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Short communication

Isolation and characterization of Shiga toxin-producing *Escherichia coli* from precooked sausages (morcillas)

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Abstract

The aim of the study was to establish the microbiological quality of morcillas, typical Argentine sausages, and to investigate the presence of Shiga toxin-producing *Escherichia coli* (STEC). Between October 2001 and October 2002, a total of 100 morcilla samples were analysed. Several samples showed high levels of total aerobic mesophilic bacteria counts, molds and yeasts. The samples analysed contained *Enterobacteriaceae* (100%) and fecal coliforms (81%), indicating inadequate application of the thermal treatment and deficient hygiene conditions during the elaboration of the product.

STEC strains were isolated from three out of 100 (3%) morcilla samples. Two strains (2%) were characterized as *E. coli* O157:H7 *stx*2 + *stx*2vh-a/*eae*/EHEC-*hly*A and one strain (1%), as *E. coli* O26:H11 *stx*1/*eae*/EHEC-*hly*A. Considering both the high microbial count in all tested samples and the presence of STEC strains in three of them, morcillas should be considered a food unsafe to consume when inadequately cooked.

Keywords: STEC; O157:H7; Blood link sausage; Morcilla; PCR

1. Introduction

Shiga toxin-producing *Escherichia coli* (STEC) is an important emerging foodborne pathogen associated with different clinical manifestations such as bloody and non-bloody diarrhea, hemorrhagic colitis (HC), and the life-threatening hemolytic-uremic syndrome (HUS) (Swerdlow et al., 1992).

Shiga toxins (Stx) are considered to be the major virulence factors of STEC, which are responsible for vascular endothelial damage. These toxins, encoded by lysogenic bacteriophages, are classified in two main types, Stx1 and Stx2. STEC strains may produce Stx1 or Stx2, or both. Variants of Stx1 and Stx2, including Stx1Ox3, Stx1c, Stx2c, Stx2d, Stx2e and Stx2f, have been reported (Beutin et al., 2004).

Another virulence factor of STEC is a 94-kDa outer membrane protein, called intimin, essential for cellular attachment. It is encoded by an *eae* gene present on a 34-kb chromosomal pathogenecity island termed the locus for enterocyte effacement (LEE) (McDaniel et al., 1995).

An additional virulence marker carried by some STEC strains is enterohemorraghic hemolysin (EHEC-Hly), encoded by a large plasmid-borne (90-kb) EHEC-*hly*A gene (Schmidt et al., 1995), which seemed to be associated with severe clinical disease in humans.

E. coli O157:H7, which was first identified as a human pathogen in 1982 associated with two major outbreaks of HC in the US (Riley et al., 1983), is the most prevalent STEC serotype. However, other STEC ser-

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otypes (O26:H11, O103:H2, O111:NM and O145:NM) have been reported to cause outbreaks or sporadic cases of HC and HUS in many countries (Kaper and O'Brien, 1998).

Accordingly, these highly virulent STEC serotypes were also designated as enterohemorrhagic *E. coli* (EHEC) (Nataro and Kaper, 1998).

In Argentina, where HUS is endemic, approximately 400 new cases are reported each year by the hospitals' nephrology units. In 2001, the estimated annual rate of HUS was 10.4 cases per 100,000 children under 5 years old. More than 7000 cases of HUS have been reported since 1965 (Rivas et al., 2003).

A variety of food vehicles have been linked to STEC infections, including beef, lettuce, cabbage, alfalfa sprouts, unpasteurized juice, dairy products and venison (World Health Organization, 1997).

Ground beef has been the main vehicle responsible for HUS outbreaks in the US. Among 183 foodborne *E. coli* O157 outbreaks reported from 1982 to 2002, 41% were linked to ground beef, but only 6% to other beef items (Rangel J, pers. comm.). In Argentina, *E. coli* O157:H7 was detected in a number of foodstuffs such as ground beef, fresh sausages, dry sausages and other meat products obtained from retail butchers (Chinen et al., 2001).

