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Molecular typing of antimicrobial-resistant Shiga-toxin-producing *Escherichia coli* strains (STEC) in Brazil

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Abstract

Antimicrobial resistance patterns and molecular characteristics were determined in thirty-two Shiga-toxin-producing *Escherichia coli* (STEC) strains previously identified in São Paulo State associated with human infections ($n = 21$) and in cattle feces ($n = 11$). The highest resistance rates were identified for tetracycline (100%), streptomycin (78%) and trimethoprim–sulfamethoxazole (56%). Eleven STEC strains showed resistance to ampicillin and carried bla_{TEM} that was confirmed as $bla_{\text{TEM-1}}$ in one representative isolate. The class 1 integrase gene (*intI*1) was detected in seven (22%) strains, and most of them belonged to the O111:H8 serotype. The class 1 integron was located on plasmids in five of the seven STEC strains, and conjugation assays confirmed the plasmid support of those resistant determinants. STEC strains were genetically classified into the B1 group, and PFGE analysis showed that most of the strains in each serogroup were grouped into the same cluster (80–97% similarity). The presence of a class 1 integron and $bla_{\text{TEM-1}}$ genes is described for the first time among STEC isolates in Brazil and clearly represents a public health concern.

Keywords: Shiga-toxin-producing *Escherichia coli*; Resistance genes; Class 1 integron; Phylogenetic background

1. Introduction

Shiga-toxin-producing *Escherichia coli* (STEC) strains are an important group of food-borne pathogens that cause a broad range of manifestations, including hemorrhagic colitis and hemolytic uremic syndrome (Nataro and Kaper, 1998). STEC colonize the gastrointestinal tract of domestic and wild animals, but ruminants are regarded as the main natural reservoirs (Beutin et al., 1993, 1997). The consumption of contaminated foods of animal origin is one of the major causes of STEC infection in humans (Cergole-Novella et al., 2006). Although antimicrobial therapy is not the primary tool for treating infections caused by STEC, some reports indicate that antimicrobial resistance of STEC is on the rise (White et al., 2002). The use of antimicrobial agents in the agricultural environment is believed to play a key role in the dissemination of resistance genes among bacteria, and it has been suggested that the overuse of antibiotics in animal husbandry creates a threat to human and animal health. Antimicrobial resistance determinants are carried mostly by mobile genetic elements such as plasmids, transposons and integrons. A possible role of integrons and conjugative plasmids in the dissemination of genes conferring antibiotic resistance from pathogenic to generic *E. coli* cells has been suggested (Nagachinta and Chen, 2009).

A high prevalence of integrons was detected in fecal *E. coli* of healthy humans, demonstrating that individuals in the community could be a reservoir of integron-containing *E. coli* isolates (Vinué et al., 2008). Several studies have demonstrated that

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there is clonal spread of resistant strains, transfer of resistance genes between human and animal bacteria and also the sharing of phylogenetic and genotypic similarities (Van Den Bogaard and Stobberingh, 2000). Extended-spectrum β-lactamases (ESBLs) have contributed to the recently noted large increase in resistance to β-lactams. Many of the ESBLs arise from point mutations in plasmid-mediated genes that encode narrow-spectrum β-lactamases such as TEM and SHV (Bradford, 2001). However, members of a newly emerging ESBL group, CTX-M, derived from class A chromosomal β-lactamases have been identified (Bonnet, 2004). In Japan, Kon et al. (2005) described an STEC O26:H11 strain resistant to cefotaxime and cefpodoxime (but not ceftazidime), producing CTX-M-3 type ESBL. Despite the fact that antimicrobial resistance in STEC has been the subject of several studies, information on the molecular basis of antimicrobial resistance among this zoonotic group of bacteria is still limited (Guerra et al., 2006; Lee, 2009; Nagachinta and Chen, 2009; Srinivasan et al., 2007; Zhao et al., 2001). The aims of this study were to further characterize the antimicrobial resistance displayed by various STEC strains isolated from human infections and the animal reservoir in Brazil, and evaluate their genetic relatedness.

