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Assessing the diversity of the virulence potential of *Escherichia coli* isolated from bacteremia in São Paulo, Brazil

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Abstract

Most of the knowledge of the virulence determinants of extraintestinal pathogenic *Escherichia coli* (ExPEC) comes from studies with human strains causing urinary tract infections and neonatal meningitis and animal strains causing avian colibacillosis. In this research, we analyzed the phylogenetic background, the presence of 20 ExPEC virulence factors, and the intrinsic virulence potential of 74 *E. coli* strains isolated in São Paulo, Brazil, from 74 hospitalized patients (43 males and 31 females) with unknown-source bacteremia. Unlike other places in the world, the bacteremic strains originated equally from phylogroups B2 (35%) and D (30%). A great variability in the profiles of virulence factors was noted in this survey. Nevertheless, 61% of the strains were classified as ExPEC, meaning that they possessed intrinsic virulent potential. Accordingly, these strains presented high virulence factor scores (average of 8.7), and were positively associated with 12 of 17 virulence factors detected. On the contrary, the non-ExPEC strains, isolated from 39% of the patients, presented a generally low virulence capacity (medium virulence factor score of 3.1), and were positively associated with only the colicin *cvaC* gene. These results show the importance of discriminating *E. coli* isolates that possess characteristics of true pathogens from those that may be merely opportunistic in order to better understand the virulence mechanisms involved in extraintestinal *E. coli* infections. Such knowledge is essential for epidemiological purposes as well as for development of control measures aimed to minimize the incidence of these life-threatening and costly infections.

Key words: Bacteremia; Extraintestinal pathogenic *Escherichia coli*; Virulence factors; Intrinsic virulence potential; Phylogenetic background

Introduction

Progress has undoubtedly been achieved in the last decade in the knowledge of bacterial pathogenesis mechanisms and the complex molecular events involved in host-pathogen interactions. Even so, infectious diseases are still the cause of significant morbidity and mortality in both human beings and animals. Among the bacterial pathogens *Escherichia coli* is a facultative, anaerobic Gram-negative rod with many facets. As many as 90% of the *E. coli* strains are commensals inhabiting the intestinal tract of humans and warm-blooded animals, and rarely cause disease. Nevertheless, some strains are provided with a wide variety of virulence factors (VFs) that enable them to be the etiological agents of various intestinal or extraintestinal diseases (1). Phylogenetic analysis has classified *E. coli* strains into four main phylogenetic groups (A, B1, B2, and D). Most of the virulent extraintestinal human strains belong to phylogroup B2, and to a lesser extent to group D (2), while many poultry pathogens belong to phylogroup A (3). On the other hand, intestinal pathogenic *E. coli* are predominantly B1 strains. Commensal *E. coli*, usually included in groups A and B1, may in fact have originated from any of the phylogenetic groups, depending on the geographical region studied (4).

The ExPEC (extraintestinal pathogenic *E. coli*) designation was created with the purpose of including all the strains that were traditionally characterized by their site of isolation rather than as specific pathotypes. Thus, the ExPEC group brings together the uropathogenic *E. coli* (UPEC), the meningitis associated with neonates *E. coli* (MNEC), and the avian pathogenic *E. coli* (APEC), as well as strains associated with bacteremia and

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septicemia but which do not belong to any of those three categories (1,3).

The virulence potential of ExPEC is largely determined by the presence of VFs, many of which are clustered in pathogenicity islands spread out along the bacterial chromosome or on plasmids. Currently, there are numerous factors (1,3), some already recognized and others putative, which are considered to play a role in ExPEC pathogenesis by enabling specific functions such as mucosal colonization, avoidance and/or subversion of local and systemic host defenses, acquisition of nutritional requirements, damaging and/or invasion of host cells or tissues and, finally, stimulation of an inflammatory response beneficial to the pathogen (1). Multiple genes encoding all these functions may be present in the ExPEC strains regardless of their serogroup, their host (human or animal), or their site of isolation. In fact, up to now there is no single factor or group of factors characterizing a pathotype, or any exclusive factor(s) that can be considered the hallmark for identification of ExPEC.

Extraintestinal infections usually occur in hospitalized patients who may be immune compromised to various degrees. Consequently, the virulence potential of the infecting pathogen may also vary, so that even bacteria with very low virulence capacity may cause extraintestinal infections in extremely compromised hosts (1).

