Heterogeneity of *Escherichia coli* STb enterotoxin isolated from diseased pigs

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To investigate the presence and frequency of *estB* variant(s), a collection of 100 STb-positive enterotoxigenic *Escherichia coli* (ETEC) strains isolated from 1980 to 2007 inclusively and randomly selected from diseased pigs in Québec, Canada, was analysed. A wide diversity of virulence gene profiles (virotypes) was detected in the strain collection. The *estB* gene was amplified by PCR using primers designed from the signal sequence and the C-terminal end, and the amplified fragment was sequenced using the forward primer. The translated DNA sequence revealed a His₁₂→Asn change in 23 of the 100 ETEC isolates tested. The STb-variant strains were observed throughout the sampling period covered in the study. No other STb-variant type was found in this study. All 23 variant strains were also positive for the STa enterotoxin and were resistant to tetracycline, as for strain 2173. The STb variant was associated with Stx2-positive strains (5/6) and STa : STb strains that did not harbour any of the tested porcine fimbrial adhesins (13/17). The remaining variant strains were associated with fimbriae F4 (1/40), F5 (1/6), F6 (1/1) and F18 (2/7; excluding F18 : Stx2 strains).

INTRODUCTION

Severe diarrhoea in humans and animals is often the result of *Escherichia coli* infections. Of the six pathotypes described for *E. coli*, enterotoxigenic *E. coli* (ETEC) is one of the most important agents of severe diarrhoea in animals such as calves and pigs (Nagy & Fekete, 2005). ETEC produce several virulence factors, for example F4 and F18 fimbriae and the adhesin involved in diffuse adherence (AIDA), which mediate bacterial adherence to the intestine, and heat-labile (LT) and heat-stable (STa and STb) enterotoxins, which cause diarrhoea. STb is mostly associated with porcine strains, although it has been found in human isolates (Lortie *et al.*, 1991; Okamoto *et al.*, 1993). This toxin is synthesized as a mature 48 aa peptide composed of two antiparallel α -helices separated by a glycine-rich loop (Sukumar *et al.*, 1995). It contains two disulfide bonds, which stabilize the peptide's tertiary structure, and both of these bonds are crucial for secretion and toxicity (Arriaga *et al.*, 1995; Dreyfus *et al.*, 1992; Okamoto *et al.*, 1995; Sukumar *et al.*, 1995). Likewise, the charged amino acid present in the glycine-rich loop also appears to be important for enterotoxicity, as are the hydrophobic residues present in the hydrophobic α -helix. In fact, mutations of these residues strongly inhibit both binding to its receptor and toxic

activity in rat ligated intestinal loops (Dreyfus *et al.*, 1992; Labrie *et al.*, 2001a). The hydrophobic α -helix has also been shown to be involved in the oligomerization of the toxin (Labrie *et al.*, 2001b).

In a recent study by Zhang *et al.* (2007), 72.6 % of 304 *E. coli* isolates from diarrhoeic pigs in the USA were found to possess the STb enterotoxin gene, indicating that STb toxin is a highly prevalent toxin. No variation in the nucleotide sequence of STb was identified prior to 2003, when Fekete *et al.* (2003) reported a variant of the toxin. This variant had two amino acid changes of His₁₂→Asn and Lys₂₃→Ile. The variant was isolated from an F18-positive ETEC, strain 2173, isolated from the small intestine of a weaned pig that died from post-weaning diarrhoea. They also showed that this STb variant was encoded along with STa enterotoxin on a pathogenicity island (PAI₂₁₇₃) present on a plasmid (pTC₂₁₇₃) that also encoded the *tetB* gene conferring tetracycline resistance.

In the present study, a collection of 100 STb-positive ETEC strains, isolated from diseased pigs from 1980 to 2007 inclusively, was analysed to determine the frequency and distribution of STb variant(s), if present, throughout this 28-year period and to determine the various ETEC virotypes.

