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High Prevalence of Serine Protease Autotransporter Cytotoxins among Strains of Enteroaggregative *Escherichia coli*

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Abstract

Enteroaggregative *Escherichia coli* (EAEC) pathogenesis is thought to comprise intestinal colonization followed by the release of enterotoxins and cytotoxins. Here, we use PCR to determine the prevalence of ten genes encoding serine protease autotransporter toxins (SPATEs) in a collection of clinical EAEC isolates. Eighty-six percent of EAEC strains harbored genes encoding one or more Class I cytotoxic SPATE protein (Pet, Sat, EspP, or SigA). Two Class II, non-cytotoxic, SPATE genes were found among EAEC strains: *pic* and *sepA*, each originally described in *Shigella flexneri* 2a. Using a multiplex PCR for five SPATE genes (*pet*, *sat*, *sigA*, *pic and sepA*), we found that most of the *Shigella* isolates also harbored more than one SPATE, whereas members of most other *E. coli* pathotypes rarely harbored a cytotoxic SPATE gene. SPATEs may be relevant to the pathogenesis of both EAEC and *Shigella* spp..

Enteroaggregative *Escherichia coli* (EAEC) has been associated with several clinical scenarios, including travelers' diarrhea [1, 2, 3], endemic pediatric diarrhea among children in industrialized [4] and developing countries [5], as well as persistent diarrhea among HIV-infected patients [6-10]. The current pathogenetic paradigm for EAEC includes colonization of the intestinal mucosa followed by the elaboration of one or more cytotoxins and enterotoxins, of which several have been described [11-18]. Enterotoxins include the enteroaggregative heat-stable toxin (EAST1) and the *Shigella* enterotoxin-1 (ShET1). The roles of these toxins in EAEC pathogenesis and epidemiology are not yet known.

Infection of human intestinal explants suggests that most EAEC strains elicit frank mucosal damage, accompanied by rounding and exfoliation of colonocytes. Henderson et al. have reported that the serine protease autotransporter (SPATE) toxin called Pet (plasmid-encoded toxin) is required for strain 042 to elicit cytotoxic effects on human explants [14]; however, only a small minority of EAEC strains carry the *pet* gene, though a much larger number of strains are toxic to explants. This paradox occurs in the context of substantial heterogeneity of EAEC adhesins and other putative virulence factors, presenting a confusing clinical and epidemiologic scenario [19].

The Pet protease is a member of the serine protease autotransporters of *Enterobacteriaceae* (SPATE) family of secreted proteases. SPATEs comprise a large group of trypsin-like serine proteases, which are secreted by *Shigella* spp., uropathogenic *E. coli*, and all of the diarrheagenic *E. coli* (DEC) pathotypes (reviewed in refs. [19] and [20]). The toxins are

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translocated across the outer membrane by the autotransporter pathway, in which translocation requires a dedicated C-terminal beta barrel domain. The N-terminal, mature SPATE toxins are 104-110 kDa in size and feature a typical N-terminal serine protease catalytic domain, followed by a highly conserved beta-helix motif, which is present in nearly all autotransporters [20]. The SPATE family has been organized phylogenetically into two classes. Members of the Class I SPATEs (which include Pet) are all cytotoxic to epithelial cells [21]. In addition to Pet, the Class I SPATEs include, prominently, EspP from enterohemorrhagic *E. coli* (EHEC), EspC from enteropathogenic *E. coli* (EPEC), SigA from *Shigella flexneri*, and Sat, from uropathogenic and diffusely adhering *E. coli* (DAEC) [16]. Class II SPATEs are more diverse with regard to phenotype, though several are known to cleave mucin. Many EAEC and *Shigella* strains encode Pic, a mucinase encoded on the bacterial chromosome [22]. Pic may promote intestinal colonization via an unknown mechanism (Harrington and Nataro, unpublished observations). Class II includes, besides Pic, SepA from *Shigella flexneri* [23] and Tsh from avian pathogenic *E. coli* [24], and several others.

We sought to resolve the paradox between observed cytotoxic effects attributed to most EAEC strains and the low carriage rate of the Pet cytotoxin. Specifically, we hypothesized that other Class I SPATE cytotoxins would be commonly found among EAEC strains. We tested this hypothesis on 55 EAEC and 10 each of ETEC, EHEC, EPEC, EIEC, and DAEC strains, as well as 12 *Shigella* strains. All strains were isolated in the course of epidemiologic studies [27, 29] and were derived from the collections of the Statens Serum Institut (SSI) in Denmark or the Center for Vaccine Development (CVD) of the University of Maryland School of Medicine (Table 1 and Table 2) [25, 26, 27, 28]. In addition, we selected 12 non-pathogenic *E. coli* strains: six strains were isolated from healthy humans in the course of the Danish antimicrobial resistance surveillance program (DANMAP), and six strains were isolated from minced meat as part of the Danish food surveillance program. *Shigella flexneri* 2a strain 2457T was used as a control[30]. Stock cultures were frozen at - 80 °C in Luria broth (LB) or SSI broth containing 10% (v/v) glycerol. All strains were grown at 37°C. Serotyping of the EAEC strains was performed at the SSI using standard methods [31].