The lack of well-defined ExPEC virulence determinants, in addition to the variable immune competence of the infected hosts, makes it difficult to assess the virulence determinants that would render *E. coli* a true extraintestinal pathogen with intrinsic virulence potential. Based on the results of Picard et al. (5) that assessed ExPEC virulence using an animal sepsis model, Johnson et al. (6) proposed that a strain would be defined as ExPEC if it possessed at least two of the following VFs: P fimbria (*papA* and/or *papC*), S and/or F1C fimbria (*sfa*/*foc*), adhesins of the AFA-DR family (*afa*/*dra*), type II capsule (*kpsMT* II), and aerobactin (*iucA/ iutA*) (6). This definition would be very useful to localize ExPEC strains within various niches and hosts, thus facilitating epidemiological studies.

In view of the world-wide importance of extraintestinal infections, many researchers are working to expand our knowledge of the virulence genes and their mechanisms of dissemination in ExPEC. So far, most of the data have been obtained from studies with human and animal *E. coli* strains characterized as UPEC, NMEC, and APEC. Furthermore, studies evaluating the virulence potential of the strains have rarely been conducted. Taking into account that *E. coli* is the most frequently isolated Gram-negative bacterium worldwide, our purpose was to investigate the phylogenetic background and the virulence profile of ExPEC strains isolated from the bloodstream of hospitalized patients in the city of São Paulo, Brazil. In this study, we also intended to determine the intrinsic virulence of the strains based on the criteria proposed by Johnson et al. (6). This information would be useful for making comparisons with ExPEC strains isolated from other infection sites as well as isolates from other countries. Moreover, the knowledge of the pathogenic characteristics of ExPEC from various geographic regions is fundamental for the development of more precise diagnostic methods and, as a consequence, the implementation of better preventive measures and therapeutics.

Material and Methods

Origin and growth conditions of the bacterial strains

This study included 74 *E. coli* bacteremia isolates from 74 patients hospitalized in a tertiary level hospital located in São Paulo city, Brazil, which is a cosmopolitan area encompassing a population of about 11 million inhabitants. The inpatients were of both genders (43 males and 31 females) with a mean age of 43 years (range, 1 month to 92 years). Most patients presented a variety of immune-debilitating conditions (29 with malignant neoplasia, 6 with renal disease, 5 with organ transplant, 2 with diabetes, 1 with pulmonary obstruction, 1 with cirrhosis, 1 HIV-infected, and 1 with cachexy). The remaining 28 patients were either immune competent (n = 19) or did not have information available (n = 9).

Primary isolation and identification of the studied strains were carried out in the hospital laboratory, and the isolated *E. coli* strains were held at –70°C in the bacterial collection of the Laboratório Especial de Microbiologia Clínica, Universidade Federal de São Paulo. For this study, a sample of each stored strain was checked for purity on MacConkey agar and kept at –20°C in Tryptic Soy Broth (TSB, Difco Laboratories, USA) with 15% glycerol. For all tests, the strains were routinely grown at 37°C in TSB or MacConkey agar (Difco).

Phylogenetic analysis

The phylogenetic origin of the *E. coli* strains was determined by the method of Clermont et al. (7) based on a triplex-PCR carried out with primers for the genes *chuA*, *yjaA*, and the TspE4.C2 anonymous fragment. PCR mixtures were prepared using boiled bacterial cultures as template DNA, primers were from IDT (Integrated DNA Technologies, USA), and other reagents from Invitrogen (USA). The reactions were performed in a thermal cycler Mastercycler gradient (Eppendorf, USA).

Detection of virulence genes

Twenty of the most relevant ExPEC VFs were detected by DNA-DNA hybridization. They included adhesins (*papC*, *sfaDE*, *afaBC* III, *iha*, *fimH*, and *clpG*), invasin (*ibe10*), elements involved in iron acquisition (*iucD*, *irp2*, and *chuA*), protectins (*iss*, *traT*, *cvaC*, *kpsMT* II, and *kpsMT* III), and toxins (*ompT*,