2. Materials and methods

2.1. Bacterial strains

A total of 32 antimicrobial-resistant STEC strains (Cergole-Novella et al., 2006) belonging to non-O157 serotypes isolated in São Paulo, Brazil, were investigated in this study. Twenty-one STEC strains were isolated from humans with diarrhea (Guth et al., 2002; Irino et al., 2007; Vaz et al., 2004), and 11 were isolated from cattle (Aidar-Ugrinovich et al., 2007; Irino et al., 2005; Leomil et al., 2003; Salvadori et al., 2003).

2.2. Antimicrobial susceptibility testing

Minimum inhibitory concentrations (MICs) were determined by agar dilution of 12 antimicrobial agents according to Clinical and Laboratory Standards Institute (CLSI, 2007) guidelines. The antimicrobials tested were: nalidixic acid (Nal), ampicillin (Amp), kanamycin (Kan), cefepime (Cef), cefotaxime (Ctx), ceftazidime (Cfz), ciprofloxacin (Cip), chloramphenicol (Chlo), streptomycin (Str), gentamicin (Gen), trimethoprim–sulfamethoxazole (Sut) and tetracycline (Tet).

2.3. Phylogenetic grouping and pulsed-field gel electrophoresis typing (PFGE)

Triplex PCR profiles specific for *E. coli* phylogenetic groups were performed with specific primers according to Clermont et al. (2000). The macrorestriction analysis of genomic DNA with XbaI described by Gautom (1997) was used with some modifications for PFGE. The digestion time was extended to 16 h, and PFGE was performed using a CHEF-DRIII PFGE apparatus (Bio-Rad). The pulse time was increased from 5 to 50 s over a 19-h period. The band patterns were analyzed by the BioNumerics program version 5.1 (Applied Maths), and the similarity between PFGE patterns was evaluated using the Dice similarity coefficient (tolerance, 1.5%).

2.4. PCR, PCR–RFLP and nucleotide sequencing of *intI*1/*qac*EΔ1 and bla_{TEM}

The presence of integrase associated with class 1 integrons (*intI*1) (5′ GTT CGG TCA AGG TTC TGG 3′ and 5′ GTA GAG ACG TCG GAA TGG 3′; 94 °C, 60 s; 67 °C, 60 s; 72 °C, 60 s; 35 cycles; 889 bp), bla_{TEM} (Rasheed et al., 1997), *intI*1/*qac*EΔ1 (Castanheira et al., 2004), *aad*A and *tet*A (Enne et al., 2008) was assayed by PCR. PCR–RFLP assay was carried out using specific primer sequences of *intI*1/*qac*EΔ1 followed by 3 h of digestion with HaeIII. Amplicons were sequenced on both strands. Nucleotide and deduced amino acid sequences were analyzed using the Lasergene software package (DNASTAR, Madison, WI) and compared with sequences available through the Internet using BLAST (http://www.ncbi.nlm.nih.gov/blast/).

2.5. Plasmid characterization and transfer of resistance

Plasmid DNA was extracted by the Birnboim and Doly (1979) alkaline lysis method and analyzed by electrophoresis in agarose gels. Plasmid masses were estimated by using *E coli* strain 39R861 with known molecular size plasmid bands as marker. In addition, Southern blot hybridization assays with an *intI*1-specific probe were also performed using Amersham ECL direct nucleic acid labelling and detection systems (GE Healthcare). To investigate the transfer of plasmids identified as carriers of resistance markers, one representative STEC strain (Ec120/00) was selected, and conjugation assays were performed using a spontaneous mutant resistant to nalidixic acid of commensal *E. coli* HS strain (Levine et al., 1978) as the recipient. The transconjugants were selected on nutrient agar containing tetracycline (25 μg/mL) and nalidixic acid (100 μg/mL), while the donor strain was selected by plating a dilution of the conjugation mixture onto nutrient agar containing tetracycline. Successful introduction of the plasmid was determined by disc diffusion, plasmid extraction and visualization by agarose gel electrophoresis, and PCR amplification.

3. Results

3.1. Antimicrobial resistance profile of STEC strains

Twenty-one (65.6%) of 32 STEC isolates were resistant to three or more classes of antimicrobials (Table 1). The highest resistance rates were noted for tetracycline (100%), streptomycin (78.1%) and trimethoprim–sulfamethoxazole (56.2%). However, resistance to ampicillin (34.4%) and kanamycin (37.5%) was also identified. The 11 STEC strains resistant to ampicillin possessed bla_{TEM}. The bla_{TEM} gene of one

Table 1
Characteristics of antimicrobial-resistant STEC strains.

