Serine protease autotransporters of Enterobacteriaceae (SPATEs) are largely distributed among *Escherichia coli* isolated from the bloodstream

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Abstract

Extraintestinal pathogenic *Escherichia coli* (ExPEC) is the major cause of Gram-negative-related sepsis. Bacterial survival in the bloodstream is mediated by a variety of virulence traits, including those mediating immune system evasion. Serine protease autotransporters of Enterobacteriaceae (SPATE) constitute a superfamily of virulence factors that can cause tissue damage and cleavage of molecules of the complement system, which is a key feature for the establishment of infection in the bloodstream. In this study, we analyzed 278 *E. coli* strains isolated from human bacteremia from inpatients of both genders, different ages, and clinical conditions. These strains were screened for the presence of SPATE-encoding genes as well as for phylogenetic classification and intrinsic virulence of ExPEC. SPATE-encoding genes were detected in 61.2% of the strains and most of these strains (44.6%) presented distinct SPATE-encoding gene profiles. *sat* was the most frequent gene among the entire collection, found in 34.2%, followed by *vat* (28.4%), *pic* (8.3%), and *tsh* (4.7%). Although in low frequencies, *espC* (0.7%), *eatA* (1.1%), and *espI* (1.1%) were detected and are being reported for the first time in extraintestinal isolates. The presence of SPATE-encoding genes was positively associated to phylogroup B2 and intrinsic virulent strains. These findings suggest that SPATEs are highly prevalent and involved in diverse steps of the pathogenesis of bacteremia caused by *E. coli*.

Keywords SPATE · Virulence factors · *Escherichia coli* · Bacteremia

Introduction

Extraintestinal pathogenic *Escherichia coli* (ExPEC) strains are usually present in the intestinal microbiota of humans and warm blood animals without causing disease. However, ExPEC is a major cause of urinary tract infections (UTI),

neonatal meningitis, and Gram-negative-related sepsis [1–4]. Accordingly, they belong to phylogroups B2 and D, which are known to be highly virulent [1].

Bacteremia usually results from two main primary sources. The first and most common is the urinary tract, involving uropathogenic *E. coli* (UPEC), especially when they ascend causing pyelonephritis. The second is the digestive tract, where ExPEC present in the intestines may find its way to the bloodstream either by active translocation or by mechanic rupture of the intestinal barrier [2, 5]. Once established, bacteremia may progress to sepsis or septic shock [6].

To survive in the bloodstream, ExPEC makes use of a variety of virulence traits, most of them originated from horizontal gene transfer. These traits encompass adhesins, invasins, protectins, iron acquisition systems, and toxins that allow the bacteria to reach and survive in the bloodstream by evading from host immune defense mechanisms. Virulence factors, such as polysaccharide capsules and complement resistance mediators (encoded by genes such as *kpsMTII*, *traT*, and *iss*), allow the bacteria to subvert mechanisms such as opsonization and phagocytosis by avoidance of macrophages

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recognition and cleavage of molecules of the complement system [2, 7]. Proteins involved in these evasion mechanisms have been identified in the past few years, including members of the serine protease autotransporters of Enterobacteriaceae (SPATE) superfamily [8, 9].

SPATE constitutes a superfamily of virulence factors secreted by the type V secretion system. These proteases have been classified into two distinct classes according to the amino acid sequence and characteristics of their passenger domains. Class 1 is known by its cytotoxic activities and class 2 by its immunomodulatory activities [10].

Sat and Vat are examples of cytotoxic SPATEs, first described in UPEC and APEC (avian pathogenic *E. coli*) strains, respectively. They are known to be involved in the host/pathogen interface, facilitating the entry of the bacteria into the bloodstream by causing tissue damage [11–13]. EspP, also a class 1-SPATE, is an important virulence factor of enterohemorrhagic *E. coli* (EHEC), presenting a role in the cytotoxic effects, adherence, and biofilm formation. Despite being usually found in diarrheagenic *E. coli* (DEC), EspP is also capable of cleaving molecules of the complement system, worsening the severity of the hemolytic-uremic syndrome caused by EHEC [9]. A similar situation involves Pic. This class 2-SPATE is a key virulence factor during enteroaggregative *E. coli* (EAEC) pathogenesis and its capability of complement cleavage has also been shown [8, 14].

