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Molecular characterization of multidrug-resistant Shiga toxin-producing *Escherichia coli* harboring antimicrobial resistance genes obtained from a farmhouse

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ABSTRACT

Shiga toxin-producing *Escherichia coli* (STEC) colonize the gastrointestinal tract of animals; however, STEC may also cause severe diarrheal diseases. Food-producing animals have been acting as reservoirs and disseminators of multidrug-resistant (MDR) bacteria and antimicrobial resistance genes (ARGs); however, there are few studies characterizing molecularly bacterial isolates from sheep. Therefore, this study aimed to characterize *E. coli* isolates obtained from feces of sheep in a Brazilian farmhouse. A total of 14 MDR *E. coli* isolates were obtained from 100 feces samples, six of which were classified as non-O157 STEC (*stx1, stx2* and *ehxA*). MDR *E. coli* isolates presented different ARGs [$bla_{CTX\text{-}M\text{-}Gp9}$, bla_{CMY}, bla_{SHV}, *qnrS, oqxB, aac(6')-Ib, tet*(A), *tet*(B), *tet*(C), *sul1*, *sul2*, and *cmlA*] and plasmids (IncI1, $IncF_{repB}$, IncFIB, IncFIA, IncHI1, IncK, and ColE-*like*). In addition, mutations in the quinolone-resistance determining region of GyrA (Ser83Leu; Asp87Asn) and ParC (Glu84Asp) were detected. PFGE showed a high genetic diversity (30.9 to 83.9%) and thirteen STs were detected (ST25, ST48, ST155, ST162, ST642, ST1247, ST1518, ST1725, ST2107, ST2522, ST3270, ST5036, and ST7100). Subtyping of the *fimH* gene showed seven *fimH-type* (25, 32, 38, 41, 54, 61, and 366). The results found in the present study showed high genetic diversity among MDR ARGs-producing *E. coli* obtained from a farmhouse. This study reports for the first time, the presence of MDR STEC and non-STEC belonging to ST25, ST162, ST642, ST1247, ST1518, ST1725, ST2107, ST3270, ST5036, and ST7100 in sheep, and contributes to the surveillance studies associated with One Health concept.

KEYWORDS

Escherichia coli; multidrug-resistant; antimicrobial resistance; Diarrheagenic virulence genes; *fimH*; MLST; sheep; One Health

Introduction

Diarrheagenic *Escherichia coli* are responsible for diarrheal diseases in animals and humans, which are classified into well-defined pathotypes. Among them, Shiga toxin-producing *Escherichia coli* (STEC) are defined as zoonotic pathogens that colonize the gastrointestinal tract of animals (e.g. sheep and bovine); however, STEC may also cause severe diarrheal diseases [1,2]. Diarrheal diseases are classified as a public health problem, which affect the developing countries and industrialized countries, causing high rates of morbidity and mortality, as well as high health care costs [3].

Resistance to antimicrobials in bacteria is a global public health problem and multidrug-resistant (MDR) bacteria, including *E. coli*, have been spreading to different sources, which is worrying. The One Health concept has been applied worldwide due to the global challenge of bacterial resistance to antimicrobials [4]. The animals have been acting as reservoirs and disseminators (e.g. for humans and the environment) of MDR bacteria carrying several antimicrobial resistance genes (ARGs), including the extended-spectrum β-lactamases (ESBL) [5–8].

Many studies related to bacterial resistance to antimicrobials are performed in food-producing animals (i.e. chickens, cattle and pigs); however, there are few studies characterizing molecularly bacterial isolates from sheep. Therefore, the present study aimed to characterize *E. coli* isolates obtained from feces of sheep in a Brazilian farmhouse regarding resistance to antimicrobials, ARGs, plasmids, diarrheagenic virulence genes and serotypes, as well as sequence types, phylogenetic groups and *fimH-type*.

Materials and methods

Obtaining and identification of isolates

A total of 100 fecal samples were obtained in a farmhouse (4 ha) from Jardinópolis City, São Paulo

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State, Brazil. The feces samples were collected using sterile recipients and transported to the laboratory on the same day. The fecal samples (1 g) were added in sterile saline solution (5 mL) (0.9% NaCl) and, subsequently, seeded on MacConkey Agar (Oxoid, UK) and incubated at 37 ºC for 24 hours. A glucose-fermenting colony from each sample was selected and stocked at –80ºC in Brain Heart Infusion broth (Oxoid, UK) plus 15 % glycerol.

Molecular identification

The GenElute™ Bacterial Genomic DNA Kit (Sigma-Aldrich, USA) was used for the extraction of genomic DNA. The sequencing of the 16S rDNA gene was performed for the identification of the isolates using the BigDye™ Terminator v3.1 Cycle Sequencing Kit (Thermo Fisher Scientific, USA) [9].

