *stx2*	5 (8.9%)
stx1 + *hlyA*	4 (7.14%)
Total	56 (100%)

and only six strains grew in the presence of tellurite, showing resistance to this substance (10.7%). Furthermore, 55 isolates displayed β-glucoronidase activity, and most of isolates showed some hemolysis (89%; 50/56): α-hemolysis was displayed by 44.6% (25/56) of isolates while 44.6% showed EHEC-hemolysin activity (25/56).

4. Discussion

STEC is an important public health concern in Chile and in the world because the microorganism can cause foodborne outbreaks and some cases evolve to life-threatening complications such as HUS ([WHO], 2015). In Chile, HUS incidence has been reported as 3.2/100000 inhabitants, and non-O157 serogroups O26 and O111 are among the most important causes of STEC infections (Vidal et al., 2010; ISP, 2014).

Since cattle are the main reservoir of STEC, beef and beef products are the most common food vehicles attributed to human disease in the world (Bettelheim, 2007; Masana et al., 2011). Official, internationally recognized methods to study the presence of STEC in meats and other food matrices are available, such as the ISO/TS 13136:2012 or the MLG 5B.06 by the USDA/FSIS (USDA, 2014). These methods focus on detecting and isolating STEC of the 5 (ISO) or 7 (USDA/FSIS) most prevalent serogroups causing human cases and that carry the gene *eae*; however, these methodologies neglect detection of STEC from other serogroups as well as *eae*-negative STEC. In this study we chose to use a combination of methodologies (Chen and Griffiths, 1998; Ju et al., 2012; Toro et al., 2013) to attempt to detect any *E. coli* serogroup carrying shigatoxin genes, because local information indicates that human cases have been caused by several different serotypes (ISP, 2014), and also because we lack current information about the STEC serogroups potentially present in local ground beef. Most of research studies on STEC in the world use adaptations of different methodologies, and therefore differences among studies need to be taken into account when comparing detection and isolation rates.

Detection and isolation rates of non-O157 STEC in ground beef greatly vary in the world. For example, studies in the U.S. detected up to 24.3% with STEC in screening (Bosilevac and Koohmaraie, 2011) and 33% in China (Bai et al., 2015), but detection rates in Argentina reached 52.2% (Brusa et al., 2012). Our overall detection rate for screening (49.3%; Table 1) was similar to the rate reported in our neighbor country, Argentina; however, our isolation rate (10%; Table 1) was similar to the reported rate in China (9.9%), somewhat superior to those reported in the U.S. (7.3% and 5.2%), but slightly below the isolation rate in Argentina (14%), the country with the highest HUS incidence in the world (Bosilevac and Koohmaraie, 2011; Brusa et al., 2012; Ju et al., 2012; Bai et al., 2015). We detected significant differences in screening rates between grocery stores and butcher shops (Table 1), but isolation rates were not different between these types of store or among areas where the samples were taken. Since we consider isolation as the main proof of presence of STEC, we concluded that there was a homogeneous distribution of ground beef contamination with STEC in Santiago, Chile. Packaged and unpackaged ground meat sample contamination rates were similar; however, we found that vacuum sealed packaged samples presented a higher contamination rate than the average (28%; 8/28. Suppl Table 1). Further studies focusing on this type of sample are required to define whether this is a common finding in vacuum packaged ground beef sold in Santiago, Chile.

Shigatoxin genes are the main virulence factor in STEC, and strains carrying stx_2 would have higher pathogenic potential than strains carrying stx_1 alone or in combination with stx_2 (Johannes and Römer, 2010). Therefore, contact with stx_2 positive STEC implies a higher risk to contract more severe cases of STEC disease, including complications such as HUS. Here we found that the gene stx_2 was the most frequently found shigatoxin gene in the isolates, either as the only shigatoxin gene (60.7%; 34/56) or in combination with stx_1 (19.6%; 11/56) (Fig. 1). Similar results were reported in Germany, Argentina and Japan where isolates carried stx_2 as the main shigatoxin gene reported (Beutin et al., 2007; Brusa et al., 2012; Hoang Minh et al., 2015). In contrast with our results, a previous study in Chile found that *stx1* was the most prevalent shigatoxin gene found in isolates from cattle (Borie et al., 1997), but there are no data on STEC isolated in beef. It is also possible that an epidemiological shift could have happened during the past 20 years, but studies of STEC isolated from cattle are needed to confirm this hypothesis.