Published surveys about the frequency of SPATE-encoding genes in ExPEC isolated from bacteremia tend to be limited to some genes that are more common in this group of pathogens, such as *sat*, *vat*, and *tsh* [2, 3, 5, 15–18], leaving behind those SPATEs that have been associated to DEC pathotypes. An exception is a study performed in India [19] that has assessed the prevalence of a diverse number of SPATEs in a collection of *E. coli* strains from neonatal septicemia isolated in India. Considering the importance of secreted serine proteases in the pathogenesis of ExPEC [20] and the limited data on their prevalence in ExPEC, the aim of this study was to assess the frequencies of all the characterized SPATE-encoding genes in a collection of *E. coli* isolated from Brazilian patients with bacteremia. We also aimed to associate this prevalence with phylogroup distribution and intrinsic virulence.

Materials and methods

Bacterial strain and growth conditions

We analyzed 278 *E. coli* strains isolated from human bacteremia [21, 22]. These strains were obtained from inpatients of both genders, various ages, and clinical conditions admitted to Hospital São Paulo (a tertiary hospital in São Paulo city, São Paulo, Brazil) in the period from 2000 to 2008. Primary isolation and identification were carried out in the hospital laboratory. These strains are part of the bacterial collection

ENTEROBACTERIALES-EXTRAINTESTINAL-EPM-DMIP maintained by the Disciplina de Microbiologia, Escola Paulista de Medicina, Universidade Federal de São Paulo. Bacterial stocks were stored at -80°C and reactivated by growth in tryptic soy broth and onto MacConkey agar plates. All cultures were incubated at 37°C for 18 h.

Polymerase chain reactions

Polymerase chain reaction (PCR) was used to detect the following SPATE-encoding genes: *eatA*, *epeA*, *espC*, *espI*, *espP*, *pet*, *pic*, *sat*, *sepA*, *sigA*, *tsh*, and *vat*. The specific primers, cycle conditions, sizes of amplified fragments, and controls are described in Table 1, respectively. Amplification was performed in a total volume of 25 μL containing 40 pmol of each primer; dATP, dTTP, dCTP, and dGTP (0.1 mM each); 1.5 U Taq DNA polymerase (Invitrogen); 5.0 μL 10 $\times$ PCR buffer (Invitrogen); MgCl_2 (1.5–2 mM); and 1.0 μL of DNA template, prepared by boiling a colony from culture on Luria-Bertani (LB) agar mixed with 500 μL of water during 10 min. The PCR products were analyzed by agarose gel electrophoresis (0.7–2%, according to the size of the products).

The phylogenetic classification was determined by the quadruplex PCR to detect *chuA*, *yjaA*, TspE4.C2, and *arpA*, followed by complementary reactions if needed, according to the revisited Clermont method [29].

DNA-DNA colony hybridization

The assessment of intrinsic virulence [30] was performed by DNA-DNA colony hybridization detecting the genes *afaBCIII*, *papA* and *papC*, *sfaDE*, *iucD*, and *kpsMT II*, using the probe fragments obtained by PCR (Supplementary Table 1). The probe fragments were purified and radiolabeled as described previously [31] and hybridization assays were carried out using high stringency conditions [22]. This assay was performed with 219 strains of our collection, since 60 strains have been previously characterized by this criterion [22].

Statistical analyses

Statistical analyses were performed using χ^2 and Fisher's exact tests.