STEC strain	Serotype	Origin[a]	*stx*	*intl*1	Antimicrobial profile[b]	Phylogenetic group
Ec36/01/87	O26:H11	HD	1	−	Tet	B1
2567/81	O26:H11	HD	1	−	StrTet	B1
16FB(12)	O55:H19	DC	1	−	AmpChloKanStrTet	B1
775/93	O111:H8	HD	1	−	AmpChloKanStrSutTet	B1
5780/76	O111:H8	HD	1	−	Tet	B1
21/85	O111:H8	HD	1	+	AmpChloStrSutTet	B1
1731-3	O111:H8	HD	1	−	StrSutTet	A
2781-8	O111:H8	HD	1	+	StrChloSutTet	B1
19/79	O111:H8	HD	1	−	Tet	B1
Ec120/00	O111:H8	HD	1	+	StrTet	B1
Ec464/01	O111:H8	HC	1 + 2	−	SutTet	B1
BP/25B1	O111:H8	DC	1	−	AmpKanStrSutTet	B1
BP/2HV4	O111:H8	DC	1	−	AmpKanStrSutTet	B1
BP/61C7-4	O111:H8	HC	1	−	AmpKanStrSutTet	B1
BP/F5-1	O111:H8	HC	1	−	StrSutTet	B1
BP/B4	O111:H8	HC	1	−	ChloStrSutTet	B1
BP/M2-1	O111:H8	DC	1	−	StrSutTet	B1
Ec256/03	O111:H8	HD	1	−	AmpChloStrSutTet	B1
3691-5	O111:H8	HD	1	+	StrTet	B1
1919/81	O111:H11	HD	1	+	StrTet	B1
490/78	O111:HNM	HD	1	−	Tet	B1
502/80	O111:HNM	HD	1	+	ChloKanStrTet	B1
Ed 19/00	O111:HNM	HD	1	−	AmpChloKanStrSutTet	B1
Ec54/87	O111:HNM	HD	1	−	Kan StrTet	B1
288/83	O111:HNM	HD	1	−	Tet	B1
27/86	O111:HNM	HD	1	−	StrSutTet	B1
BY-1	O118:H16	DC	1	+	AmpChloKanStrSutTet	B1
937BG[c]	O118:H16	DC	1	−	AmpKanStrSutTet	B1
1157/89	O118:H16	HD	1	−	StrSutTet	B1
44/01	ONT:H2	HD	1	−	AmpKanStrTet	B1
Ec586/03	ONT:H2	HD	1	−	KanStrSutTet	B1
Ec248/02	ONT:H49	HC	1 + 2	−	Tet	B1

[a] HD, human with diarrhea; DC, feces from diarrheic cattle; HC, feces of healthy cattle.
[b] Amp, ampicillin; Chlo, chloramphenicol; Kan, kanamycin; Str, streptomycin; Tet, tetracycline; Sut, trimethoprim–sulfamethoxazole.
[c] Multidrug-resistant O118:H16 STEC strain previously described in South America (Castro et al., 2003).

representative ampicillin-resistant STEC strain (BY-1) was sequenced confirming the presence of $bla_{\text{TEM-1}}$.

3.2. Phylogenetic grouping and PFGE

Phylogenetic grouping classified all strains except one, O111:H8, into the B1 group (Table 1). The PFGE typing of O111 and O118 STEC strains showed a diversity of profiles (Fig. 1), but O111 STEC strains were grouped into three distinct clusters with more than 80% similarity.

3.3. Presence of the intl*1* gene in the STEC strains

The integrase gene associated with class 1 integrons was found in 22% of STEC. Six human STEC strains that carry the class 1 integron belonged to the O111 serogroup, and one bovine STEC strain belonged to the O118 serogroup (Table 1). Analysis of integrons by PCR–RFLP revealed identical profiles in four (Ec120/00, 3691-5, 1919/81 and 502/80) of the seven STEC strains (data not shown). These four integrons had a uniform size and contained a single gene cassette, *aad*A1 (streptomycin resistance), in their variable region (*intl*1F–*aad*A1–*qac*EΔ1R), as determined by nucleotide sequencing. In addition, these strains showed resistance to streptomycin and had high MICs (≥64 μg/mL).