PCR was used to detect the presence of genes corresponding to known SPATE sequences. Primers used are listed in Table 3. DNA template was obtained as previously described [25]. The accuracy of a subset of PCR reactions was verified by nucleotide sequencing in the Biopolymer Laboratory Core Facility, University of Maryland School of Medicine. A multiplex PCR was designed to detect the most common SPATE genes; the Multiplex PCR kit was used according to manufacturer's instructions (Qiagen inc., Valencia, Ca, USA). Products were amplified by using the Eppendorf Mastercycler Gradient thermal cycler (Eppendorf North America Inc, Westbury, NY, USA). The specific temperatures and primers are listed in Table 3. Monoplex PCR reactions were performed as previously described [25]. Multiplex PCR cycles comprised (a) 15 min. denaturation at 95°C, (b) 30 sec. denaturation (depending on the size of the product, with a 30 sec. increase for each 500 bp) at 94°C, (c) annealing for 1.5 min, and (d) extension 1.5 min. at 72°C with 35 cycles from step (b). The final extension was 10 min. at 72°C.

Results of monoplex PCR for ten SPATE-encoding genes (*pet*, *sigA*, *espC*, *espP*, *sat*, *vat*, *pic*, *sepA*, *tsh* and *eatA*) are presented in Table 1. In order to corroborate results of the PCR assays, the supernatants of several strains which were positive for SPATE sequences by PCR were separated by 17.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE); strains positive for up to three SPATEs by PCR also showed the expected number of supernatant proteins at the predicted molecular mass (104-130 kDa). Several protein bands were excised and subjected to tryptic digest and mass spectrometry in a Finnigan LCQ Advantage Ion Trap Mass Spectrometer in the Protein Analysis Core of the Center for Vascular and Inflammatory Diseases, University of Maryland School of Medicine. These analyses

yielded protein identifications that correlated completely with PCR assays for *pic*, *sat* and *sepA* (Figure 1).

Out of the 55 EAEC strains tested, 94.5% harbored genes for one or more of the SPATE proteins, including members of Class I (Pet, Sat, SigA, EspP) and/or Class II (Pic, SepA, Tsh). No EAEC strain yielded a product corresponding to *vat*, *espC*, *eatA*, or *tsh*. 18.2% of the strains had only a Class I SPATE and no Class II sequence, 9.1% of the strains had only a Class II, and 67.3% were positive for both a Class I and II SPATE (Fig. 2A). Interestingly, the single most common SPATE among the EAEC strains was Sat (74.5% of strains), which was first described in uropathogenic *E. coli* strains, but which has more recently been reported among DAEC. Interestingly, we found *pet* in only 9.1% of our strains, and one strain (H92-1, Fig. 2A, Table 1) also harbored the *sat* gene. The mature Sat and Pet toxins are 52% identical at the amino acid level and thus they may be allelic toxins which fulfill the same roles in pathogenesis [32]. Sat is cytotoxic to urinary epithelial cells in vitro [32], and Guignot et al. have suggested that Sat induces cytoskeletal perturbation in intestinal epithelium accompanied by rearrangement of tight junction proteins [33]. Though the fundamental mode of action of Sat is unknown, Maroncle et al. have suggested that the protein enters epithelial cells and directly cleaves spectrin[34], an effect also attributed to its closest homolog, Pet [35]. Further experiments are required to determine whether the two toxins induce identical effects.

Some EAEC strains carried neither *pet* nor *sat*, but interestingly, the majority of these strains harbored another Class I SPATE (Fig. 2B). 7.3 % of the EAEC strains harbored *sigA*, a cytotoxin originally described in *S. flexneri*, and 3.6% harbored *espP*, reported as a cytotoxin in Shiga toxin-producing *E. coli* strains. Overall, nearly 85% of EAEC strains harbored a gene encoding a Class I cytotoxic SPATE protein.

The Class II SPATEs were also common among the EAEC collections: 63.6 % of the strains harbored *pic* and 38.2 % carried *sepA*. Pic, originally described in strains of *S. flexneri* 2a, has been shown to cleave submaxillary mucin [22], and our data suggest that Pic promotes intestinal colonization in a mouse model (S. Harrington and J. Nataro, unpublished). SepA, also originally reported in *S. flexneri* 2a, may promote intestinal inflammation induced by *Shigella* strains, but its mode of action has not been described [23].

We hypothesized that the distribution of SPATE proteases would correlate with that of the AAF adhesins and/or with EAEC phylogeny as previously published [27]. All five strains positive for the AAF/II pilin also carried the gene encoding Pet; none of these strains carried genes encoding Sat or SigA. Interestingly, these five Pet-encoding strains were distributed into three distantly related clusters on the EAEC phylogram, suggesting either horizontal co-transmission of AAF/II with Pet-encoding genes, or functional linkage of these factors. In contrast, strains harboring *aggA* (encoding the pilin of AAF/I) or *aag4A* (the pilin of AAF/IV, also called Hda) were commonly found to encode *sat* or *sigA* but not *pet*.

