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Shiga Toxigenic and Atypical Enteropathogenic *Escherichia coli* in the Feces and Carcasses of Slaughtered Pigs

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Abstract

Escherichia coli is a pathogen of major importance in swine and public health. To determine the prevalence of Shiga toxigenic *E. coli* (STEC) and enteropathogenic *E. coli* (EPEC), samples were collected from the feces and carcasses of swines. In total, 441 samples were collected in four samplings, of which 141 samples tested positive for either the *stx*1, *stx*2, and/or *eae* genes. From the positive samples, one STEC and 15 atypical EPEC (aEPEC) isolates were obtained, and all originated from the same sampling. In addition to *eae*, $lpfA_{O157/OI\text{-}141}$, *ehx*A, *tox*B, and $lpfA_{O113}$ were present in the aEPEC isolates. The only *stx*2-containing isolate carried *stx*2*e* and belonged to serotype O103:HNT. Resistance to four or more antimicrobials was found in almost half of the isolates, and some isolates shared the same fingerprint patterns by enterobacterial repetitive intergenic consensus–polymerase chain reaction (ERIC-PCR). The presence of certain virulence genes and the high level of resistance to antimicrobials, as well as the possible fecal contamination of carcasses showed that some of the isolates are of public health concern.

Introduction

Escherichia coli is an important pathogen in swine medicine and public health (Bertschinger and Fairbrother, 1999; Kaper *et al.*, 2004). There are several pathotypes of *E. coli*, which are characterized by the presence of different virulence factors. The genes responsible for these factors are located on chromosomes, plasmids, or phages (Teng *et al.*, 2004; Trabulsi, 1999). Shiga toxin–producing (STEC) and enteropathogenic *Escherichia coli* (EPEC) represent two of the six different categories of diarrheagenic *E. coli* that can cause disease in humans (Kaper *et al.*, 2004).

STEC, which is defined by the production of two Shiga toxins, Stx1 and/or Stx2, is a zoonotic pathogen that is a major cause of diarrhea worldwide. In humans, STEC can cause hemorrhagic colitis (HC), which can progress and cause severe extraintestinal complications, such as hemolytic-uremic syndrome (HUS) (Paton and Paton, 1998). Stx2 is more closely related to these diseases than Stx1 (Miceli *et al.*, 1999; Siegler *et al.*, 2003). Although *E. coli* O157:H7 is the most widely recognized pathogenic STEC serotype, non-O157 STEC serogroups, including O26, O103, O111, and O113, are also commonly associated with this disease (Nataro and Kaper, 1998; Acheson *et al.*, 2000).

There are three Stx1 subtypes (Stx1a, Stx1c, and Stx1d) and seven Stx2 subtypes (Stx2a, Stx2b, Stx2c, Stx2d, Stx2e, Stx2f, and Stx2g) according to the subtyping nomenclature proposal put forth at the 7th International Symposium on Shiga Toxin (Verocytotoxin)–Producing *Escherichia coli* Infection, held in Buenos Aires, in 2009.

EPEC, which is characterized by the presence of the *eae* gene and the absence of the toxin Stx, can be typical (tEPEC) or atypical (aEPEC). The former, tEPEC, contains both the plasmid EAF and the gene cluster *bfp*, whereas the latter, aEPEC, lacks both EAF and *bfp* (Trabulsi *et al.*, 2002). A previous report demonstrated that aEPEC is the second major cause of *E. coli*–related infantile diarrhea in Brazil (Araujo *et al.*, 2007).

Other virulence factors that may contribute to the pathogenicity of *E. coli* have been found. These include the *ehx*A gene, which encodes a hemolysin that is responsible for the formation of pores in host cells (Schmidt *et al.*, 1996), and putative adhesins encoded outside of the loci of enterocyte effacement (LEE), such as *iha*, *efa*1, *tox*B, $lpfA_{O157/OI\text{-}141}$,

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lpfA$_{O157/OI-154}$, *lpfA*$_{O113}$, and *saa* (Toma *et al.,* 2004). Several recent studies demonstrated that some genes, such as *efa*1, *lpf*A, *paa*, and *ehx*A (Afset *et al.,* 2006; Narimatsu *et al.,* 2010), are associated with EPEC isolated from humans with diarrhea and could therefore act as zoonotic potential predictors.