3.4. Plasmid characterization and transfer of resistance by conjugation of STEC strains carrying intl*1*

The plasmid profile analysis showed that all strains carrying *intl*1 had 2–8 bands of high (>15 kb) and low (<15 kb) molecular weights. Southern blot hybridization assays indicated that a class 1 integron was located on plasmids in five of the seven strains (Fig. 2A,B). To see if hybridization signal was detectable in chromosomal DNA, genomic DNA incorporated in agarose plugs was digested with I-CeuI and DNA fragments were separated by PFGE (Shu-Lin et al., 1993). Southern blot hybridization indicated that *intl*1 was not present in chromosomal DNA (data not shown). To further confirm *intl*1-carrying plasmid, conjugation experiments were performed using STEC strain Ec120/00 as donor and *E. coli* HS as recipient. STEC strain Ec120/00 showed the presence of *intl*1 and resistance to streptomycin and tetracycline (Table 1). Likewise, the transconjugant strain

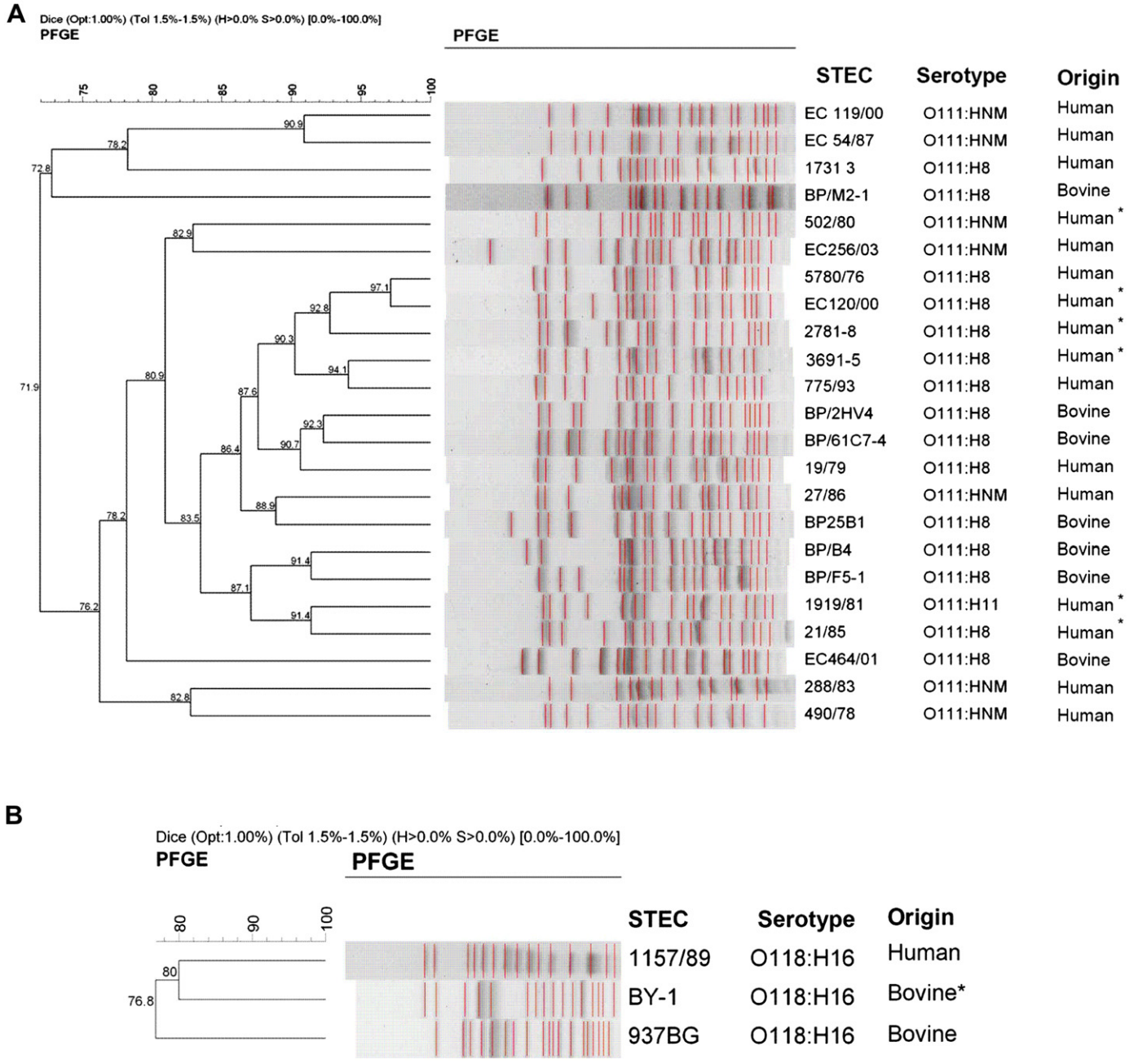

Fig. 1. Dendrogram outlining the relationship of antibiotic-resistant O111 (A) and O118 (B) STEC strains from human and animal sources, determined by macrorestriction analysis of genomic DNA with XbaI. *STEC strains carrying *int*I1.

possessed *intI*1 (Fig. 3), *aadA* and *tetA*, responsible for streptomycin and tetracycline resistance as detected by PCR and disc diffusion (data not shown).

4. Discussion