Results

The initial screening of the 278 *E. coli* strains detected 170 (61.2%) strains harboring at least one SPATE-encoding gene (SPATE⁺). Individual analysis showed that *sat* was the most frequent gene found in 95 (34.2%) strains, followed by *vat* (79 strains/28.4%); *pic* (23 strains/8.3%); *tsh* (13 strains/4.7%);

Table 1 Primers, PCR conditions, and controls for detection of SPATE-encoding genes

Gene	Primer sequences (5'–3')	Annealing temperature (°C)	Amplicon (bp)	Positive control ^a	Reference
<i>espI</i>	(F) ATGGACAGAGTGGAGACAG (R) GCCACCTTATTCTCACCA	54	560	EHEC EH250	[23]
<i>espC</i>	(F) GACAGTTTACGTTAGCTGG (R) TCCTGCCGAAAACCGAAGC	55	1099	EPEC E2348/69	[24]
<i>espP</i>	(F) GTCCATGCAGGGACATGCCA (R) TCACATCAGCACCGTTCTCTAT	55	547	EHEC EDL933	[25]
<i>sepA</i>	(F) GGTGTGATGTTTCTATTATGAACA (R) CGCATCATATTCCACTG	55	1031	<i>Shigella flexneri</i> M90T	This study (GenBank accession number: Z48219.1)
<i>pic</i>	(F) GGAAGTGACAGGGCATTTG (R) CTTCGTATTGCCACCACTG	56	1011	EAEC 042	[26]
<i>eatA</i>	(F) TTGGCGTTCTGTCATCAGGA (R) CCTTACCAGAGAGTGGATG	57	1068	EAEC BA179	[24]
<i>epeA</i>	(F) GGGAGAGTTCAGGCATTTA (R) CAGCGTTACCTTACTTGAG	57	783	<i>E. coli</i> EH41	[27]
<i>pet</i>	(F) GGCACAGAATAAAGGGGTGTTT (R) CCTCTGTGTTCCACGACATAC	58	302	EAEC 042	[25]
<i>sigA</i>	(F) CCGACTTCTCACTTTCTCCCG (R) CCATCCAGCTGCATAGTGTGTTG	58	430	<i>Shigella flexneri</i> M90T	[28]
<i>vat</i>	(F) AACGGTTGGTGGCAACAATCC (R) AGCCCTGTAGAATGGCGAGTA	58	420	<i>E. coli</i> RS218	[25]
<i>tsh</i>	(F) CCGTACACAAATACGACGG (R) GGATGCCCTGCAGCGT	59	300	APEC O1	[25]
<i>sat</i>	(F) TCAGAAAGCTCAGCGAATCATTG (R) CCATTATCACCAGTAAAACGCACC	59	930	DAEC FBC114	[28]

^a EHEC enterohemorrhagic *E. coli*, EPEC enteropathogenic *E. coli*, EAEC enteroaggregative *E. coli*, APEC avian pathogenic *E. coli*, DAEC diffusely adherent *E. coli*

espP (5 strains/1.8%); *espI*, *eatA*, *sepA*, and *sigA* (3 strains/1.1% each); *espC* (2 strains/0.7%); and *pet* (1 strain/0.4%), while *epeA* was not detected in any of the strains (Fig. 1). Among the 170 SPATE-positive strains, 124 (72.9%) harbored only one SPATE gene and the remaining strains presented several distinct gene profiles (Table 2).

Considering the phylogenetic analysis of the entire *E. coli* collection, the predominant phylogroup was B2 (103 strains/37.1%), followed by phylogroups E (43 strains/15.5%), B1 (38 strains/13.7%), A (33 strains/11.9%), D (23 strains/8.3%), F (22 strains/7.9%), and C (9 strains/3.2%). Seven strains (2.5%) were not classified in any phylogroup (unknown phylogroup). In general, the presence of SPATE-encoding genes was statistically more frequent among phylogroup B2 than in non-B2 phylogroups ($p < 0.0001$). A negative association between the presence of these genes and strains belonging to phylogroups A and B1 ($p < 0.001$ and $p < 0.0001$, respectively) was observed (Fig. 2a). Phylogroups B2, E, F, and D were more frequent among the 170 SPATE-positive strains, although the presence of SPATE-encoding genes in phylogroups D, E, and F was not statistically significant ($p > 0.05$).