ehxA, *espP*, *hlyA*, and *cnf1*). The probe fragments were obtained by PCR as described previously (8-10) using the primers and reaction conditions presented in Supplementary Table S1. The following bacterial prototype strains were used as DNA templates for the amplification of the indicated virulence genes: *E. coli* RS218 (*sfaDE*, *fimH*, *kpsMT* II, *traT*, *ibe10*, and *ompT*); *E. coli* J96 (*papC*, *kpsMT* III, *hlyA*, *cnf1*, and *chuA*); *E. coli* EDL933 (*iha*, *ehxA*, and *espP*); *E. coli* A30 (*afaBC* III); *E. coli* 042 (*irp2*), *Shigella flexneri* SA101 (*iucD*), *Klebsiella pneumoniae* CF504 [*clpG*] (8-10), and *E. coli* EC-40 (*cvaC* and *iss*), an isolate characterized in our laboratory. After resolution on 2.0% agarose gels, the probe fragments were purified and radiolabeled as described previously (8). DNA-DNA hybridization assays were carried out by colony hybridization using high-stringency conditions as described by Okeke et al. (11). The prototype strains mentioned above were used as positive controls according to their genotype. Laboratory *E. coli* strains HB101, DH5α (Gibco BRL, USA), and J53 were used as negative controls in all hybridization experiments.

Statistical analysis

Comparison of frequencies among different groups was made by the Fisher exact test. The threshold for statistical significance was a P value <0.05. The VF scores of the strains are reported as the sum of the virulence markers they possessed.

Results

Phylogenetic classification

The *E. coli* strains studied belonged mainly to phylogroups B2 (35.1%) and D (29.7%); phylogroups B1 (18.9%) and A (16.2%) were less frequent (Table 1).

Detection of virulence genes

Seventeen of 20 VFs were detected among the 74 *E. coli* isolates (Table 1). The most frequent VFs were the adhesins (97%), followed by the protectins (86%) and the iron acquisition systems (82%), while toxins, and invasins were seldom detected (35 and 12%, respectively). The majority of the VFs occurred in at least one of the strains, the only exceptions were *clpG*, *espP* and *ehxA*, which were not detected. The VFs most frequently found were *fimH* (93%), *irp2* (76%), *chuA*, and *kpsMT* II (65% each), while *ibe10* and *kpsMT*III were rarely detected (Table 1). The number of VFs present in each strain was variable; the overall mean was 6.4 (ranging from 0 to 13). This variability was reflected in the virulence profiles of the strains, given that 42 of 52 profiles were unique, and the other 10 profiles were found in not more than 5 strains each (data are presented in Supplementary Table S2).

Distribution of virulence factors among phylogenetic groups

The diversity of VFs observed in the isolates belonging to phylogroups B2 and D was higher (16 and 14 VFs of

Table 1. Frequency of virulence factors and their distribution among phylogenetic groups.

Traits	Gene[a]	Total	Phylogenetic groups			
			A (n = 12)[b]	B1 (n = 14)	D (n = 22)	B2 (n = 26)
Adhesins/invasin	*fimH*	69 (93)	10 (83)	12 (86)	22 (100)	25 (96)
	papC	39 (53)	2 (17)[+]	1 (7)[+]	15 (68)	21 (81)*
	iha	33 (45)	4 (33)	0 (0)[+]	14 (64)*	15 (58)
	sfaDE	11 (15)	0 (0)	0 (0)	0 (0)[+]	11 (42)*
	afaBC III	8 (11)	1 (8)	0 (0)	6 (27)*	1 (4)
	ibe10	1 (1)	0 (0)	0 (0)	0 (0)	1 (4)
Iron acquisition system	*irp2*	56 (76)	8 (67)	4 (29)[+]	18 (82)	26 (100)*
	chuA	48 (65)	0 (0)[+]	0 (0)[+]	22 (100)*	26 (100)*
	iucD	47 (64)	8 (67)	2 (14)[+]	18 (82)*	19 (73)
Protectins	*kpsMT* II	48 (65)	4 (33)[+]	0 (0)[+]	19 (86)*	25 (96)*
	traT	45 (61)	5 (42)	5 (36)	16 (73)	19 (73)
	ompT	45 (61)	2 (17)[+]	5 (36)	13 (59)	25 (96)*
	iss	16 (22)	3 (25)	2 (14)	5 (23)	6 (23)
	cvaC	16 (22)	4 (33)	6 (43)	2 (9)	4 (15)
	kpsMT III	2 (3)	0 (0)	0 (0)	2 (9)	0 (0)
Toxins	*hlyA*	19 (26)	0 (0)[+]	0 (0)[+]	4 (18)	15 (58)*
	cnf1	9 (12)	0 (0)	0 (0)	0 (0)[+]	9 (35)*

Data are reported as number with percent in parentheses. [a]The genes *clpG*, *ehxA* and *espP* were not detected. [b]Total number of strains in the group. * $P<0.05$, frequency of virulence factors in each phylogenetic group compared to the frequency in all others (Fisher exact test). [+]Negative association between the phylogroup and the virulence factor.