Resistance genes can be associated with mobile DNA plasmids, transposons and integrons, which are known to facilitate their distribution (Guerra et al., 2006; Jacoby, 1994). In the present study, almost 22% of STEC strains displaying resistance to antimicrobials possessed a plasmid-carried class 1 integron (Fig. 2B), and most of these strains were isolated from human infections and belonged to the O111 serogroup. This is the first description of a class 1 integron among STEC strains isolated in Brazil. The presence of multi-drug resistance, β-lactamase and class 1 integron-associated genes was also recently described in some STEC serotypes isolated from cattle feces and soil samples in Ireland (Scott et al., 2009). In addition, Nagachinta and Chen (2008) determined that integron-mediated streptomycin and sulfisoxazole resistance genes can be transferred from STEC strains belonging to serotypes O157:H7 and O111:H8 to a susceptible *E. coli* K-12 strain in storm water and bovine feces.

The prevalence of the *aadA*1 gene cassette among class 1 integrons observed in the present study confirms previous observations (Martinez-Freijo et al., 1998, 1999; Nagachinta

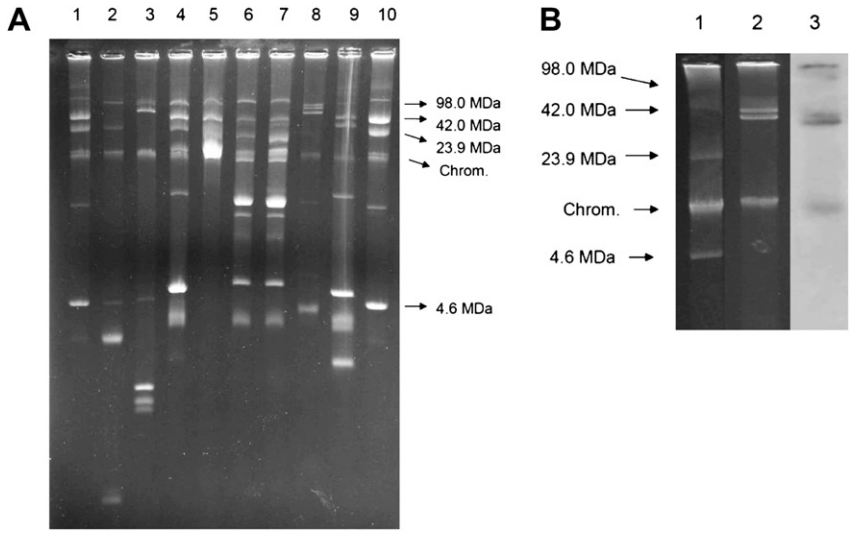

Fig. 2. Plasmid profiles of STEC strains carrying *intI*1. (A) Lanes 1 and 10, *E. coli* 39R861 strain carrying plasmids of known molecular sizes; 2, 502/80; 3, 1919/81; 4, Ec120/00; 5, 3691-5; 6 and 7, 21/85; 8, BY-1; 9, 2781-8 STEC strains. (B) Representative Southern blot hybridization assay with *intI*1-specific probe. Lane 1, *E. coli* 39R861; lane 2, plasmid profile of STEC 1919/81 strain; lane 3, corresponding Southern blot hybridization.

and Chen, 2009). Moreover, integrons carrying the *aad*A1 gene were found to be widely spread among STEC strains isolated from humans, cattle, and food and belonging to serogroups O26 and O111 (Morabito et al., 2002).