'Morcilla' is the Spanish term for a typical Argentine blood link sausage which is quite similar to the English black pudding. It is a mixture of blood of animals approved for consumption, pork fat, ground pigskin, salt, onion and spices, stuffed into a bovine gut or a synthetic casing, tied manually and immersed in a hot water bath at 90 °C for a certain period of time, which may vary from one producer to another. However, deficient hygienic conditions during processing or insufficient thermal treatment may render this product unsafe. In Argentina, morcillas are either eaten directly, without any further preparation, or previously cooked for some minutes.

The aim of the study was to establish the microbiological quality of morcillas, and to investigate the presence of STEC.

2. Materials and methods

2.1. Samples

Between October 2001 and October 2002, a total of 100 samples of morcilla (approximately 100 g each), denominated M1–M100, were purchased in local retail stores in La Plata City, Argentina. They were aseptically collected, placed into sterile containers and immediately sent to the laboratory.

2.2. Microbiological characterization

Samples were analysed to assess their microbial quality and safety. Subsamples of 20 g were randomly taken from each sample and aseptically weighed within a sterile stomacher bag with 180 ml of sterile 0.1% (w/v) peptone water (Merck KGaA, Darmstadt, Germany), and homogenized for 1 min in a Model 400 Stomacher (Seward Medical, London, UK). Appropriate serial dilutions were used to determine different microorganisms according to the following procedures:

(1) Total aerobic mesophilic bacteria counts were enumerated following pour plate technique in plate count agar (PCA) (Merck) and incubated at 37 °C for 48 h.
(2) Molds and yeasts counts were enumerated via surface plating on YGC agar (yeast extract, glucose, chloramphenicol) (Merck) and incubated at 25 °C for 5 days.
(3) *Enterobacteriaceae* counts were enumerated via surface plating on violet red bile glucose agar (VRBGA) (Merck) and incubated at 37 °C for 24–48 h.
(4) *Staphylococcus aureus* was enumerated via surface plating on Baird–Parker agar (Merck) and incubated at 37 °C for 48 h. Representative colonies with typical black appearance and surrounded by clear zone were picked, and subjected to catalase and coagulase tests (rabbit plasma EDTA, Difco Laboratories, Detroit, Michigan, USA).
(5) Total and fecal coliforms were analysed according to AOAC Method 46016 (AOAC, 1984).

2.3. STEC isolation

A 25-g portion of each morcilla sample was added to 225 ml of modified EC broth (Difco) containing novobiocin (ICN Biomedicals Inc., Ohio, USA) (*m*EC + *n*) (final concentration, 20 μg/ml). After homogenization in a stomacher for 1 min, the samples were incubated at 37 °C for 6, 12, 18 and 24 h, with and without shaking, to determine the best enrichment condition.

After the enrichment step, 1 ml of the culture was plated onto sorbitol-MacConkey agar (SMAC, Difco) and onto SMAC supplemented with cefixime (50 ng/ml) and potassium tellurite (bioMérieux, Marcy l'Etoile, France) (25 mg/ml) (CT-SMAC). The plates were incubated at 37 °C for 18–24 h. The confluent growth zones were screened for the presence of stx_1, and stx_2 genes by a multiplex polymerase chain reaction (PCR) using the oligonucleotide primers described by Pollard et al. (1990). From each PCR-positive sample, 10–20

colonies were selected, and PCR for the amplification of *stx*1 and *stx*2 genes was performed as described above.

Isolates harboring stx_1 or stx_2 genes were confirmed as *E. coli* by biochemical tests (Ewing, 1986). The *stx*-positive colonies were sent to the National Reference Laboratory (NRL) at the ANLIS 'Dr. Carlos G. Malbrán', for further characterization.

2.4. STEC characterization and subtyping

At NRL, the isolates were confirmed by a multiplex PCR for stx_1, stx_2 and rfb_{O157} genes, using the primers described by Pollard et al. (1990) and Paton and Paton (1998), respectively.