SigA was first reported to be encoded on a large chromosomal pathogenicity island in *S. flexneri*, which also encoded the Pic protease. In our collection, four EAEC strains harbored *sigA*; two of the four also carried *pic*. In contrast, 33 strains carried *pic* but not *sigA*. These data suggest that the linkage of *pic* and *sigA* as originally described is uncommon. No other correlations among toxins or adhesins were observed and no correlation of toxin genes with the phylogenetic clusters was apparent.

To facilitate later analysis for the five SPATEs commonly found in EAEC strains (*pet, sat, sigA, pic and sepA*), we developed a multiplex PCR. The multiplex assay yielded clear products at the appropriate mass in all relevant controls, with no erroneous bands detected. All EAEC strains positive for these genes by monoplex were re-tested with the multiplex, revealing perfect correlation between the multiplex and monoplex PCR assays. A representative image of

multiplex PCR products derived from a subset of the EAEC and *Shigella* strains is shown in Fig. 3. Use of this assay as a tool for prospective epidemiologic analysis to address the potential role of SPATEs in human disease will be reported elsewhere.

We performed our multiplex PCR analysis on sets of ETEC, EHEC, EPEC, EIEC, DAEC, *Shigella* and non-pathogenic *E. coli* strains (Table 2). The prevalence of the genes varied markedly by pathotype. In contrast to EAEC, we did not find Pic, SepA, Sat, SigA or Pet-encoding genes among any of the 10 ETEC strains (Table 2). Similarly, only two of 12 non-pathogenic *E. coli* strains were positive for any of these five SPATEs (one for *sat* and one for *sigA*; Table 2). One EPEC strain was positive for Pic, two EHEC strains were positive for Sat and either Pic or SigA, three DAEC strains harbored Sat and one Pic (Table 2). Previous studies have reported the presence of *sat* in the diarrheagenic *E. coli* pathotypes DAEC, EHEC and EPEC [32, 36]. Since several of the SPATEs have previously been reported among *Shigella* strains [22, 23, 37, 38], we subjected 12 clinical *Shigella* isolates (11 *S. flexneri* and one *S. sonnei*) to our multiplex PCR assay. As we observed in EAEC strains, most of the *Shigella* strains harbored more than one SPATE protein (Fig. 3). Whereas none of the *Shigella* strains harbored the *pet* gene, all were positive for *sat* and/or *sigA*, with four strains harboring both genes. Interestingly six out of the ten EIEC strains harbored SigA as the only SPATE protein. EIEC is distinguished from *Shigella* by biochemical tests, but EIEC strains and *Shigella* spp. share essential virulence factors [39, 40] Thus it is plausible that SigA plays an important role in the pathogenesis of both EIEC and *Shigella*.

In our hands, the cytotoxic SPATE proteins were found almost exclusively among microbial pathogens that have been shown to induce mucosal damage and inflammation. Clinically, *Shigella* are more virulent in this regard than EAEC, and they commonly carried more than one cytoxic SPATE toxin. Whether the SPATE toxins exhibit primary or secondary roles in pathogenesis by *Shigella* and EAEC is under further investigation in our laboratory.

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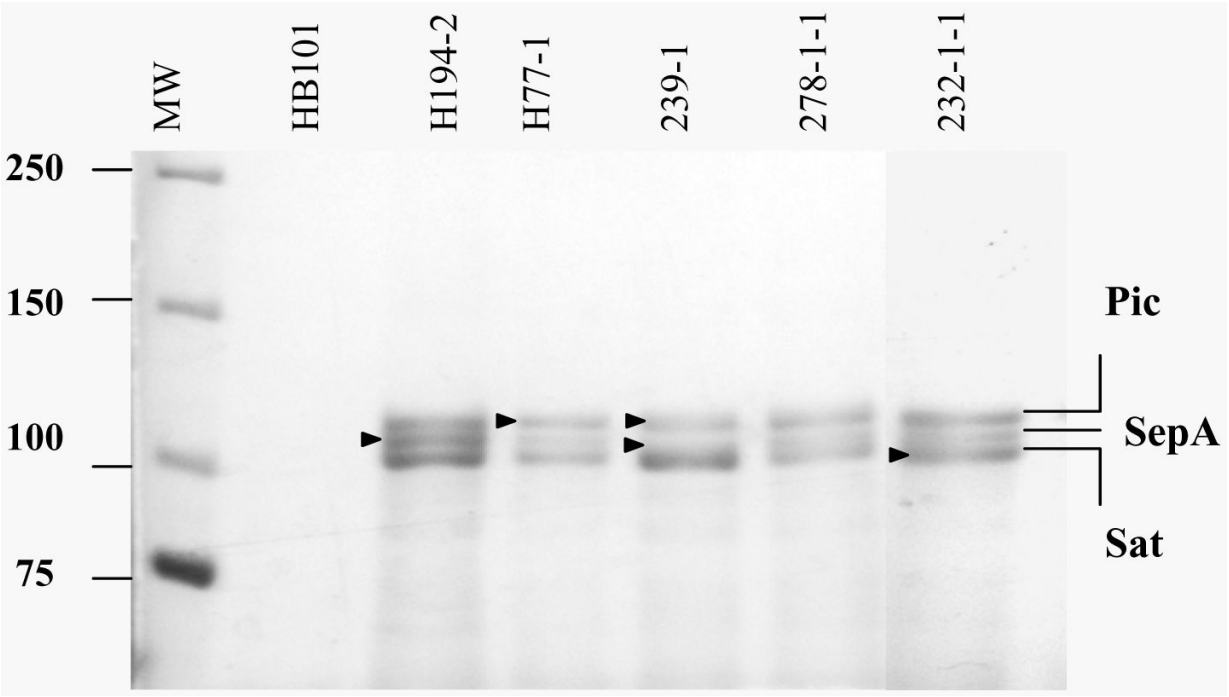