The presence of at least two genes among *afa/dra*, *papA* and/or *papC*, *sfa/foc*, *iucD/iutA*, and *kpsMT II* indicates that an *E. coli* strain has the potential to cause disease in a healthy subject and can be classified as ExPEC [30]. Following this criterion, 173 (62.2%) strains of our collection were considered as ExPEC. As shown in Fig. 2b, SPATE⁺/ExPEC were more frequent than SPATE⁺/non-ExPEC strains ($p < 0.0001$).

A description of all SPATE genes found in each strain, according to their ExPEC and phylogroups classification, is presented in Supplementary Table 2.

Discussion

SPATEs are important virulence factors with cytotoxic and immunomodulatory activities that have been extensively studied in different pathotypes of *E. coli* [10]. However, the survey for SPATE-encoding genes in ExPEC isolates in previous works was inserted in a general virulence factor analysis context and usually focused on ExPEC-related SPATEs genes. For this reason, this work aimed to provide a comprehensive analysis of the frequency of all characterized SPATE-encoding genes in an ExPEC collection isolated from the blood.

Fig. 1 Individual SPATE-encoding gene frequencies among *E. coli* strains isolated from bacteremia

We found in our collection a high frequency of *sat* (34.2%), followed by *vat* and *pic*. In accordance, several authors have also reported *sat* in high frequencies among bacteremia strains, ranging from 26.7 to 70% [2, 3, 5, 15–18]. However, only three works reported the presence of *vat* in *E. coli* isolated from bacteremia, ranging from 51.4 to 70%, higher frequencies than the found in our collection (28.4%) [16, 17, 19]. Only one study from India [19] reported a more complete survey about SPATEs in ExPEC strains from neonatal sepsis, finding similar results, as *vat* and *sat* were the most frequent genes followed by *espP*, *sepA*, *pet*, and *pic*.

Usually, most of the SPATE-encoding genes associated with intestinal *E. coli* pathotypes are not surveyed in ExPEC. *pic* is an exception, reported in lower frequency herein (8.3%) in comparison with other two studies [2, 19]. *espC*, *eatA*, and *espI* were also detected in our screening and to our knowledge, this is the first study to detect the presence of these genes in ExPEC strains. *EspC* is an enterotoxin first described in enteropathogenic *E. coli* [32] that is capable of cleaving hemoglobin and factor V of blood coagulation [33, 34]. *EatA* and *EspI* are members of the class of SPATEs with immunomodulatory activities, based on phylogenetic analysis of their passenger domains [10]. In enterotoxigenic *E. coli* (ETEC) strains, *EatA* has a role in the cleavage of a glycoprotein adhesin present in ETEC, which in turn increases the delivery of the heat-labile toxin [35]. *EspI* secreted by Shiga toxin-producing *E. coli* (STEC) causes degradation of plasma proteins, but its role in pathogenesis remains to be elucidated [23]. In a sepsis context, these virulence traits could be useful for iron acquisition during bacterial growth and intensifying

the coagulation disorders that may occur in the course of such pathology [6, 36].

Our group has previously published surveys on SPATE-encoding genes in two different collections of DEC [24, 27]. SPATEs that were first described in intestinal pathotypes, such as *pic*, *pet*, *sepA*, and *espC*, were significantly more frequent in these studies, in agreement with other authors [25, 37]. On the other hand, *sat* was the most frequent gene found in other two studies involving DEC strains isolated in different countries, but *pic* and *sepA* were also detected [28, 38]. These findings reinforce the evidence that some SPATEs are virulence factors that are usually associated with certain *E. coli* pathotypes.