The *eae* and EHEC-*hly*A genes were detected by PCR using the primers AE9–AE10 (Gannon et al., 1993) and hlyA1–hlyA4 (Schmidt et al., 1995), respectively.

Strain serotyping was conducted with somatic and flagellar antisera of INPB-ANLIS 'Dr. Carlos G. Malbrán' (Ørskov and Ørskov, 1984).

To determine the Stx production, bacterial supernatant fluids and periplasmic cell extracts were used in cytotoxicity assays on Vero cells (Karmali et al., 1985) using Stx1- and Stx2-specific monoclonal antibodies (MAb 13C4 and BC5BB12, respectively), provided by Dr. N.A. Strockbine, Centers for Disease Control and Prevention, Atlanta, Georgia, USA (CDC). The enterohemolysis was determined on sheep blood agar plates (Beutin et al., 1989). Antibiotic susceptibility patterns were assayed by Kirby–Bauer method for ampicilin, amikacin, ciprofloxacin, colistin, chloramphenicol, gentamicin, nalidixic acid, nitrofurantoin, streptomycin, tetracycline and trimethoprim-sulfamethoxazole (National Committee for Clinical Laboratory Standards, 2000).

The genotyping of stx_2 variants was established by restriction fragments length polymorphism analysis of a B-subunit-encoding DNA fragments obtained by PCR (PCR-RFLP) (Tyler et al., 1991).

Phage typing was performed by the method described by Khakhria et al. (1990). The *E. coli* O157:H7 typing phages used were provided by R. Ahmed of the National Microbiology Laboratory, Canadian Centre for Human and Animal Health, Winnipeg, Manitoba, Canada. The macrorestriction fragment separation by pulsed-field gel electrophoresis (PFGE) was performed using the 24-h CDC, with minor modifications (Foodborne and Diarrheal Branch and Centers for Disease Control and Prevention, 1998). Digestion was carried out with 25 U of *Xba*I (Promega Corp., Madison, Wisconsin, USA) at 37 °C for 18 h. The standard strain used was *E. coli* O157:H7 G5244, provided by the CDC. DNA fragments were resolved in 1% agarose gel in 0.5 × Tris borate EDTA electrophoresis buffer at 14 °C, in a contour clamped homogeneous electric field DR-III electrophoresis chamber (Bio-Rad Laboratories, Hercules, California, USA). The run time was 19 h, with a constant voltage of 200 V, using a linear pulse ramp of 2.2–54.2 s. The *Xba*I–PFGE patterns were analysed by BioNumerics Ver. 3.0 (Applied Maths). The relatedness among the patterns was estimated by the proportions of shared bands, applying DICE coefficient, and a phenogram was obtained based on the UPGMA method.

3. Results and discussion

3.1. Microbiological characterization

Microbiological values found in 100 morcilla samples are presented in Table 1. The total aerobic mesophilic bacteria counts ranged from 10^3 to 10^8 cfu/g, while 94% of the samples contained molds and yeasts at levels higher than 10^3 cfu/g. These high values would indicate an inadequate application of the thermal treatment and inappropriate hygienic conditions during the elaboration of this type of product. Eighty-one percent of the samples analysed contained fecal coliform counts between 3.0 and 5.3×10^3 MPN/g. Although all samples