METHODS

Bacterial strains. A total of 100 STb-positive *E. coli* strains were randomly selected from the *E. coli* culture collection of the reference

Abbreviations: AIDA, adhesin involved in diffuse adherence; EAE, *E. coli* attaching-and-effacing factor; EAST1, enteroaggregative *E. coli* heat-stable enterotoxin 1; ETEC, enterotoxigenic *Escherichia coli*; LT, heat-labile toxin; Paa, porcine attaching- and effacing-associated protein; ST, heat-stable enterotoxin; Stx, Shiga toxin.

laboratory for *Escherichia coli* (EcL), part of the diagnostic services of the Faculté de Médecine Vétérinaire, Université de Montréal, Québec, Canada. Isolates had been recovered from samples from nursing or weaned pigs from 1980 to 2007 inclusively. Farms from which the pigs originated were distributed throughout the various regions of Québec. The *estB* gene encoding STb was identified using colony hybridization, and serotyping was performed by standard techniques using a slide agglutination test with antisera produced in rabbits (Harel *et al.*, 1991). Strains were stored at -80°C in tryptic soy broth with 10% glycerol and were plated on tryptic soy agar supplemented with 5% sheep blood before testing.

Virotyping using colony hybridization. Strains were tested for the following virulence or putative virulence factors using colony hybridization as described previously (Harel *et al.*, 1991): LT, STa, STb, enteroaggregative *E. coli* heat-stable enterotoxin 1 (EAST1), porcine attaching- and effacing-associated protein (Paa), AIDA, Shiga toxins 1 and 2 (Stx1 and Stx2), *E. coli* attaching-and-effacing factor (EAE) and fimbriae F4 (K88), F5 (K99), F6 (987P), F17, F18 and F41. DNA probes were derived from recombinant plasmids or from PCR products (Fecteau *et al.*, 2001; Ngeleka *et al.*, 2003).

Isolation, amplification and sequencing of DNA. Total bacterial DNA was isolated using a DNeasy tissue system kit (Qiagen) according to the manufacturer's instructions. The *estB* gene was amplified by PCR using the primers 5'-CCACTGGTATAA-GTTTATTGCTTATAG-3' and 5'-TTAGCATCCTTTTGCTGCA-3'. PCRs were carried out in a total reaction volume of 45 μl in a Biometra T3 thermocycler containing 2.78 mM MgCl_2 , 0.33 mM each primer, 280 μM each dNTP, 2 U *Taq* recombinant polymerase (Invitrogen) and 1.5 μl template. The PCR cycle program consisted of initial denaturation at 94°C for 5 min, followed by 30 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s and extension at 72°C for 30 s, followed by a final extension at 72°C for 5 min. The PCR product was resolved by electrophoresis on a 3% agarose gel in $1 \times$ Tris/borate/EDTA buffer along with a 1 kbp DNA ladder as marker. Sequencing was carried out at the Faculté de Médecine Vétérinaire using an ABI 310 genetic analyser (Applied Biosystems) with a BigDye Terminator kit.

Tetracycline susceptibility. The tetracycline susceptibility of *E. coli* isolates was determined by the Kirby-Bauer disc diffusion method (Bauer *et al.*, 1966). Readings were performed according to NCCLS (1990) guidelines.

RESULTS

The *estB* gene encoding the STb toxin was amplified and sequenced for the 100 tested strains. The gene sequence was identical to the sequence originally described by Lee *et al.* (1983) in 77 strains. The remaining 23 strains differed from the previously described sequence (wild-type STb) by one base at codon 34 (CAT to AAT), resulting in a change in the deduced amino acid residue at position 12 (His $\rightarrow$ Asn). This variant was observed in strains dating from 1980 to 2007 and was associated with specific virotypes found in ETEC (Table 1). These 23 variant strains were determined to be resistant to tetracycline. The STb variant was associated mainly with STa:STb strains harbouring none of the tested porcine fimbriae (13/23). Serotyping revealed that 10 of these 13 STa:STb variant strains belonged to the O?:K48 group, whilst the other three were not typable (Table 1). Thus the most common strain isolated with the STb variant was STa:STb:O?:K48.