Detection of diarrheagenic virulence genes and *E. coli* serotyping

Detection of diarrheagenic virulence genes (*ipaH, stx1, stx2, ehxA, aaiC, aatA, eae, bfpA, aggR, elt, est, aap, aggR*, and *AA probe*) was performed by PCR [10–14]. Serotyping was performed by agglutination assays in 96-well Microtiter™ microplates (Thermo Fisher Scientific, USA), using rabbit antisera against 1 to 187 somatic (O) and 53 flagellar (H) antigens (SERUNAM, registered trademark in Mexico, 323,158/2015) [15,16].

Antimicrobial susceptibility testing

Antimicrobial susceptibility testing was performed by disk diffusion [17]. Thirty-two antimicrobials were tested, including aminoglycosides (streptomycin, gentamicin, tobramycin, amikacin), β-lactams (ampicillin, amoxicillin-clavulanate, ampicillin-sulbactam, piperacillin-tazobactam, cefoxitin, cefazolin, cefuroxime, cefaclor, cefepime, cefotaxime, ceftriaxone, ceftazidime, ertapenem, meropenem, imipenem, aztreonam), (fluoro) quinolones (nalidixic acid, ciprofloxacin, levofloxacin, ofloxacin, norfloxacin, lomefloxacin), tetracyclines (minocycline, doxycycline, tetracycline), nitrofurans (nitrofurantoin), sulfonamides (trimethoprim-sulfamethoxazole), and phenicols (chloramphenicol). The isolates were classified as multidrug-resistant when presented non-susceptibility to ≥ 1 antimicrobial in ≥ 3 antimicrobial categories [18].

Detection of ARGs and plasmid replicon typing

The ARGs were detected by PCR in non-susceptible (resistant or intermediate) isolates for (fluoro) quinolones (*oqxAB, qepA, qnrA, qnrB, qnrS*), tetracyclines [*tet* (A) to (E), *tet*(G), *tet*(J), *tet*(L), *tet*(M), *tet*(O), *tet*(P), *tet*(Q), *tet*(S), and *tet*(X)], β-lactams [$bla_{CTX\text{-}M}$ (groups 1, 2, 8 and 9), bla_{CMY}, bla_{VEB}, bla_{PER}, $bla_{OXA\text{-}1\text{-}like}$, bla_{SHV}], phenicols (*floR, cmlA*), sulfonamides (*sul1, sul*2, *sul*3), and aminoglycosides [*aph(3')-Ia, aph(3')-VI, aac(6')-Ib, aac (6')-Ih, aac(3')-Ia, aac(3')-IIa, ant(2")-Ia*] [19–28]. The amplicons were sequenced for confirmation.

Detection of mutations in the quinolone-resistance determining region (QRDR) of GyrA (encoded by *gyrA* gene) and ParC (encoded by *parC* gene) was also performed [29,30]. Plasmids were detected by PCR-based replicon typing for twenty plasmid families ($IncF_{repB}$, IncFIB, IncFIA, IncFIC, IncU, IncR, IncHI1, IncHI2, IncK, IncY, IncI1, IncL/M, IncW, IncP, IncN, IncA/C, IncT, IncX, ColE-*like*) [31,32].

Pulsed-field gel electrophoresis (PFGE)

Genetic relatedness of *E. coli* isolates was performed by PFGE using 50U of *XbaI* restriction enzyme (Thermo Fisher Scientific, USA). *Salmonella* Braenderup H9812 was used as a molecular mass standard. The electrophoresis was performed on the CHEF-DR III system (Bio-Rad, USA) at 14 °C (Voltage: 6 V; Initial switch time: 6.76 seconds; Final switch time: 35.38 seconds; Included angle: 120°; Run time: 19 hours). A similarity dendrogram was constructed on the BioNumerics v. 7.6 (Applied Math, Belgium) using unweighted pair group method with arithmetic mean (UPGMA) and DICE similarity coefficient (optimization: 1.5%; band position tolerance: 1.5%). Discrimination index (DI) was evaluated using the Simpson's diversity [33].

Phylogenetic groups, multilocus sequence typing (MLST) and fimH subtyping

The phylo-typing method was used to determine phylogenetic groups (A, B1, B2, C, D, E, and F) using five target genes (*chuA, yjaA, TspE4.C2, arpA*, and *trpA*) [34]. MLST analysis was performed as described by Achtman scheme (housekeeping genes [*adk, gyrB, icd, fumC, purA, mdh*, and *recA*]) available in *Escherichia* MLST website (https://pubmlst.org/escherichia/) and subtyping of *fimH* gene was performed using the FimTyper 1.0 [35,36].