It is well known that UTI are the main source for bloodstream infections and sepsis caused by *E. coli* [2, 36]. Therefore, some genetic similarity would be expected among strains isolated from UTI and sepsis. Although, as observed for ExPEC isolated from the bloodstream, most of the published studies comprise data on the prevalence of SPATE-encoding genes related to extraintestinal pathotypes such as *sat*, *vat*, and/or *tsh*, with only a few of them searching for *pic/picU* [39–42]. Considering all the available data in these studies, *sat* was the most prevalent gene in UPEC strains, followed by *vat*, *pic*, and *tsh*. Restieri et al. [25] performed a large screening of SPATE-encoding genes in UPEC, and the same prevalence profile mentioned above was observed, which is also in accordance with our results. Although SPATEs are known for their role in intestinal and uropathogenesis, it is possible that they also play a role in the early stages of the bloodstream infection, such as the acquisition of iron, immune

Table 2 Genetic profiles and phylogroups of *E. coli* strains isolated from bacteremia

No. (%) of strains in each phylogroup	SPATE-encoding genes	No. (%) of positive strains	No. (%) of ExPEC trains	No. (%) of non-ExPEC strains
A	<i>espI/pic/sepA</i>	1 (3.0)	1 (100)	
33 (11.9)	<i>sepA/sat</i>	1 (3.0)	1 (100)	
	<i>sat</i>	4 (12.1)	3 (75)	1 (25)
	<i>espP</i>	2 (6.1)		2 (100)
	<i>espI</i>	1 (3.0)	1 (100)	
	<i>sigA</i>	1 (3.0)		1 (100)
	None	23 (69.7)	4 (17.4)	19 (82.6)
B1	<i>pet/pic/sepA/sat</i>	1 (2.6)		1 (100)
38 (13.7)	<i>vat</i>	1 (2.6)	1 (100)	
	<i>tsh</i>	4 (10.5)	1 (25)	3 (75)
	<i>espC</i>	2 (5.3)		2 (100)
	<i>sigA</i>	1 (2.6)		1 (100)
	None	29 (76.3)	1 (3.4)	28 (96.6)
B2	<i>pic/sat/vat</i>	11 (10.7)	11 (100)	
103 (37.1)	<i>sat/vat</i>	18 (17.5)	18 (100)	
	<i>pic/vat</i>	9 (8.7)	8 (88.9)	1 (11.1)
	<i>tsh/vat</i>	3 (2.9)	3 (100)	
	<i>sat</i>	10 (9.7)	7 (70)	3 (30)
	<i>vat</i>	36 (35.0)	30 (83.3)	6 (16.7)
	<i>tsh</i>	2 (1.9)	1 (50)	1 (50)
	<i>espI</i>	1 (1.0)	1 (100)	
	None	13 (12.6)	11 (84.6)	2 (15.4)
C	<i>tsh</i>	2 (22.2)		2 (100)
9 (3.2)	None	7 (77.8)		7 (100)
D	<i>sat</i>	16 (69.6)	16 (100)	
23 (8.3)	None	7 (30.4)	1 (14.3)	6 (85.7)
E	<i>sigA/sat</i>	1 (2.3)	1 (100)	
43 (15.5)	<i>sat</i>	18 (41.9)	18 (100)	
	<i>tsh</i>	1 (2.3)		1 (100)
	<i>espP</i>	2 (4.7)		2 (100)
	<i>eatA</i>	3 (7.0)	2 (66.7)	1 (33.3)
	None	18 (41.9)	11 (61.1)	7 (38.9)
F	<i>pic/vat</i>	1 (4.5)		1 (100)
22 (7.9)	<i>sat</i>	14 (63.6)	14 (100)	
	<i>tsh</i>	1 (4.5)	1 (100)	
	<i>espP</i>	1 (4.5)	1 (100)	
	None	5 (22.7)	1 (20)	4 (80)
Unknown	<i>sat</i>	1 (14.3)	1 (100)	
7 (2.5)	None	6 (85.7)	3 (50)	3 (50)

Classification of *E. coli* as ExPEC or non-ExPEC based on the presence or absence, respectively, of at least two of the following genes: *afa/dra*, *papA* and/or *papC*, *sfa/foc*, *iutA*, and *kpsMT II* [30]

system evasion, and intensification of coagulation disorders [36].