Previous studies reported the presence of STEC and EPEC in the feces of pigs (Fratamico *et al.,* 2004; Vu-Khac *et al.,* 2007; Oporto *et al.,* 2008) and STEC in their carcasses (Botteldoorn *et al.,* 2003; Leung *et al.,* 2001). However, these studies did not focus on EPEC from carcasses. A number of studies report the prevalence of putative adhesins in STEC and EPEC from humans and several animal species (Afset *et al.,* 2006; Leotta *et al.,* 2006; Toma *et al.,* 2006; Aidar-Ugrinovich *et al.,* 2007; Cergole-Novella *et al.,* 2007; Islam *et al.,* 2008; Narimatsu *et al.,* 2010). It would therefore be of great interest to investigate the prevalence of *E. coli* in pigs.

Due to the importance of *E. coli*–related foodborne diseases, this study focused on pig feces and carcasses, which were examined for the presence of both STEC and EPEC. We also characterized the isolates for virulence genes, enterobacterial repetitive intergenic consensus (ERIC)–polymerase chain reaction (PCR) fingerprint patterns, and antimicrobial resistance levels.

Materials and Methods

Sampling and initial procedures

A total of 441 samples were collected from pig feces and carcasses between March and August 2010 at three slaughterhouses in São Paulo State, Brazil. In slaughterhouse 1, two samplings were performed on two different days (S1a and S1b), while at the other two slaughterhouses, a single sampling (S2 and S3) was performed. In total, 96 samples (52 from feces and 44 from carcasses), 139 samples (70 from feces and 69 from carcasses), 122 samples (62 from feces and 60 from carcasses), and 84 samples (42 from feces and 42 from carcasses) were collected from S1a, S1b, S2, and S3, respectively. Feces were collected directly from the rectum of each animal using sterile swabs. Carcass samples from slaughtered pigs were collected with a sterile sponge rubbed over the leg, ribs, shoulder, and neck, for a total of 100 cm^2. All swabs were deposited in tubes containing peptone water (5 mL), and all sponges were placed in bags containing 15 mL of the same medium. All samples were transported to the laboratory in thermal boxes with ice.

Detection of STEC and EPEC by PCR

For the detection of STEC- and EPEC-positive samples, 1 mL of peptone water from the tubes and bags containing feces and carcass samples, respectively, were transferred to tubes containing 5 mL of Brilliant Green broth, incubated at 37°C for 24 h under static conditions. After incubation, 100 μL of each culture were streaked onto MacConkey agar (MA) plates and incubated at 37°C for 24 h. The DNA template was prepared from confluent MA cultures by thermal lyses (available at www.apzec.ca/en/APZEC/Protocols/pdfs/ECL_PCR_Protocol.pdf). Multiplex PCR was performed on the samples to detect the presence of the *stx*1, *stx*2, and *eae* genes (China *et al.,* 1996). The following parameters were used in the PCR: 4 μL of DNA template was added to a mixture containing 0.4 μL of 10 mM dNTPs, 2 μL of 10× buffer (100 mM Tris-HCl, pH 8.8 at 25°C, 500 mM KCl, 0.8% [v/v] Nonidet P40), 1.6 μL of 25 mM $MgCl_2$, 0.8 μL of each 10 pM primer, and 1 unit of *Taq* DNA polymerase (Fermentas, Europe). MilliQ water was added for a total volume of 20 μL. The amplification cycles were performed in an Eppendorf Mastercycler Gradient thermocycler under the following conditions: t1, 5 min at 94°C; t2, 30 sec at 94°C; t3, 45 sec at 50°C; t4, 1 min at 72°C; t2–t4, 25 repeated cycles; and t5, 7 min at 72°C. Ten individual colonies from each positive sample, which was defined by the presence of at least one of the three studied genes, were also tested by PCR to isolate STEC and EPEC strains (a methodological approach from the Reference Laboratory for *Escherichia coli* [EcL] Université de Montréal). From these isolates, the remaining tests were performed.