Results

Isolates, diarrheagenic virulence genes and serotypes

In this study, 14 MDR *E. coli* isolates were obtained from feces of sheep from a Brazilian farmhouse. These isolates were identified by sequencing of the 16S rDNA (GenBank access no. MN147823-MN147836). Three diarrheagenic virulence genes (*stx1, stx2* and *ehxA*) related to STEC pathotype were detected in six isolates (SJA6, SJA11, SJA29, SJA31, SJA80, and SJA92). The other diarrheagenic virulence genes (*ipaH, aaiC, aatA, eae, bfpA, aggR, elt, est, aap, aggR*, and *AA probe*) were not detected. Thirteen

serotypes were assigned in *E. coli* isolates, including O153:H12 (2), O8:H21 (1), O127:H43 (1), O173:HNM (1), O100:HNM (1), O147:H19 (1), O154:H9 (1), O54:H21 (1), ONT:H10 (1), O102:H21 (1), O8:H19 (1), O78:HNM (1) (Figure 1).

Antimicrobial resistance profile

All isolates were classified as MDR and presented several ARGs for β-lactams, aminoglycosides, tetracyclines, (fluoro) quinolones, phenicols, and sulfonamides (Table 1). All isolates were resistant to ampicillin, tetracycline and doxycycline, 12 (85.7%) to cefazolin, cefuroxime, cefaclor and trimethoprim-sulfamethoxazole, 5 (35.7%) to streptomycin, gentamicin and tobramycin, three (21.4%) to ampicillin-sulbactam, nalidixic acid, ciprofloxacin, levofloxacin, norfloxacin, lomefloxacin and ofloxacin, and two (12.3%) to chloramphenicol (Table 1).

ARGs and plasmids

Twelve different ARGs were detected in the MDR *E. coli* isolates and all presented at least two ARGs investigated. All isolates presented *tet*(B), followed by *tet*(A) (11), $bla_{CTX\text{-}M\text{-}Gp9}$ (7), *sul1* (6), *sul2* (6), bla_{SHV} (3), *qnrS* (2), *cmlA* (2), *aac(6')-Ib* (2), bla_{CMY} (1), *tet*(C) (1), and *oqxB* (1) (Table 1). Among the fluoroquinolone-resistant *E. coli* isolates (SJA29, SJA91 and SJA92), only SJA29 showed mutations in QRDR of GyrA (Ser83Leu; Asp87Asn) and ParC (Glu84Asp) (GenBank accession no. MN148169-MN148182). Among the plasmid families, the IncI1 (11) was the most prevalent, followed by ColE-*like* (5), $IncF_{repB}$ (4), IncFIB (4), IncFIA (3), IncHI1 (3), and IncK (1) (Table 1).

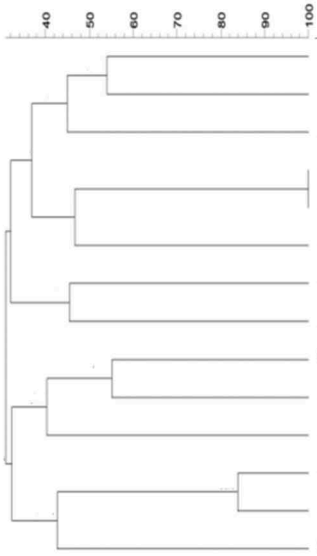
Epidemiological analysis

PFGE showed a high genetic diversity (30.9 to 83.9%) among the MDR *E. coli* isolates and thirteen sequence types (STs) belonging to six clonal complexes (CCs) were detected (ST25, ST48/CC10, ST155/CC155, ST162/CC469, ST642/CC278, ST1247, ST1518/CC206, ST1725, ST2107, ST2522, ST3270, ST5036/CC86, and ST7100). Subtyping of the *fimH* gene showed seven *fimH-type* (*fimH*25, 32, 38, 41, 54, 61 and 366) (Figure 1) (GenBank access no. MN148183-MN148196). Three phylogenetic groups [B1 (8), A (3) and Unknown (3)] were detected. Interestingly, the isolates SJA80 and SJA92 presented 100% of genetic similarity and the same diarrheagenic virulence genes, pathotype, serotype, ST, phylogenetic group and *fimH-type*; however, they presented different resistance profile as well as ARGs and plasmids (Table 1; Figure 1).