Shigatoxin production is necessary but not sufficient for STEC virulence. In this study we analyzed the presence of other two main virulence factors: intimin (*eae*) and enterohemolysin (*hlyA*). Intimin is an adhesin with a role in bacterial attachment to the intestinal cell; it has been frequently described in highly virulent isolates (McWilliams and Torres, 2014). None of the isolates carried the gene *eae* in the present study. Similar results were obtained in the United States (Ju et al., 2012) and in Argentina (Brusa et al., 2012) where the *eae* gene was absent in non-O157 STEC. In contrast, other studies have found the gene *eae* in beef samples (Llorente et al., 2014; Hoang Minh et al., 2015). As mentioned above, strains carrying *eae* are considered potentially pathogenic, yet the pathogenicity potential of *eae*-negative isolates cannot be predicted (EFSA, 2013), considering that *eae*-negative strains have caused diarrhea, HUS and outbreaks of disease in the world (EFSA, 2013). Studies have discovered that other virulence genes related to adherence—such as *saa*, *aidA, agn43*, *ehaA*, or *iha* (Colello et al., 2016)—might replace the *eae* function, allowing *eae*-negative strains to cause disease (EFSA, 2013). In this study we did not survey the isolates for the presence of other adhesins that could be substituting for *eae*, but we plan to address these questions in further studies.

hlyA is a gene that codifies for α-hemolysin, a toxin that lyses mammalian erythrocytes (Lorenz et al., 2013); studies have associated the presence of *hlyA* and other hemolysins with human clinical STEC isolates (Vidal et al., 2010; Lorenz et al., 2013). A third of our isolates (37.7%) carried the *hlyA* gene, and we detected hemolysin activity in almost 90% of the isolates, either produced by EHEC-enterohemolysin or α-hemolysin activity. Since this characteristic is associated with medical complications, it is worrying that a large portion of the STEC isolated in this study have these virulence factors.

Human disease has been more frequently associated with some STEC serogroups. In Chile, three serogroups are the major cause of disease caused by STEC: O157, O26 and O111; however, there are multiple isolates causing disease that could not be serotyped or belong to other serogroups (ISP, 2014). Therefore, although we did not find isolates of serogroup O157 or any of the big six serogroups, the isolates found could still have public health impact.

The STEC are a heterogeneous group, and their biochemical

characteristics are an example of this. We studied biochemical characteristics of the isolates and found that the predominant biochemical profile was sorbitol fermenter, β-glucoronidase positive and susceptible to potassium tellurite. Some of these substances are components of culture media used worldwide for the isolation and identification of non-O157 STEC (Verhaegen et al., 2015; Kerangart et al., 2016). In our study, only 10% of isolates grew in the presence of potassium tellurite, indicating that media containing this substance would not be suitable to isolate all non-O157 STEC in beef samples, as indicated in another study (Verhaegen et al., 2015).

The present study has some shortcomings that must be considered when analyzing our results. First, we tested 30 colonies for each positive sample at screening, while other studies analyzed up to 50 colonies per positive sample, or used detection techniques with higher sensitivity (Ju et al., 2012). Consequently, it is possible that the STEC isolation rate reported in this study is underestimating the real contamination rate of ground beef sold in Santiago, Chile. The presence of other adhesion genes with potential pathogenicity roles in *eae*-negative STEC was not investigated and the serotype of the isolates was not determined. Although this information is important, we consider that the main aim of this study was reached since we were able to determine whether ground beef is a vehicle for STEC in Chile. We expect to describe the isolates in detail in future studies.

5. Conclusion

We conclude that beef is a food vehicle for STEC in Santiago, Chile. STEC isolates obtained in this study were of serogroups different from the most frequent disease-causing serogroups in the world (O157 and "big six") and *eae*-negative. Considering that *eae*-negative isolates from a variety of serotypes have caused severe disease and outbreaks in the world, we cannot disregard our isolates as potential causes of human disease. Consequently, the population of Santiago might be at risk of acquiring STEC infection by manipulating ground beef. Further studies fully characterizing of the isolates are required to understand the real potential pathogenicity of our isolates.

Conflict of interest

No conflict of interest declared.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.fm.2017.10.015.

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