Table 1
Range of values of the analysed microorganisms in 100 morcilla samples

Groups of bacteria	Cell count (cfu/g or MPN/g)	No. of samples ($n = 100$)
Total aerobic mesophilic bacteria	2.4×10^3–7.3×10^3	4
	1.2×10^4–8.1×10^4	10
	1.0×10^5–9.3×10^5	16
	1.1×10^6–7.0×10^6	34
	2.1×10^7–9.1×10^7	23
	1.3×10^8–3.2×10^8	13
Molds and yeast	1.6×10^2–7.3×10^2	6
	1.2×10^3–8.1×10^3	16
	1.0×10^4–9.3×10^4	42
	1.4×10^5–7.2×10^5	36
Total coliforms	<3.0	8
	3.0–9.2×10	5
	2.0×10^2–7.3×10^2	16
	1.2×10^3–8.1×10^3	22
	1.3×10^4–3.3×10^4	49
Fecal coliforms	<3.0	19
	3.0–8.2×10	22
	1.1×10^2–6.2×10^2	38
	1.6×10^3–5.3×10^3	21
Enterobacteriaceae	2.0×10^2–7.3×10^2	9
	1.2×10^3–8.1×10^3	18
	1.0×10^4–9.3×10^4	27
	1.1×10^5–8.2×10^5	46
S. aureus	$<1 \times 10^2$	100

Table 2
Microbiological properties of STEC-positive morcilla samples

Microorganisms	Averages		
	M7	M12	M54
Total microbial counts	1.12×10^8 cfu/g	3.20×10^8 cfu/g	2.22×10^6 cfu/g
Molds and yeast	8.72×10^3 cfu/g	4.15×10^4 cfu/g	3.66×10^3 cfu/g
Total coliforms	1.36×10^3 MPN/g	3.29×10^4 MPN/g	1.58×10^3 MPN/g
Fecal coliforms	1.20×10^2 MPN/g	6.12×10^2 MPN/g	8.0×10 MPN/g
Enterobacteriaceae	6.44×10^4 cfu/g	8.22×10^5 cfu/g	8.36×10^3 cfu/g
S. aureus	$<01^2$ cfu/g	$<01^2$ cfu/g	$<01^2$ cfu/g
STEC	O157:H7	O157:H7	O26:H11

contained *Enterobacteriaceae*, counts higher than 1×10^4 cfu/g were detected in 73% of them. The high microbial counts in some samples, and the presence of micro-organisms indicating fecal contamination, such as fecal coliforms and *Enterobacteriaceae*, make this type of precooked sausage (morcilla) hazardous.

A previous study in this type of food (Oteiza et al., 2003) showed that generic *E. coli* was detected in 76.6% of the 30 samples analysed. The chemical composition of ground sausages (15% protein, 32% fat, 1% carbohydrates and 52% water, pH 6.2) was suitable for the survival of pathogenic micro-organisms. However, *S. aureus* was not found in any of the samples analysed.

3.2. STEC isolation

Among the different incubation conditions assayed, the best STEC recovery was obtained by incubation in *m*EC + *n* broth at 37 °C during 6 h without shaking.

STEC strains were isolated from three out of 100 (3%) morcilla samples. Two strains were characterized as STEC O157:H7/stx_2 (M7 and M12) and another strain as STEC O26:H11/stx_1 (M54). The positive-O157:H7 samples were purchased during January 2002, and the positive-O26:H11 sample, during October 2002. Salami and other types of sausages are known causes of STEC outbreaks outside Argentina, but they are relatively uncommonly recognized as vehicles (Centers for Disease Control and Prevention, 1995; Paton et al., 1996). In Argentina, Chinen et al. (2001), using selective enrichment in *m*EC + *n* followed by immuno-magnetic separation (IMS) and selective plating onto CT-SMAC, found that four out of 83 (4.8%) fresh sausages and one out of 30 (3.3%) dry sausages tested were positive for *E. coli* O157:H7. In France, a study conducted by Vernozy-Rozand et al. (1997) showed that one out of 250 (0.4%) pork sausages tested was positive for *E. coli* O157:H7. However, no bibliographical information on the presence of STEC O157 and non-O157 strains in precooked sausages, such as morcillas, is currently available.

The total aerobic mesophilic bacteria counts of the samples M7, M12 and M54 were 1.12×10^8, 3.20×10^8 and 2.22×10^6 cfu/g, respectively. The three samples showed molds and yeasts counts higher than 10^3 cfu/g, and the values of fecal coliforms and *Enterobacteriaceae* ranged from 8×10 to 6.1×10^2 MPN/g, and from 8.4×10^3 to 8.2×10^5 cfu/g, respectively (Table 2).