Discussion

STEC produces Shiga toxins [Stx1 (*stx1*) and Stx2 (*stx2*)], which may be associated with the enterohemolysin (*ehx*A), a virulence marker. STEC can cause diarrheal disease and outbreaks by STEC have been reported and are principally related to the consumption of contaminated products [1,2,37,38]. Infections by non-O157 STEC belonging to several serogroups (O8, O78, O100, O127, O153, and O154) have been increasing significantly over time in the United States, mainly in children (1 to 4 years) [39,40].

Resistance to β-lactams (ampicillin and cephalosporins), tetracyclines and sulfonamides have been increasingly detected in *E. coli* isolates obtained from different



IST	DVG	PTT	Serotype	PGroup	ST/CC	*fimH-type*
SJA6	*stx1, stx2*	STEC	O8:H21	B1	1725	61
SJA44	-	-	O127:H43	Unknown	1247	25
SJA11	*stx1*	STEC	O173:HNM	B1	642/278	25
SJA80	*ehxA, stx1*	STEC	O153:H12	B1	2522	38
SJA92	*ehxA, stx1*	STEC	O153:H12	B1	2522	38
SJA45	-	-	O100:HNM	A	48/10	54
SJA43	-	-	O147:H19	A	1518/206	41
SJA49	-	-	O154:H9	A	3270	54
SJA7	-	-	ONT:H10	Unknown	5036/86	25
SJA31	*stx1, stx2*	STEC	O54:H21	B1	25	32
SJA29	*stx2*	STEC	O102:H21	B1	7100	38
SJA81	-	-	O8:H19	B1	162/469	32
SJA91	-	-	O8:H19	Unknown	2107	32
SJA1	-	-	O78:HNM	B1	155/155	366

Figure 1. Dendrogram based on PFGE *XbaI* fingerprints representing the genetic relatedness among the MDR *E. coli* isolates obtained from sheep. IST, isolate; DVG, diarrheagenic virulence genes; PTT, pathotype; STEC, Shiga toxin-producing *E. coli*; PGroup, phylogenetic group; ST, sequence type; CC, clonal complex.

Table 1. STEC and non-STEC isolates according to antimicrobial resistance profile, ARGs and plasmids found.

	Isolate	Antimicrobial resistance profile[a]	ARGs[b]	Plasmid incompatibility (Inc) groups
STEC	**SJA6**	AMP, STP, GEN, TOB, TET, DOX, SXT, CLO	*tet*(A), *tet*(B), *sul2, cmlA*	FIA, FIB, ColE-*like*
	SJA11	AMP, CFZ, CRX, CFC, STP, GEN, TOB, TET, DOX	*tet*(A), *tet*(B), *aac(6')-Ib*	I1, ColE-*like*
	SJA29	AMP, ASB, CFZ, CRX, CFC, STP, GEN, TOB, TET, DOX, NAL, CIP, LVX, NOR, LMX, OFX, SXT	$bla_{CTX\text{-}M\text{-}Gp9}$, bla_{CMY}, *tet*(A), *tet* (B), *aac(6')-Ib, sul2*	F_{repB}, FIA, I1
	SJA31	AMP, CFZ, CRX, CFC, TET, DOX	*tet*(B), *tet*(C)	F_{repB}, FIA, I1
	SJA80	AMP, CFZ, CRX, CFC, TET, DOX, SXT	$bla_{CTX\text{-}M\text{-}Gp9}$, *tet*(B), *sul2*	I1
	SJA92	AMP, CFZ, CRX, CFC, STP, GEN, TOB, TET, DOX, NAL, CIP, LVX, NOR, LMX, OFX, SXT	bla_{SHV}, *tet*(B), *qnrS, oqxB, sul1*	HI1
Non-STEC	**SJA1**	AMP, TET, DOX, SXT, CLO	*tet*(A), *tet*(B), *sul1, cmlA*	FIB
	SJA7	AMP, ASB, CFZ, CRX, CFC, TET, DOX, SXT	$bla_{CTX\text{-}M\text{-}Gp9}$, *tet*(A), *tet*(B), *sul1*	FIB, I1, ColE-*like*
	SJA43	AMP, CFZ, CRX, CFC, TET, DOX, SXT	$bla_{CTX\text{-}M\text{-}Gp9}$, *tet*(A), *tet*(B), *sul2*	F_{repB}, I1
	SJA44	AMP, CFZ, CRX, CFC, TET, DOX, SXT	$bla_{CTX\text{-}M\text{-}Gp9}$, *tet*(A), *tet*(B), *sul1*	I1, ColE-*like*
	SJA45	AMP, CFZ, CRX, CFC, TET, DOX, SXT	$bla_{CTX\text{-}M\text{-}Gp9}$, bla_{SHV}, *tet*(A), *tet* (B), *sul1*	I1, K, ColE-*like*
	SJA49	AMP, CFZ, CRX, CFC, TET, DOX, SXT	*tet*(A), *tet*(B), *sul1*	F_{repB}, I1
	SJA81	AMP, CFZ, CRX, CFC, TET, DOX, SXT	*tet*(A), *tet*(B), *sul2*	HI1, I1
	SJA91	AMP, ASB, CFZ, CRX, CFC, STP, GEN, TOB, TET, DOX, NAL, CIP, LVX, NOR, LMX, OFX, SXT	$bla_{CTX\text{-}M\text{-}Gp9}$, bla_{SHV}, *qnrS, tet*(A), *tet*(B), *sul2*	FIB, HI1, I1