Figure 1. Secretion of SPATE proteins from EAEC strains

Supernatants from 5 representative EAEC strains are shown. Supernatant proteins were precipitated with 10% TCA and separated by SDS-PAGE followed by Coomassie Staining. SPATE proteins represented by bands directly identified by mass spectrometry were SepA from strains H194-2 and 239-1; Pic from strains H77-1 and 239-1; Sat from strain 232-1-1 (indicated by arrows). MW, Molecular weight; HB101, negative control strain.

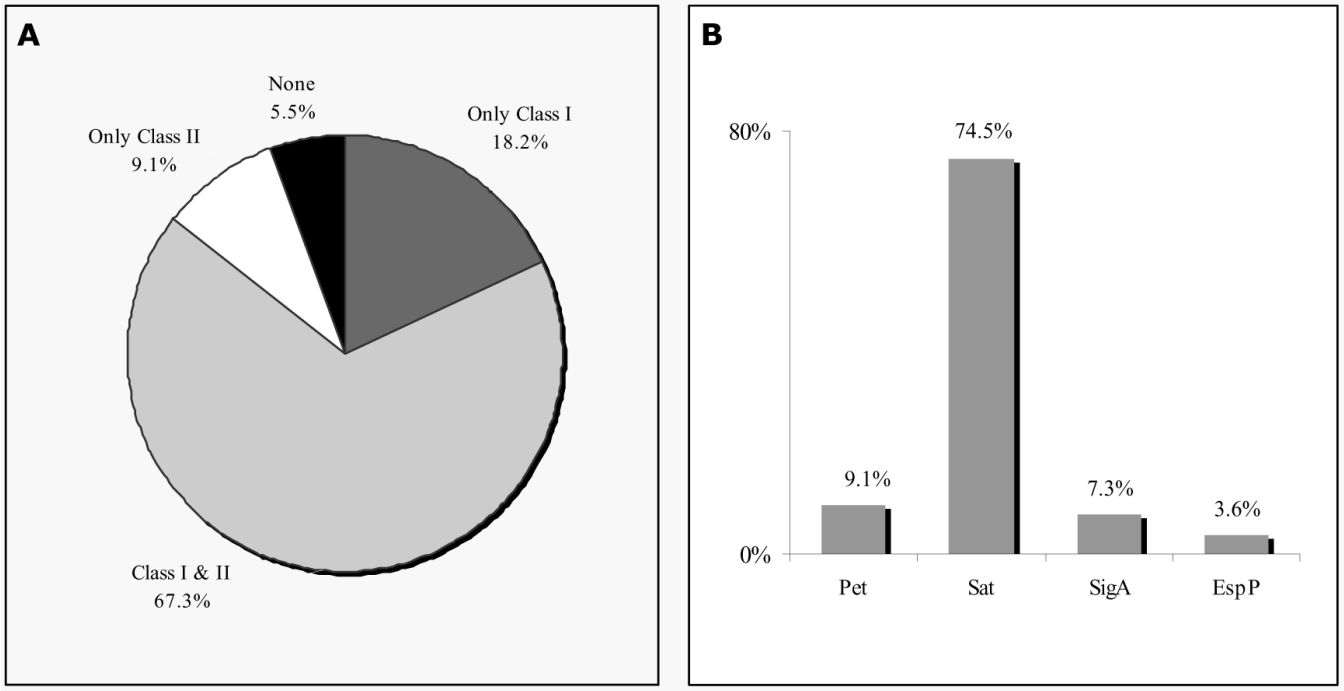

Figure 2. Distribution of Class I and Class II SPATEs among EAEC strains

The distribution of the SPATE genes among 55 EAEC strains was determined by PCR. (A) Distribution of Class-I and Class-II SPATEs among the EAEC collection. (B) Frequency of Pet, Sat, SigA, and EspP.