We assessed whether the presence of individual toxin genes (STa, Stx2, LT and EAST1) could be correlated with the presence of wild-type or variant STb (Table 2). Five of the six Stx2-positive strains (83.3%) were associated with the STb variant and the frequency of Stx2 was higher in the strains harbouring the STb variant. Thus there was a correlation between the presence of Stx2 and the STb variant. Conversely, the LT and EAST1 toxins were more often associated with the wild-type STb sequence (94 and 87.1%, respectively), and the frequency of LT and EAST1

Table 1. Incidence of the His₁₂ $\rightarrow$ Asn STb variant in porcine *E. coli* strains

Virotype*	No. of strains tested	No. of STb variants	OK group†
F4:LT:STa:EAST1 (Paa)	10	0	
F4:LT:Paa:EAST1 (AIDA)	10	0	
F4:LT (EAST1, AIDA)	17	0	
F4 (EAST1, AIDA, Paa)	2	0	
F4:STa	1	1	O149
F18:Stx2 (LT, STa, EAST1, AIDA, Paa)	4	3	O149, O?:KV17, NT
Stx2 (LT, STa, EAST1 AIDA, Paa)	2	2	O141, NT
F18 (LT, STa, EAST1, AIDA, EAE)	7	2	O157 (2)
F5:STa	6	1	O149
F6:STa:EAST1:Paa	1	1	O9
STa (EAST1, AIDA, Paa)	18	13	O?:K48 (10), NT (3)
LT (EAST1, AIDA) and EAST1:AIDA (Paa) and AIDA	22	0	
Total	100	23	

*Virulence factor profile of STb-positive strains. The presence or absence of virulence factor(s) is indicated in parentheses. Strains were tested for the following virulence factors: LT, STa, STb, EAST1, Paa, AIDA, Stx1 and Stx2, EAE and fimbriae F4, F5, F6, F17, F18 and F41.

†Only strains with the His₁₂ $\rightarrow$ Asn variant were tested for the OK group. The number of strains with the OK group is given in parentheses. NT, Non-typable.

Table 2. Association of wild-type STb and STb-variant strains with virulence factors

Virulence factor*	Wild-type STb (%)†	STb variant (%)‡
STa (45)	22 (28.5) 48.9	23 (100) 51.1
Stx2 (6)	1 (1.3) 16.7	5 (21.7) 83.3
LT (50)	47 (61) 94	3 (13) 6
EAST1 (70)	61 (87.1) 87.1	9 (39.1) 12.9
F4 (40)	39 (50.6) 97.5	1 (4.3) 2.5
F5 (6)	5 (6.49) 83.3	1 (4.3) 16.7
F6 (1)	0 (0) 0	1 (4.3) 100
F18 (11)	6 (7.8) 54.5	5 (21.7) 45.5
Paa (27)	23 (29.9) 85.2	4 (17.4) 14.8
AIDA (31)	28 (36.4) 90.3	3 (13) 9.7

*The total number of strains positive for the virulence factor is shown in parentheses.

†The percentage of the 77 wild-type STb strains is shown in parentheses and the percentage of wild-type STb strains with the virulence factor indicated is in bold.

‡The percentage of the 23 STb-variant strains is shown in parentheses and the percentage of STb-variant strains with the virulence factor indicated is in bold.

was higher in the strains harbouring wild-type STb. Thus there was a correlation between the presence of EAST1 or LT and wild-type STb. All 23 STb-variant ETEC strains possessed the STa enterotoxin, whilst only 28.5 % of the wild-type STb-positive ETEC strains possessed STa. However, an equal number of STa-positive strains were found with the wild-type STb gene or its variant. There was therefore a weaker correlation between the presence of STa and the STb variant.