3.3. STEC characterization

The O157:H7 strains (M7 and M12) did not ferment sorbitol and were β-glucuronidase-negative. Genotypic characterization showed that the isolates harbored *stx*2 + *stx*2vh-a/*eae*/EHEC-*hly*A genes. STEC O26:H11 strain was sorbitol fermenting, and β-glucuronidase-positive, and harbored *stx*1/*eae*/EHEC-*hly*A genes. The three strains were susceptible to all antibiotic assayed. Cytotoxicity assays on Vero cells confirmed the *stx* results obtained by genotyping techniques.

Although Stx is considered the primary virulence factor, a combination of virulence factors is required for a complete pathogenic process. Meng et al. (1998) have pointed out that the combination of both *eae* and EHEC-*hly*A would seem to be a more important indicator of virulence than either factor alone. All strains isolated in this study harbored this gene combination; therefore, these strains represent a potential risk to consumers.

The genotype *stx*2 + *stx*2vh-a is considered highly virulent by several authors (Nishikawa et al., 2000). In Argentina, this genotype is prevalent in STEC O157 strains of human and food origin (Chinen et al., 2003), and most of the *E. coli* O157:H7 strains isolated from HUS cases harbor the *eae*/*stx*2 + *stx*2vh-a/EHEC-*hly*A genes (Miliwebsky et al., 1999).

Strains M7 and M12 belonged to phage types 24 and 49, respectively, and yielded two different restriction *Xba*I–PFGE profiles: pattern #I (M7 strain) and pattern #II (M12 strain). They shared 94.1% of the *Xba*I–PFGE restriction fragments, with only two bands difference (Fig. 1).

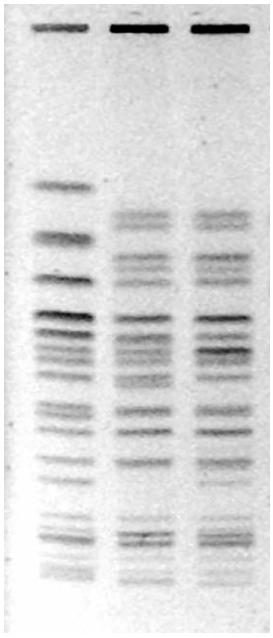

Fig. 1. *Xba*I–PFGE patterns. Lane 1: standard strain *E. coli* O157 G5244; lane 2: pattern #I-M7; lane 3: pattern #II-M12.

When the patterns were compared with former patterns entered into the Argentine STEC O157 database, the *Xba*I–PFGE pattern #I was identified in only one strain isolated from an HUS case in 1996; whereas the *Xba*I–PFGE pattern #II was identified in 14 strains of human origin and in seven strains of food origin (meat, minced beef, barbecue-type fresh sausage, bovine hamburger, cooked chicken hamburger and chicken nuggets) isolated between 1996 and 2002.

The presence of STEC strains in morcillas represents a risk to consumers' health taking into account the low infectious dose necessary to cause illness. As in Argentina morcillas are usually eaten either cold or grilled for a few minutes, their center does not reach 71.1 °C to kill the STEC strains. This behavior is especially risky since many pediatricians recommend feeding cooked morcilla to young children due to its high levels of iron and proteins.

In Argentina, it is common to add starch to the product to increase its weight, and thus its yield. Although not approved by the sanitary authorities, this practice increases the risk to consumers because this component has a protective effect on the thermal inactivation of the micro-organism (Oteiza et al., 2003).

To our knowledge, time-temperature standards for cooking have not been established in any national or international legislation. Currently, Section No. 317 of the Argentine Food Law provides that this kind of products must be free from pathogens, without laying down any additional microbiological criteria.

Further studies using more sensitive methods, such as IMS for *E. coli* O157, are necessary to determine the prevalence of STEC strains in this kind of products, in order to establish control and prevention strategies for STEC-associated diseases.

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