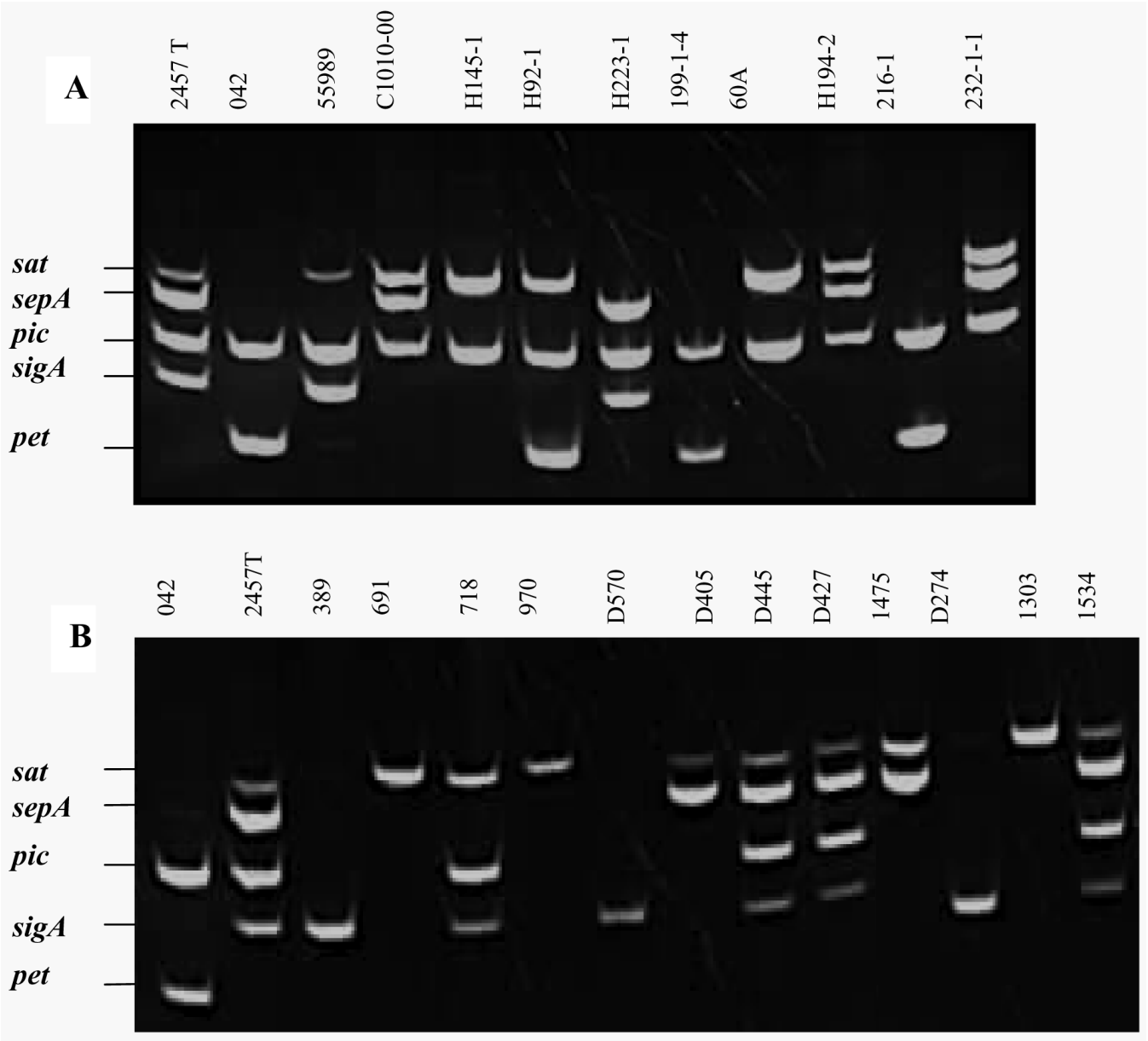

Figure 3. Identification of SPATE sequences from EAEC and *Shigella* isolates by multiplex PCR

PCR products representing genes for Sat, SepA, Pic, SigA and Pet were separated in 1.2% agarose gels. (A) PCR products from 11 EAEC strains (B) PCR products from 12 *Shigella* strains. EAEC strain 042 is the positive control for *pet* and *pic*; *S. flexneri* 2a strain 2457T is the positive control for *sat*, *sepA*, *pic* and *sigA*.

Table 1

Fifty-five EAEC strains used in this study including, origin, serotype and monoplex PCR results.