[a] AMP, ampicillin; ASB, ampicillin-sulbactam; CFZ, cefazolin; CRX, cefuroxime; CFC, cefaclor; STP, streptomycin; GEN, gentamicin; TOB, tobramycin; TET, tetracycline; DOX, doxycycline, SXT, trimethoprim-sulfamethoxazole; NAL, nalidixic acid; CIP, ciprofloxacin; LVX, levofloxacin; NOR, norfloxacin; LMX, lomefloxacin; OFX, ofloxacin; CLO, chloramphenicol.
[b] ARGs, antimicrobial resistance genes.

spheres. This MDR profile is associated with the presence of β-lactamases (CTX-M-*like*, SHV and CMY), acquired efflux pumps (TetABC) and changed dihydropteroate synthase (Sul) [41,42]. For other antimicrobials, such as aminoglycosides, phenicols and (fluoro) quinolones, the resistance is correlated to the presence of aminoglycoside-modifying enzymes (APH, ANT and AAC), efflux pumps (ClmA and FloR), and mutation in QRDR and/or plasmid-mediated quinolone resistance genes (PMQR), respectively [42–44].

Detection of antimicrobial-resistant *E. coli*, including MDR non-O157 STEC, obtained from animals (e.g. sheep) have been reported worldwide, including in Brazil [45–49]. Many studies characterize only the antimicrobial resistance profile of non-O157 STEC obtained from animals; however, there are few studies reporting different ARGs related to this phenotype. Srinivasa et al. [50], Ferdous et al. [51] and Bai et al. [52] reported non-O157 STEC carrying ARGs for tetracyclines (*tet* genes), (fluoro) quinolones (*qnrS* and *oqxA*) and sulfonamides (*sul1* and *sul2*).

CTX-M-*like* β-lactamases have extended-spectrum against β-lactams antimicrobials and have been increasingly detected worldwide, including non-O157 STEC from animals [51,53–56]. The ARGs detected in the present study are commonly reported in plasmids [e.g. IncI1, IncF (F_{repB}, FIA and FIB), HI1, K, and ColE-*like*], which carrying principally encoding genes for β-lactamases, PMQR and efflux pumps [56–59]. IncI1 plasmids were the most detected in this study and have been reported carrying several ARGs, including *tet*(A), *tet* (B), $bla_{CTX\text{-}M\text{-}like}$, *sul1* and *sul2* in *E. coli* isolates obtained from humans and animals [8,60,61].

The association of molecular typing and subtyping methods (i.e. phylogenetic group, MLST and *fimH-type*) has been used for epidemiological studies related to antimicrobial resistance and virulence in *E. coli* isolates. From this association, is possible to differentiate within the same ST/CC [34,36,62]. According to Enterobase Database (https://enterobase.warwick.ac.uk/), the ST48/CC10, ST155/CC155 and ST2522 have already been detected in sheep; however, all the other STs detected in the present study have been reported carrying ARGs in different sources (e.g. human, animal, food and the environment).

Curiously, *E. coli* isolates assigned as B1-ST2522-*fimH38*, B1-ST642-*fimH25* and B1-ST155-*fimH366* were previously reported in food, human, animals (i.e. food-producing animal, companion animal and wild animal), and in the environment (i.e. soil and water). Besides, ESBL-producing *E. coli* belonging to CC10 and CC155 are commonly reported causing infections in humans [63,64]. Therefore, to the best of our knowledge, this is the first report in the world of MDR STEC and non-STEC belonging to ST25, ST162/CC469, ST642/CC278, ST1247, ST1518/CC206, ST1725, ST2107, ST3270, ST5036/CC86, and ST7100 in sheep.

In conclusion, the results found in the present study showed high genetic diversity among MDR ARGs-producing *E. coli*, including non-O157 STEC, obtained from a farmhouse. These results contribute to the surveillance studies associated with One Health concept and call attention to the monitoring of MDR *E. coli* in animals, mainly in sheep.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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