Detection of other virulence genes

Other virulence genes were also detected by PCR. The conditions were the same as above except that the annealing temperatures were 53°C, 52°C, 39°C, 49°C, 59°C, 55°C, 47°C, and 56°C, respectively, for duplex PCR of *iha*, *tox*B genes, and simple PCR of *saa*, *lpfA*$_{O113}$, *lpfA*$_{O157/OI-141}$, *lpfA*$_{O157/OI-154}$, *ehx*A, *efa*1, and *bfp*A genes. The primers used have been described previously (Schmidt *et al.,* 1995, 2001; Nicholls *et al.,* 2000; Doughty *et al.,* 2002; Szalo *et al.,* 2002; Paton and Paton 2002; Tarr *et al.,* 2002; Toma *et al.,* 2004; Vidal *et al.,* 2004). For the differentiation of *stx*2 variants, RFLP-PCR method was carried out as previously described (Cergole-Novella *et al*., 2006). Identification of *stx*2e subtype was performed with the primers and PCR conditions described by Pièrard *et al.* (1998).

ERIC–PCR

E. coli isolates were subjected to Enterobacterial Repetitive Intergenic Consensus (ERIC-PCR) reactions with the primers ERIC1R (5′ ATGTAAGCTCCTGGGGATTCAC-3′) and ERIC2 (5′ AAGTAAGTGACTGGGGTGAGCG-3′) (Versalovic *et al.,* 1991). Each ERIC-PCR reaction was performed in a total volume of 20 μL, as described previously. The PCR amplification thermal profile was based on a previous report (Silveira *et al.,* 2002) with minor modifications: t1, 5 min at 94°C; t2, 30 sec at 94°C; t3, 45 sec at 52°C; t4, 1 min at 72°C; t2–t4, 30 repeated cycles; and t5, 7 min at 72°C. The similarities in fragments were compared using a Dice coefficient at 1% tolerance and 0.5% optimization, and a dendrogram was constructed with the Unweighted Pair Group with Arithmetic Mean clustering method using the software BioNumerics.

Biochemical characteristics and antimicrobial susceptibility testing

All STEC and EPEC isolates were tested for lactose fermentation, indole production, methyl red reactions, Voges-Proskauer, and citrate utilization, as well as for the production of urease and hydrogen sulfide (H_2S) (Mac Faddin, 1976). Antimicrobial disk susceptibility tests were performed using the disk diffusion method (CLSI, 2009). The antimicrobials tested were ampicillin (10 μg), cephalothin (30 μg), streptomycin (10 μg), gentamicin (10 μg), nalidixic acid (30 μg), ciprofloxacin (5 μg), chloramphenicol (30 μg), tetracycline (30 μg), nitrofurantoin (300 μg), and sulfamethoxazole + trimethoprim (25 μg).

Serotyping

The determination of the EPEC serogroup was performed with the slide agglutination technique, using anti-O sera against the classical EPEC somatic antigens O26, O55, O111, O119, O114, O125, O142, O158, O86, O126, O127, and O128 (Probac, São Paulo, Brazil). STEC serotyping with all somatic and flagellar antigens was performed at a reference laboratory (Instituto Adolfo Lutz, São Paulo, Brazil).

Results

After testing the confluent growth from fecal and carcass samples, we found differences in the prevalence of genes related to STEC (*stx*) and EPEC (*eae*) in the different slaughterhouses. The samples from S1b had the greatest percentage of *eae,* whereas the samples from S3 showed the highest percentage of *stx*2. In addition, *eae* was more prevalent than *stx2* in all samplings, except in S3. In all samplings, *stx*1 was not found. Furthermore, samples that were positive for at least one gene were found more often in the fecal rather than the carcass samples. The prevalence of these genes is shown in Table 1. After testing ten colonies from each of the 141 positive samples for the presence of the *stx*1, *stx*2, and *eae* gene, we found only one STEC isolate (*stx*2+*stx1- eae*-) in a carcass sample and none in the feces. Therefore, the prevalence of STEC isolates was 0.4% of the total carcasses sampled (215), and no STEC isolates were detected in the feces. Moreover, eight fecal and seven carcass samples each yielded a single isolate of aEPEC (*stx*1-, *stx*2-, *eae*+). The prevalence of aEPEC was 3.5% and 3.2% in the fecal and carcasses, respectively, relative to the total number of feces (226) and carcasses (215) samples. All sixteen isolates described in this work originated from the second sampling of slaughterhouse 1 (S1b).