				SPATE proteins					Aggregative Adherence Fimbriae (AAF)			
				Class I			Class II		AAF/I	AAF/II	AAF/III	AAF/IV
Species	**Strain**	**Origin**	**Serotype**	***pet***	***sat***	***sigA***	***pic***	***sepA***	***aggA***	***aafA***	***agg3A***	***agg4A***
EAEC	**144-1-1**	Thailand (H)	O36:H18	−	+	−	+	−	−	−	−	+
	6-1-1	Thailand (H)	ORough:H2	−	+	−	−	−	−	−	−	+
	199-1-4	Thailand (H)	ORough:H1	+	−	−	+	−	−	+	−	−
	103-1-1	Thailand (H)	O148:H28	−	+	−	−	−	−	−	−	−
	44-1-1	Thailand (H)	O77:H−	−	+	−	−	−	−	−	−	−
	435-1-1[a]	Thailand (H)	O33:H16	−	−	−	+	+	−	+	−	−
	501-1-1	Thailand (H)	O148:H53	−	+	−	−	+	−	−	−	+
	253-1-1	Thailand (H)	O3:H2	−	+	−	−	−	+	−	−	−
	309-1-1	Thailand (H)	O130:H27	−	+	−	+	−	+	−	−	+
	133	Peru (H)	ONT:H1	−	+	−	+	−	−	−	−	+
	H46-2	Peru (H)	O130:H26	−	+	−	+	−	+	−	−	+
	H92-1	Peru (H)	O33:H16	+	+	−	+	−	−	−	−	+
	H32-1a	Peru (H)	O15:H−	−	+	−	+	+	−	−	−	+
	H223-1 [b]	Peru (H)	O101:H33	−	−	+	+	+	−	−	−	+
	H232-1	Peru (H)	O20:H1	−	+	−	+	−	−	−	−	+
	H145-1	Peru (H)	O130:H27	−	+	−	+	−	+	−	−	+
	191-1	Peru (H)	O101:H9	−	+	−	+	+	−	−	−	−
	H38-1	Peru (H)	O15:H−	−	+	−	−	+	+	−	−	−
	H32-1b	Peru (H)	O73:H16	−	+	+	−	+	+	−	−	−
	DS65-R3	Philipines (H)	O103:H43	−	+	+	−	+	+	−	−	−
	DS67-R2	Philipines (H)	O25:H4	−	+	−	−	+	−	−	−	+
	DS244- R3	Philipines (H)	O103:H43	+	−	−	+	−	−	+	−	+
	60A[c]	Mexico (H)	O20:H+	−	+	−	+	−	−	−	−	−
	H194-2[c]	Peru (H)	O175:H27	−	+	−	+	+	−	−	−	+
	H77-1	Peru (H)	O92:H33	−	+	−	+	+	−	−	−	−
	239-1	Thailand (H)	ORough:H21	−	+	−	+	+	−	−	−	−

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				SPATE proteins					Aggregative Adherence Fimbriae (AAF)			
				Class I			Class II		AAF/I	AAF/II	AAF/III	AAF/IV
Species	**Strain**	**Origin**	**Serotype**	***pet***	***sat***	***sigA***	***pic***	***sepA***	***aggA***	***aafA***	***agg3A***	***agg4A***
	278-1-1	Thailand (H)	O125ac:H21	−	+	−	+	+	−	−	−	+
	216-1	Thailand (H)	ORough:H27	+	−	−	+	−	−	+	−	
	495-1	Thailand (H)	O130:H27	−	+	−	+	−	+	−	−	+
	232-1-1	Thailand (H)	O127:H21	−	+	−	+	+	−	−	−	+
	99-1	Japan (H)	O5:H10	−	+	−	+	+	−	−	−	−
	96-1	Japan (H)	O5:H10	−	+	−	+	+	−	−	−	−
	86-1	Japan (H)	O5:H10	−	+	−	−	−	−	−	−	+
	101-1	Japan (H)	O36:H10	−	−	−	−	−	−	−	−	+
	C585-00 [*]	Denmark (H)	O86:H−	−	+	−	−	−	+	−	−	−
	C790-00	Denmark (H)	O+:H−	−	−	−	−	−	−	−	−	−
	C809-00	Denmark (H)	O86:H1	−	+	−	−	−	+	−	−	−
	C1010-00 [d]	Denmark (H)	ORough:H−	−	+	−	+	+	−	−	−	+
	C1046-00	Denmark (H)	O44:H18	−	+	−	−	−	+	−	−	−
	C1059-00	Denmark (H)	O73:K5:H18	−	−	−	+	+	−	−	−	+
	C1082-00	Denmark (H)	O107, O117:H32	−	−	−	−	−	−	−	−	+
	C246-01	Denmark (H)	O34:H9	−	−	−	−	+	−	−	−	+
	C247-01 [*]	Denmark (H)	ONT:H−	−	−	−	−	−	−	−	−	−
	C252-01	Denmark (H)	O111:H21	−	+	−	+	+	−	−	−	−
	C254-01	Denmark (H)	ONT:H10	−	−	−	+	−	−	−	−	+
	C390-01	Denmark (H)	O134:H27	−	+	−	+	−	+	−	−	−
	C763-01	Denmark (H)	O153:H2	−	+	−	+	−	+	−	−	−
	C798-01	Denmark (H)	O92:H33	−	+	−	+	−	+	−	−	−
	C1239-01	Denmark (H)	O86:H18	−	+	−	+	−	+	−	−	−
	C1275-01	Denmark (H)	O91:H1	−	−	−	−	+	+	−	−	−
	C274-02	Denmark (H)	O86:H2	−	+	−	+	−	+	−	−	−
	JM221	Mexico (H)	O92:K-:H33	−	+	−	+	−	+	−	−	−
	17-2	Serbia (H)	O3:H2	−	+	−	−	−	+	−	−	−