20, respectively) than that detected among strains from phylogroups A and B1 (10 and 8 VFs, respectively). The number of VFs per strain also differed among the groups, and the VF score (i.e., the average number of VFs per strain) was higher in groups B2 (8.9) and D (7.5) than in groups A (4.1) and B1 (2.3). It is worth noting that, among the 16 VFs detected in phylogroup B2, two were exclusive to that group (*sfaDE* and *cnf1*), and six others (*papC*, *irp2*, *kpsMT* II, *hlyA*, *ompT*, and *chuA*) showed a statistically significant association ($P \leq 0.001$) with that group (Table 1). To a lesser extent, group D was also closely associated with some virulence markers. On the other hand, groups A and B1 had a negative association with some VFs. Overall, with the exception of genes *sfaDE* and *cnf1*, all the genes that were positively associated with the virulent phylogroups B2 and/or D were negatively associated with the nonvirulent phylogroups A and/or B1 (Table 1).

ExPEC status

By applying the Johnson criteria (6) for a molecular definition of intrinsic virulence in this survey, only 45 (61%) of the strains were classified as ExPEC while the remaining 29 (39%), although isolated from patients with bacteremia, were classified as non-ExPEC. Accordingly, ExPEC strains had higher VF scores (mean of 8.7 per strain) than the non-ExPEC (mean of 3.1 per strain). Moreover, 12 virulence genes were prevalent in the ExPEC isolates while only the *cvaC* gene was prevalent among the non-ExPEC strains (Table 2). Furthermore, the ExPEC group that harbored most of the strains included the B2 (24 of 26) and D (18 of 22) phylogroups that are considered virulent, as well as a few (3 of 12) from group A. On the other hand, none of the virulence factors characteristic of ExPEC were found in any B1 strain.

Table 2. Distribution of virulence factors according to the extraintestinal pathogenic *Escherichia coli* (ExPEC) status[a].

Virulence factor	ExPEC (n = 45)[b]	Non-ExPEC (n = 29)
irp2	44 (97.8)*	12 (41.4)
kpsMT II	44 (97.8)*	4 (13.8)
fimH	43 (95.6)	26 (89.6)
chuA	42 (93.3)*	6 (20.7)
iucD	38 (84.4)*	9 (31)
papC	38 (84.4)*	1 (3.4)
ompT	34 (75.5)*	11 (37.9)
traT	33 (73.3)*	12 (41.4)
iha	31 (68.9)*	2 (6.9)
hlyA	19 (42.2)*	0 (0)
sfaDE	11 (24.4)*	0 (0)
iss	10 (22.2)	6 (20.7)
cnf1	9 (20)*	0 (0)
afaBC III	8 (17.8)*	0 (0)
cvaC	6 (13.3)	10 (34.4)*
kpsMT III	1 (2.2)	1 (3.4)
ibe10	1 (2.2)	0 (0)

Data are reported as number with percent in parentheses. [a]Defined according to the Johnson (6) criterion. [b]Total number of strains in the group. *$P < 0.05$, frequency of the virulence factor compared between ExPEC and non-ExPEC groups (Fisher exact test).

Discussion

In the present study, we assessed the phylogenetic background, virulence genes, and virulence potential of 74 *E. coli* strains isolated from the bloodstream of 74 individuals of all ages and both sexes who were inpatients of a tertiary level hospital in São Paulo city, Brazil.

The higher prevalence of the virulent B2 and D phylogroups compared to the nonvirulent A and B1 phylogroups observed in this study is in accordance with recent reports from our region as well as other countries (12-14), although with some unique results. In comparison with our results, the reported frequencies of B2 strains are higher in other countries (60 *vs* 35%), while D strains appear in lower frequencies (20 *vs* 30%) (12,13). One exception is a report from Spain by Martinez et al. (15), where 52% of the isolates belonged to group D and only 18% were from group B2. Isolates belonging to phylogroups A and B1 were more frequent in São Paulo than in other regions around the world (12,13,15).