The $lpfA_{O113}$ (46.6%) gene was the most prevalent in the aEPEC isolates. The $lpfA_{O157/OI\text{-}141}$ (13.3%), *ehx*A (13.3%), and *tox*B (13.3%) genes were also detected in the aEPEC isolates. There was no aEPEC isolate that contained the $lpfA_{O157/OI\text{-}154}$, *saa*, *efa*1, *iha*, or *bfp* gene. The absence of *bfp* indicated that all of the EPECs isolated in this study were considered atypical EPEC (aEPEC). The only *stx*2-containing isolate presented *stx*2*e* subtype and was serotyped as O103:HNT. None of the aEPEC isolates belonged to the classical EPEC serogroups tested.

All of the isolates in this study were tested against ten antimicrobial agents. The most common resistances were to tetracycline (93.7%), nalidixic acid (81.3%), and ampicillin (50.0%). Less frequent resistances were to chloramphenicol (33.3%), streptomycin (31.2%), cephalothin (25.0%), gentamicin (18.7%), sulfamethoxazole/trimethoprim (12.5%), nitrofurantoin (0%), and ciprofloxacin (0%). Multidrug resistance was found in 43.8% of the isolates, which was defined as resistance against four or more antimicrobials. Some isolates showed the same fingerprint pattern by ERIC-PCR, and two of these samples, one from carcass (46C) and one from feces (25R), also shared the same virulence genes and antimicrobial resistance profile (Fig. 1). All isolates showed typical characteristics of *Escherichia coli* in biochemical tests.

Discussion

In the present study, we identified STEC and aEPEC isolates from swine feces and carcasses. Some of these isolates showed similar genetic profiles by ERIC-PCR and virulence genes, including putative adhesins.

According to Table 1, the percentage of samples containing *stx*1, *eae,* or *stx*2/*eae* varied between slaughterhouses, which could possibly be attributed to the batch of animals that each slaughterhouse had received. The animals were likely to have been exposed to different sanitary managements at the farms of origin. The PCR sampling yielded more positive results when compared to the PCR from the isolates. These findings may be attributed to the PCR being performed with confluent cultures from MacConkey Agar. These results would represent all Gram-negative microbiota from each sample, thereby enhancing the detection levels of STEC and EPEC. Because only a few PCR-positive isolates were detected from these samples, it is apparent that STEC and EPEC isolates occur at a much lower level in swine compared to *E. coli* of different pathotypes.

In this study, the prevalence of STEC isolates, both in carcasses and feces, was similar to those reported in other studies conducted in pigs, which found frequencies ranging from 0% to 1% (Leung *et al.,* 2001; Lindblad 2007; Oporto *et al.,* 2008). Using the same methodology described in this study, we

Table 1. Prevalence of *Stx*1, *Stx*2, and *eae* in Samples of Feces and Carcasses of Pig from Different Slaughterhouses

	S1a[1]				*S1b*[2]				*S2*[3]				*S3*[4]			
	(n=*96*)				(n=*139*)				(n=*122*)				(n=*84*)			
	Feces		*Carcass*		*Feces*		*Carcass*		*Feces*		*Carcass*		*Feces*		*Carcass*	
	52		*44*		*70*		*69*		*62*		*60*		*42*		*42*	
	n	%	n	%	n	%	n	%	n	%	n	%	n	%	n	%
*stx*2	0		0		13	18.6	4	5.8	3	4.8	2	3.3	15	35.7	4	9.5
eae	17	32.7	3	6.8	25	35.7	25	36.2	13	20.9	3	5.0	2	4.7	1	2.4
*stx*2/*eae*	5	9.6	0		2	2.8	1	1.4	0		0		2	4.7	1	2.4

[1]Slaughterhouse 1, sampling a.
[2]Slaughterhouse 1, sampling b.
[3]Slaughterhouse 2.
[4]Slaughterhouse 3.
*stx*1 was not found in any slaughterhouse.