Similar antibiotic resistance patterns could be observed in some of the animal and human isolates presently studied. It is noteworthy that, although ampicillin and sulfonamides are old antimicrobial compounds, they are still widely used. Although these antimicrobial agents are more commonly used in the treatment of humans than animals, more animal isolates exhibited resistance (Maynard et al., 2004). Since 1983, when the first bla_{TEM} allele was identified (Sirot et al., 1987), several variants of bla_{TEM} that differ in amino acid sequence have been identified (www.lahey.org/studies). However, TEM-1 is the most commonly encountered β-lactamase in gram-negative bacteria, and up to 90% of ampicillin resistance in *E. coli* is due to the production of TEM-1 (Livermore, 1995). There are few studies in the literature describing the presence of β-lactamase in STEC. Ahmed et al. (2005) and Ishii et al. (2005) reported for the first time a β-lactamase TEM-1 gene in EHEC O157 and an ESBL CTX-M18-producing STEC isolate, respectively. Roest et al. (2007) reported the presence of β-lactamase in *E. coli* O157 isolated from chicken. In the present study, bla_{TEM} was carried by 34% of the STEC strains, and it was confirmed as the β-lactamase TEM-1 gene by sequence analysis of one representative STEC isolate. To the best of our knowledge, the presence of the bla_{TEM-1} gene in ampicillin-resistant STEC strains isolated from human infections has not been previously described in our community.

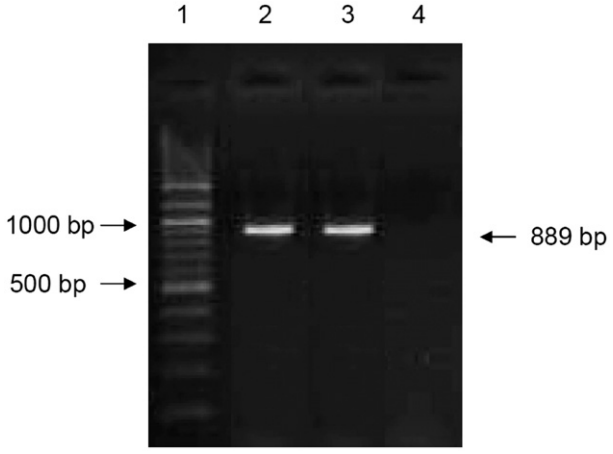

Fig. 3. Representative agarose gel of amplified PCR products confirming the transfer of *intI*1 by conjugation assay. Lane 1, 100-bp DNA ladder; 2, transconjugant strain carrying *intI*1; 3, donor STEC strain Ec120/00; and 4, recipient commensal strain *E. coli* HS.

Several PFGE profiles were identified among the O111 and the O118 isolates studied. However, most of the strains in each serogroup were grouped into the same cluster (80%–97% similarity) and were classified into the phylogenetic group B1. Interestingly, most of the O111 strains carrying *intI*1 were clustered into two subgroups that showed more than 90% similarity. In relation to the O118:H16 STEC isolates, conclusions are more difficult to draw due to the low number of strains, but it is interesting to note that the bovine strain with *intI*1 displayed 80% similarity with the human isolate. In addition, it should be mentioned that these two O118:H16 isolates were more distantly related (76.8%) to the multidrug-resistant O118:H16 STEC strain previously described in South America (Castro et al., 2003).

Although antimicrobial therapy is generally not recommended for the treatment of STEC infections in humans, the selection of multiresistant strains may contribute to the increase in emerging antimicrobial-resistant pathogens, and may facilitate the spread of mobile resistance elements to other bacteria. The presence of antimicrobial resistance genes among STEC strains is worrisome, and no doubt plays a significant role in the

acquisition and dissemination of new antimicrobial resistance. Increased surveillance and the development of adequate prevention strategies are warranted.

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