				SPATE proteins					Aggregative Adherence Fimbriae (AAF)			
				Class I			Class II		AAF/I	AAF/II	AAF/III	AAF/IV
Species	**Strain**	**Origin**	**Serotype**	***pet***	***sat***	***sigA***	***pic***	***sepA***	***aggA***	***aafA***	***agg3A***	***agg4A***
	042	Peru (H)	O44:H18	+	−	−	+	−	−	+	−	−
	55989	CAR (H)	O104:H4	−	+	+	+	−	−	−	+	−

ONT, O not typeable; H+, motile but not typeable; H−, non motile; (H), human origin; (A)

*Strains positive for EspP

[a]EAEC strain 435-1 was found probe positive for Pet in a previous study [27]

[b]EAEC strain H223-1 was found probe negative for SigA in a previous study [27]

[c]EAEC strains 60A and H194-2 were found probe positive for AggA in a previous study [27]

[d]Reported in a previous study as ORough:H1 41

Table 2

Shigella, ETEC and non-pathogenic *E. coli* strains used in this study.

				SPATE proteins				
				Class I			Class II	
Species	**Strain**	**Origin**	**Serogroup/type**	***pet***	***sat***	***sigA***	***pic***	***sepA***
Shigella	**389**	Chile (H)	*S. flexneri* 6	−	−	+	−	−
	691	Chile (H)	*S. flexneri* 1b	−	+	−	−	−
	718	Chile (H)	*S. flexneri* 2a	−	+	+	+	−
	970	Chile (H)	*S. flexneri* 2b	−	+	−	−	−
	D570	Chile (H)	*S. sonnei*	−	−	+	−	−
	D405	Chile (H)	*S. flexneri* 3a	−	+	−	−	+
	D445	Chile (H)	*S. flexneri* 2a	−	+	+	+	+
	D427	Chile (H)	*S. flexneri* 2a	−	+	+	+	+
	1427	Chile (H)	*S. flexneri* 2b	−	+	−	−	+
	D274	Chile (H)	*S. flexneri* 6	−	−	+	−	−
	1303	Chile (H)	*S. flexneri* 1b	−	+	−	−	−
	1534	Chile (H)	*S. flexneri* 2a	−	+	+	+	+
ETEC	**24337/A**	(H)	ND	−	−	−	−	−
	1392/752A	(H)	ND	−	−	−	−	−
	B7A-7318	(H)	ND	−	−	−	−	−
	H10407	(H)	ND	−	−	−	−	−
	E11881D	(H)	ND	−	−	−	−	−
	E11881A	(H)	ND	−	−	−	−	−
	E11881L	(H)	ND	−	−	−	−	−
	214-4	(H)	ND	−	−	−	−	−
	60R75	(H)	ND	−	−	−	−	−
	TD225	(H)	ND	−	−	−	−	−
EHEC *	**84-067**	(H)	O126:K7:H8	−	−	−	−	−
	83-377	(H)	O2:K1:H1	−	−	−	−	−

				SPATE proteins				
				Class I			Class II	
Species	**Strain**	**Origin**	**Serogroup/type**	***pet***	***sat***	***sigA***	***pic***	***sepA***
	83-378	(H)	O1:K1:H−	−	+	+	−	−
	83-408	(H)	O145:H−	−	−	−	−	−
	86-420	(H)	O6:H1	−	+	−	+	−
	10433-88	(H)	O157:H7	−	−	−	−	−
	8350-86	(H)	O157:H7	−	−	−	−	−
	8504-86	(H)	O157:H7	−	−	−	−	−
	8868-86	(H)	O157:H7	−	−	−	−	−
	23379-85	(H)	O157:H7	−	−	−	−	−
EPEC	**E2348/69**[42]	England (H)	O127:K63:H6	−	−	−	−	−
	50869	(H)	O114	−	−	−	−	−
	1	(H)	ND	−	−	−	−	−
	6	(H)	O55	−	−	−	+	−
	7	(H)	O111	−	−	−	−	−
	9	(H)	O55	−	−	−	−	−
	10	(H)	O119	−	−	−	−	−
	11	(H)	O55	−	−	−	−	−
	13	(H)	O111	−	−	−	−	−
	18	(H)	O119	−	−	−	−	−
EIEC	**E134**	(H)	O28ac:H−	−	−	+	−	−
	439-1	Thailand (H)	O114:H25	−	−	−	−	−
	705-1	Thailand (H)	O114:H25	−	−	−	−	−
	949-1	Thailand (H)	O114:H25	−	−	−	−	−
	1109-1	Thailand (H)	O170:H−	−	−	−	−	−
	205-1	Thailand (H)	O170:H−	−	−	+	−	−
	145-1	Thailand (H)	O164:H−	−	−	+	−	−
	905-2	Thailand (H)	O28ac:H−	−	−	+	−	−