The positive association observed between strains harboring SPATE-encoding genes and strains belonging to phylogroup B2 ($p < 0.0001$) was similar to the results obtained in India [19]. Accordingly, our work shows that the presence

of SPATE-encoding genes was negatively associated with strains belonging to phylogroups A and B1 ($p < 0.05$). These inferences and the high frequency of SPATE-positive strains in groups B2, D, E, and F, detected in our work, corroborate the association of the SPATE-encoding genes with highly virulent phylogroups. Concerning the intrinsic virulence, the

Fig. 2 Distribution of SPATE-encoding genes (%) according to **a** phylogroups classification, ****p* values < 0.001; *****p* values < 0.0001; **b** intrinsic virulence assessment, *****p* value < 0.0001

positive association between the presence of SPATE-encoding genes and ExPEC+ strains ($p < 0.0001$) was expected and is reinforced by the fact that 80% of these strains harbor at least one of these genes.

In our study, no associations or statistical significance were observed between the patients' characteristics, meaning demographics and underlying or chronic diseases, and the frequency of any SPATE-encoding gene. Nevertheless, the high prevalence of SPATEs among bacteremia strains suggests that these proteases may play an important role in the virulence potential of those strains prompting them to succeed beyond the primary site of infection. It is worth to note that sepsis is usually a secondary infection originated from other infected sites, and that the sepsis state is defined by a set of clinical parameters and manifestations [6].

Some interesting SPATE-profiles were observed in the analyzed strains. Those from phylogroup B2 showed the most diverse SPATE-profiles harboring up to three SPATE-encoding genes (Table 2). One strain (EC092) belonging to phylogroup B1 and not considered as an ExPEC [30] was found to harbor the genes *pet*, *pic*, *sepA*, and *sat*. The classification into phylogroup B1, the presence of *pet* and *pic* (SPATEs associated to EAEC), and the lack of genes associated with ExPEC suggested that such strain was in fact an intestinal pathotype. Considering the presence of *pet* and *pic* (SPATEs firstly described in EAEC) and the description of some bacteremia cases caused by EAEC [43], we investigated the presence of five EAEC specific genetic markers [44, 45] as well as the adherence pattern on HeLa cells [46]. In fact, EC092 harbors the following EAEC genes: *aggR*, *aata*, *aap*, and *aaiA-aaiG*, which encode respectively the master regulator AggR; one protein of a specific ATP-binding cassette transporter system—formerly known as the EAEC probe; dispersin; and two components of a type VI secretion system [47–49]. It is worth mentioning that this strain produces the aggregative adherence (AA) pattern on HeLa cells and production of Pet, Pic, Sat, and SepA was detected by immunoblotting of culture supernatant (data not shown).

pic was detected in four different associations (*espI-pic-sepA*, *pet-pic-sepA-sat*, *pic-sat-vat*, and *pic-vat*), mostly among strains classified as ExPEC and belonging to virulent phylogroups. It was recently demonstrated that this SPATE is capable of cleaving C3/C3b, C4, and C2 molecules of the complement system, reducing its activation [8, 26]. Considering that complement proteins are one main host defense against bacteria reaching the bloodstream, their cleavage is an important mechanism of immune evasion, raising the chances of bacterial survival in the blood.

The low frequency of the remaining genes *tsh*, *espP*, *eata*, *espI*, *sepA*, *sigA*, *espC*, and *pet* searched herein was expected, considering that they are associated with APEC and intestinal *E. coli* pathotypes, respectively [50]. In addition, most of the strains positive for only one of these genes were not classified as ExPEC and distributed among phylogroups that are characteristic of intestinal pathotypes. Since DEC and ExPEC are initially cohabiting the intestine, horizontal gene transfer events among them may have favored the acquisition of these genes by the bloodstream isolates.

Altogether, our data highlight that the high presence of SPATE-encoding genes is associated with the diversity of genetics attributes of *E. coli* isolated from bacteremia and that these serine proteases may be involved in different steps of the pathogenesis of sepsis, improving the ability of these strains to survive in the bloodstream.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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