It has been proposed (16) that the climate, population habits, and use of antibiotics influence the phylogenetic composition of *E. coli* in the intestinal microbiota. If that is the case it could explain the differences observed in the phylogeny of the *E. coli* strains involved in extraintestinal infections in diverse geographical areas, since these pathogens may be part of the intestinal microbiota, as already demonstrated (16; and Santos MVM, Santos ACM, Silva RM, unpublished results).

Along with *chuA*, which defines phylogroups B2 and D, the virulence factors *fimH*, *irp2*, and *kpsMT* II were the most frequent among the strains identified in this study, and they seem to be the most frequently involved in many other *E. coli* extraintestinal infections (3,10,12,13,17). Moreover, *iucD*, *traT*, *papC*, *iha*, *afaBC* III, and *kpsMT* III occurred in frequencies similar to those already reported for *E. coli* strains isolated from bacteremia (10,12,13). On the contrary, the VFs *hlyA*, *sfaDE*, *cnf1*, and *ibe*10 have been reported in higher frequencies, and *cvaC* and *iss* in lower frequencies than those found in this study (10,12,13). A similar survey of *E. coli* strains isolated from the blood stream of patients with sepsis has been conducted in Campinas, Brazil, a large city of approximately 1 million inhabitants about 140 km from São Paulo. In that study, Ananias and Yano (17) found that *papC*, *ibe10*, *irp2*, *iucD*, and *cnf1* were more frequent, and *afaBC*, *traT*, and *cvaC* were less frequent than in our

study. It has to be emphasized that, in the study by Ananias and Yano, all of the 60 patients died in 2 days after hospitalization. However, in our study only 15 of 74 patients were diagnosed with clinical sepsis, and only 7 went on to die. This difference could be due to the heterogeneity in the virulence potential of the strains in both studies rather than to the susceptibility of the patients, who seemed to present very similar overall characteristics. Altogether, the discussion above reveals important differences on the virulence repertoire of bacteremic *E. coli* strains around the world, and even in neighboring geographical regions.

As already reported by others (3,12), the present study detected many virulence factors that were positively associated with the phylogroups B2 (*papC*, *sfaDE*, *irp2*, *kpsMT* II, *hlyA*, *ompT*, and *cnf1*) and D (*iha*, *afaBC* III, *iucD*, *kpsMT* II, and *kpsMT* III). In addition, some of these virulence genes were negatively associated with phylogroup B1 (*iha*, *irp2*, and *iucD*) or with both phylogroups A and B1 (*papC*, *kpsMT* II, and *hlyA*). Nonetheless, there was such a variety of virulence genes detected in the bacteremia strains in this study that it is not possible to define virulence profiles within this group of isolates based on what is currently known of either established or putative virulence factors of ExPEC. Overall, our results are in agreement with other studies that have not yet considered it possible to assign pathotypes to the ExPEC strains. In fact, the VFs mentioned above as associated with the more virulent strains isolated from bacteremia are the same ones present in ExPEC isolates from other body sites (3).

Johnson et al. (6) proposed that the presence of at least two of the following virulence factors: *papA* and/or *papC*, *sfa/foc*, *afa/dra*, *kpsMT* II, and *iuc/iutA*, would be sufficient for molecular characterization of an *E. coli* strain as an ExPEC. According to this assumption, only 61% of the isolates in this study would be classified as ExPEC. In fact, this classification seems to embrace the strains with a greater array of virulence genes, since as many as 12 of the 17 VFs detected here were prevalent among the ExPEC group compared to the remaining 39% of non-ExPEC isolates. Accordingly, the B1 strains had the lowest number of VFs and none of them was classified as ExPEC.

Although in the present study the virulence profiles of the *E. coli* isolates were not analyzed in regard to the immune status of the patients, the occurrence of 39% of non-ExPEC strains causing infections may be due to the immune deficiencies present in the hosts. In fact, many investigators have addressed immune status as a key factor to explain the involvement of less virulent *E. coli* strains in blood infections (18,19). On the other hand, there are still controversies on the correlation of virulence as defined by phylogeny, virulence in mice and/or molecular intrinsic virulence potential, and the severity of human blood infections (18,20).

It is conceivable that individuals with varied degrees of immune deficiencies would be prone to infection by strains with different degrees of virulence. Therefore, it is necessary to recognize among the infecting strains those that present intrinsic virulence potential, because they are the ones that should be classified as true ExPEC.

Supplementary Material

Click here to view [pdf]

Acknowledgments

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