Next, we assessed whether the presence of individual fimbrial genes (F4, F5, F6 and F18) could be correlated with the presence of wild-type or variant STb (Table 2). A total of 40 STb-positive strains were also positive for the F4 fimbriae. F4-positive strains harbouring different combinations of enterotoxins (STa, STb, LT and/or EAST1), with or without Paa, are classic causes of post-weaning diarrhoea (Fairbrother *et al.*, 2005). However, the STb variant was found in only one (2.5 %) of the F4-positive strains. Thus the presence of F4 was strongly correlated with that of wild-type STb. In fact, 97.5 % of the F4-positive strains were associated with wild-type STb and 50.6 % of the strains positive for wild-type STb also had F4, whereas this frequency dropped to 4.3 % for strains

positive for the STb variant. A total of 11 strains in the collection were positive for the F18 fimbriae. Five of these strains showed the Asn₁₂ variation, corresponding to 21.7 % of the STb-variant strains. None of the other fimbrial genes exhibited such a correlation with wild-type or variant STb.

With regard to the other virulence factors involved in adherence of ETEC to host cells (Paa and AIDA), both were found to correlate with the presence of wild-type STb, as 85.2 or 90.3 % of strains positive for Paa or AIDA, respectively, were associated with wild-type STb (Table 2).

DISCUSSION

In this study, we screened a total of 100 ETEC strains for possible variants of the STb enterotoxin gene. Unexpectedly, we found 23 STb-variant strains, all bearing a unique variation at codon 34, but no other variant type was observed. The observed modification is responsible for a change of His₁₂ to Asn. Two amino acid changes were observed in strain 2173 by Fekete *et al.* (2003). However, when sequenced in our laboratory, the *estB* gene from strain 2173 corresponded to a single amino acid change and the sequence was identical to the one found for the reported variant in the current study. This result was confirmed by P. Fekete and B. Nagy (personal communication). The change observed in our study was located in the amphipathic helix of the toxin, a highly hydrophilic region. The side chain of His₁₂ points towards the solvent (Sukumar *et al.*, 1995) and thus could be involved in receptor recognition and/or toxicity. Studies using a point-mutation approach have determined the role of some amino acid residues in toxicity and binding of STb to its receptor (Dreyfus *et al.*, 1992; Fujii *et al.*, 1991; Labrie *et al.*, 2001a). However, the histidine at position 12 has not been investigated so far. Thus we cannot hypothesize about the effect of the amino acid substitution found in the variant identified in our study.

It appears that the STb variant is associated with unusual ETEC virotypes. The STb variant was associated with STa-positive ETEC strains, as all of the 23 variants also hybridized with the STa enterotoxin gene. Most of the ETEC strains positive for the STb variant were associated with two ETEC groups, STa- and STb-positive strains harbouring none of the tested porcine fimbriae, and Stx2-positive strains. Stx2-positive strains also positive for one or a combination of enterotoxins are also called ETEC/STEC (shiga toxin-producing *E. coli*) strains and are responsible for oedema disease and/or diarrhoea (Fairbrother *et al.*, 2005). STa:STb strains without porcine-associated fimbriae are less well characterized and could be associated with diarrhoea, as this type of ETEC strain has been isolated from diarrhoeic pigs (J. M. Fairbrother, personal communication). The STb variant was not associated with F4-positive ETEC, these strains being the main cause of post-weaning diarrhoea in pig

production. Classical post-weaning diarrhoea-associated F4-positive ETEC generally harbour at least the STb and LT enterotoxins and are associated with serogroup O149 (Fairbrother *et al.*, 2005). In the current study, the only F4-positive strain in which the STb variant was detected was not a classical post-weaning diarrhoea-associated ETEC strain, as it was positive for STa, STb and F4, but negative for LT.

As all 23 variant strains were STa-positive and resistant to tetracycline, as also observed by Fekete *et al.* (2003), this could indicate that the observed variant is also present on the pTC₂₁₇₃ plasmid where STb, STa and resistance to tetracycline are found. This could be the result of selection due to preferred colonization of swine by strains carrying the plasmid and the ensuing development of enteric disease.

In conclusion, we demonstrated that the frequency of an STb variant, identified in this study, is quite high and is distributed among diverse ETEC virotypes, but mainly in STa:STb:Stx2-positive ETEC and STa:STb ETEC harbouring no porcine-associated fimbriae. It would be interesting to determine the impact of this genetic variation on the toxicity of the STb variant *in vivo*.

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