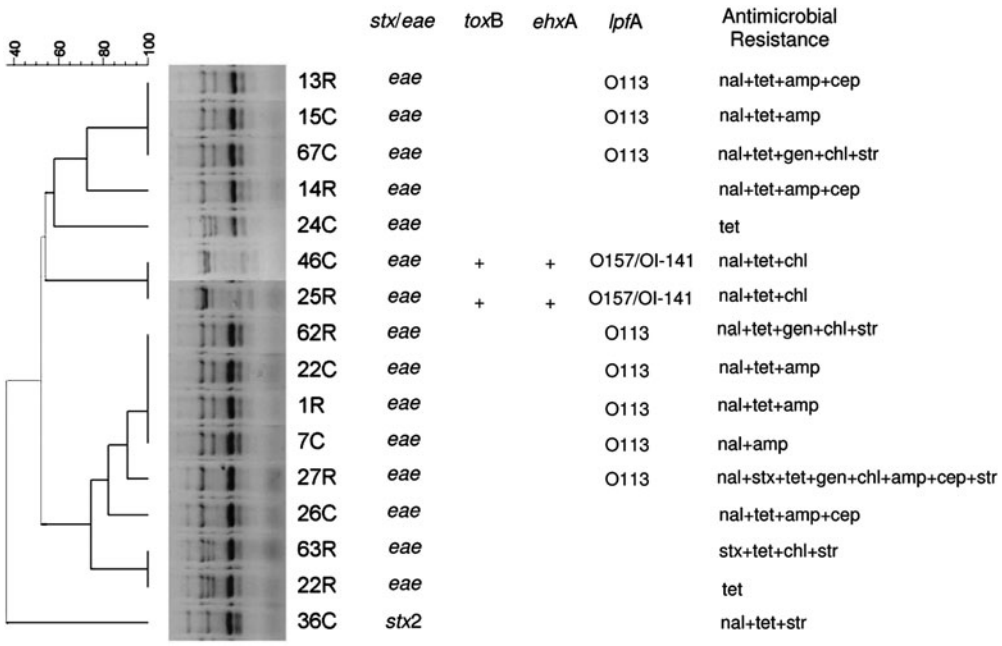

FIG. 1. Dendrogram of enterobacterial repetitive intergenic consensus (ERIC)–polymerase chain reaction (PCR) profiles showing virulence genes and antimicrobial resistance of the 16 isolates of *Escherichia coli* from pigs. R, feces; C, carcass; AMP, ampicillin; GEN, gentamicin; STR, streptomycin; CEP, cephalothin; STX, trimethoprim/sulfamethoxazole; CHL, chloramphenicol; NAL, nalidixic acid; TET, tetracycline.

found that the prevalence of STEC isolates from sheep and buffalo samples were greater than the prevalence in pigs (unpublished data). These findings suggest that pigs might not be an important reservoir for STEC.

The STEC isolate identified in this study was positive for Stx2e, a subtype commonly associated with edema disease in pigs but is rarely detected in human infections (Friedrich *et al*., 2002; Zweifel *et al*., 2006; Vu-Khac *et al*., 2007). We also found that this strain belonged to the serogroup O103, which is not common in swine but is considered a serious public health threat because it has been recovered from infected patients. The serotype usually related to human infection is O103:H2 and possess another stx subtype (Mariani-Kurkdjian *et al*., 1993; Guth *et al*., 2005). However, these findings suggest that healthy pigs cannot be excluded as a potential source of human infection.

We did not find any O157:H7 isolates in our study. These results were similar to the findings from studies conducted in France, Belgium, and Ireland using swine carcasses (Bouvet *et al*., 2001; Botteldoorn *et al*., 2003; Lenahan *et al*., 2009). However, the use of a more specific technique for O157:H7, such as immunomagnetic separation (IMS), may improve detection of this microorganism.