				SPATE proteins				
				Class I				Class II
Species	**Strain**	**Origin**	**Serogroup/type**	***pet***	***sat***	***sigA***	***pic***	***sepA***
	164-3	Thailand (H)	O28ac:H−	−	−	+	−	−
	183-4	Thailand (H)	O28ac:H−	−	−	+	−	−
DAEC	**C1845**[43]	(H)	O75:H−	−	+	−	−	−
	DS55R3	(H)	ND	−	−	−	−	−
	DS112R3	(H)	ND	−	−	−	−	−
	DS126R1	(H)	ND	−	−	−	−	−
	1	Mexico (H)	ND	−	+	−	−	−
	4	Mexico (H)	ND	−	−	+	−	−
	5	Mexico (H)	ND	−	−	−	−	−
	17	Mexico (H)	ND	−	−	−	−	−
	18	Mexico (H)	ND	−	+	−	−	−
	20	Mexico (H)	ND	−	−	−	−	−
Non-pathogenic *E. coli*	**C 123-01** [a]	Denmark (H)	O73:H18	−	+	−	−	−
	C 129-01 [a]	Denmark (H)	O8:H9	−	−	−	−	−
	C 130-01 [a]	Denmark (H)	O81:H27	−	−	+	−	−
	C 131-01 [a]	Denmark (H)	O9:H19	−	−	−	−	−
	C 133-01[a]	Denmark (H)	O125ab:H4	−	−	−	−	−
	C 134-01[a]	Denmark (H)	O40:H−	−	−	−	−	−
	C 107-01[b]	Denmark (MM)	O129:H31	−	−	−	−	−
	C 108-01[b]	Denmark (MM)	O35:H29	−	−	−	−	−
	C 109-01A[b]	Denmark (MM)	O150:H8	−	−	−	−	−
	C 109-01B[b]	Denmark (MM)	O140:H21	−	−	−	−	−
	C 110-01[b]	Denmark (MM)	O25:H28	−	−	−	−	−
	C 111-01[b]	Denmark (MM)	O32:H37	−	−	−	−	−

ONT, O not typeable; H+, motile but not typeable; H−, non motile; ND, not determined; (H), human origin

*All EHEC strains positive for Shiga toxin (Stx); (MM) Minced meat

[a]Non-pathogenic *E. coli* from healthy humans isolated as part of the Danish antimicrobial resistance surveillance program DANMAP

[b]Non-pathogenic *E. coli* from minced meat isolated as part of the Danish food surveillance program.

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Table 3

Primers used for monoplex PCR and multiplex PCR

Factor class	Gene	Primer (pmol/μL)	Primer sequence (5′-3′)	PCR product in bp [a]	Accession no.[b]	Annealing temp	Reference
SPATE-gene	*sat*	25	TCAGAAGCTCAGCGAATCATTG CCATTATCACCAGTAAAACGCACC	930	AE014075	59 °C	This study
	sigA	25	CCGACTTCTCACTTTCTCCCG CCATCCAGCTGCATAGTGTTTG	430	NC_004337	58 °C	This study
	pet	25	GGCACAGAATAAAGGGGTGTTT CCTCTTGTTTCCACGACATAC	302	AF056581	58 °C	44
	pic	25	ACTGGATCTTAAGGCTCAGGAT GACTTAATGTCACTGTTCAGCG	572	AF097644	58 °C	44
	sepA	25	GCAGTGGAAATATGATGCGGC TTGTTCAGATCGGAGAAGAACG	794	Z48219	58 °C	44
	tsh	25	CCGTACACAAATACGACGG GGATGCCCCTGCAGCGT	900	AF218073	59 °C	44
	vat	25	AACGGTTGGTGGCAACAATCC AGCCCTGTAGAATGGCGAGTA	420	AY151282	58 °C	44
	eatA	25	CAGGAGTGGGAACATTAAGTCA CGTACGCCTTTGATTTCAGGAT	743	AY163491	60 °C	44
	espP	25	GTCCATGCAGGGACATGCCA TCACATCAGCACCGTTCTCTAT	547	NC_002128	60 °C	44
	espC	25	TAGTGCAGTGCAGAAAGCAGTT AGTTTTCCTGTTGCTGTATGCC	839	AF297061	59 °C	44
AAF gene	*aggA*	25	AAATATGAGAAGAAAGAA AAAAATTAATTCCGGTATGG	500	ECU12894	50 °C	41
	aafA	25	CAGAATGTTTGCGATTGCTAC TTTGTCACAAGCTCAGCATT	468	AF012835	50 °C	41
	agg3A	25	GTATCATTGCGAGTCTGGTATTCAG GGGCTGTTATAGAGTAACTTCCAG	462	AF411067	50 °C	45
	agg4A	25	TCCATTATGTCAGGCTGCAA GGCGTTAACGTCTGATTTCC	411	EU637023	59 °C	41

[a] bp, Base pair

[b] GenBank database accession number for the SPATE and fimbrial genes