Previous reports demonstrated a prevalence range between 1.4% and 18.5% of aEPEC in pigs with diarrhea (Frydendahl *et al*., 2002; Malik *et al*., 2006; Vu-Khac *et al*., 2007). We found that the prevalence of aEPEC isolated from the feces was much lower compared to another study that was conducted in healthy pigs. This study found that 14% of the samples were positive for aEPEC (Malik *et al*., 2006), suggesting that the occurrence of aEPEC varies with the batch rather than health status. However, these studies used different methods and approaches to detect aEPEC, and it is therefore possible that the differences in methods could impact the results.

In Korea, previous studies demonstrated that STEC and EPEC strains were identified in 2.0% and 2.5%, respectively, of the isolates collected from pork meat. A similar study conducted in New Zealand found that 4.0% were STEC isolates, whereas in the United States, the prevalence of this pathotype was 18% (Samadpour *et al*., 1994; Brooks *et al*., 2001; Lee *et al*., 2009). These data demonstrated that meat, including pork, could potentially act as a reservoir for pathogenic *E. coli* in humans. A study conducted in cows concluded that *E. coli* that contaminates meat can originate from feces (Aslam *et al*., 2003). It is likely that *E. coli* from feces have contaminated the meat in this study. The isolates that showed 100% similarity by ERIC-PCR and shared the same genetic profile suggest that this ocurred.

In contrast to other studies on *E. coli* (Paton *et al*., 2001; Cergole-Novella *et al*., 2007; Vu-Khac and Cornick, 2008), we did not find isolates possessing both the *ehx*A and *saa* genes. Instead, our isolates contained only the *ehx*A gene, which had already been reported from EPEC isolates obtained from healthy cattle (Aidar-Ugrinovich *et al*., 2007). Other reports (Osek *et al*., 2003; Tatarczak *et al*., 2005) identified the presence of $lpfA_{O113}$ loci in 100% and 61% of STEC from pigs. Osek *et al*. (2003) suggest that LPFO113 contributed to the colonization of the porcine host. As this gene was also found in our study, it is possible that a similar event occurs in porcine aEPEC.

Previous studies did not find $lpfA_{O157/OI-141}$ and *tox*B in STEC collected from pigs (Tatarczak *et al*., 2005; Osek *et al*., 2006). To the best of our knowledge, this is the first report of the $lpfA_{O157/OI-141}$, $lpfA_{O113}$ and *tox*B genes being found in aEPEC isolated from swine.

The *ehx*A gene has been previously found in *E. coli* from humans with diarrhea, hemorrhagic colitis and hemolytic uremic syndrome (Cookson *et al*., 2007; Pradel *et al*., 2008). The

lpfA$_{O113}$ gene was also associated with aEPEC that causes diarrhea in humans (Afset *et al.,* 2006). Thus, the isolates reported in our study that had these genes (13R, 15C, 25R, 46C, 67C) may have zoonotic potential.

Multidrug resistance was found in almost half of the *E. coli* isolates from pigs, which were resistant against four or more antimicrobials. This result is in agreement with other studies conducted in Brazil, Germany, Switzerland, and the United States that reported multidrug resistance of *E. coli* isolated from healthy pigs (Stephan and Schumacher, 2001; Fratamico *et al.,* 2004; Von Müffling *et al.,* 2007; Costa *et al*., 2010). This result is not surprising because antimicrobials have been used for many years for prophylactic purposes as well as for growth promotion. The widespread use of antibiotics has probably contributed to high rates of resistance (Mathew *et al*., 1999; Stephan and Shumacher, 2001).

Vieira *et al.* (2011) reported strong and significant correlations between resistances to aminoglycosides (streptomycin), aminopenicillins (ampicillin), and fluoroquinolones (nalidixic acid) in *E. coli* isolates from humans and pigs in many European countries. Our results also demonstrated increased resistance of these isolates to the same class of antimicrobials. Because these are important antimicrobials used in human medicine, the results from this study are of great public health concern (Collignon *et al*., 2009).

In conclusion, this study showed that pig carcasses and feces carry aEPEC and STEC, as well as some putative adhesins. The presence of certain genes, together with the high level of resistance to antimicrobials and possible fecal contamination of carcasses, demonstrate that *E. coli* from pork may pose a concern to public health.

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Disclosure Statement

No competing financial interests exist.

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