Molecular and Phenotypic Characterization of Diarrheagenic *Escherichia coli* Strains Isolated from Bacteremic Children

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Abstract. *Escherichia coli* is an important cause of Gram-negative bacteremia. The aim of this study was to characterize at the molecular and phenotypic levels *E. coli* strains belonging to different diarrheagenic pathotypes [diarrheagenic *E. coli* (DEC)] isolated from bacteremia in children younger than 5 years of age. Seventy bacteremia *E. coli* strains were collected in a prospective study in 12 hospitals in Lima, Peru. The presence of virulence genes associated with DEC [enterotoxigenic (*lt* and *st*), enteropathogenic (*eaeA*), shiga toxin-producing (*stx1* and *stx2*), enteroinvasive (*ipaH*), enteroaggregative (*aggR*), and diffusely adherent (*daaD*)] was determined by multiplex real-time polymerase chain reaction (PCR). Those positive *E. coli* strains were further analyzed for 18 additional virulence factors encoding genes and others phenotypic features. Virulence genes associated with DEC were identified in seven bacteremic children (10%), including: one *aggR*-positive [enteroaggregative *E. coli* (EAEC)], one *eaeA*-positive [enteropathogenic *E. coli* (EPEC)], one *st*-positive [enterotoxigenic *E. coli* (ETEC)], one *daaD*-positive [diffusely adherent *E. coli* (DAEC)], and three strain positive for *aggR* and *daaD* (EAEC/DAEC) at the same time. All strains, except EPEC, had the Ag43 adhesin, and all, except ETEC had the siderophore gene *fyuA*. The phylogenetic profile of these strains was variable, two (B2), two (D), two (A), and one (B1) strain. These isolates were susceptible to all tested antibacterial agents except to ampicillin and gentamicin. The three EAEC/DAEC strains showed biofilm formation and aggregative adhesion and had the same repetitive extragenic palindromic-PCR patterns. These findings suggest that some DEC strains, especially *aggR* and *daaD* positive, might cause bacteremia in children.

INTRODUCTION

According to their set of virulence factors and clinical properties, *Escherichia coli* isolates can be classified as commensals, intestinal, or extraintestinal pathogens. Extraintestinal pathogenic *E. coli* (ExPEC) are responsible for urinary tract infections (UTIs), intraabdominal and soft tissue infections, meningitis, pneumonia, and osteomyelitis, and are often associated with bacteremia in special populations.¹ Bacteremia represents the tenth major cause of death in developed countries; *E. coli* is among the most important Gram-negative bacteria involved.² Bloodstream infections are associated with substantial morbidity, mortality, and healthcare costs in adults. However, children admitted to the hospital usually have higher rates of bloodstream infections than adults.

In general, ExPEC infections are caused by strains that are transitorily present in the fecal microbiota and bear specific groups of genes encoding virulence factors.¹ ExPEC infections strains usually display a wide array of putative virulence factors, such as adhesins, iron acquisition systems, host defense-subverting mechanisms, and toxins.² Adherence and biofilm formation has been considered an important factor for the maintenance of bacteria on the mucosal surface of the host organism. Among the *E. coli* pathogens, there are six well-described categories: enteropathogenic *E. coli* (EPEC), enterohemorrhagic *E. coli* (EHEC), enterotoxigenic *E. coli* (ETEC), enteroaggregative *E. coli* (EAEC), enteroinvasive *E. coli* (EIEC), and diffusely adherent *E. coli* (DAEC). Moreover,

recently has been described the presence of diarrheagenic *E. coli* (DEC) presenting mixed characteristics.³ Most of them have the ability to adhere to the human intestinal epithelium. DEC could have one of four adherence patterns in HEp-2 cells: localized adherence (LA), localized adherence-like (LAL), aggregative adherence (AA), and diffuse adherence (DA).² Phylogenetic analyses have shown that *E. coli* strains fall into four main phylogenetic groups (A, B1, B2, and D); virulent extraintestinal strains belong mainly to group B2 and, to a lesser extent, to group D, whereas most commensal and less virulent strains belong to A or B1 groups.⁴ Over the last decade, there has been a marked increase in infections caused by antibiotic-resistant *E. coli* that could impact the outcome in patients with bacteremia.⁵

Therefore, although some of these DEC pathotypes have been described in UTIs,⁶ no studies have addressed their relevance as potential cause of bacteremia. These studies are of special relevance in low- and middle-income countries both because the high burden of DEC which potentially increase the risk of its involvement in ExPEC disease including bacteremia and the fact that in these countries bloodstream infections are a leading cause of febrile illness, with an special affection on neonates and children under 5 years.⁷

Therefore, the aims of this study were to determine the presence of DEC genes among bacteremia *E. coli* strains in Peruvian children and to characterize these isolates at the molecular and phenotypic levels.

MATERIALS AND METHODS

Bacterial strains. A total of 70 *E. coli* strains from bloodstream infections were isolated from children less than 5 years of age, 40 samples from neonates (0–28 days), 22 infants (28 day to 1 year), and eight older children. Sixty strains were isolated in 12 hospitals in Lima between 2008 and 2009 as part

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of a prospective study aimed at studying antimicrobial resistance in Lima.⁵ From 2009 to 2010, 10 strains additionally were collected, from the same hospitals.

Ethical aspects. The study was approved by the institutional review boards of the Universidad Peruana Cayetano Heredia in Lima.

DNA isolation. *Escherichia coli* strains were subcultured from peptone stocks onto MacConkey agar plates. After overnight incubation, a single colony was carefully removed from the plate using a sterile toothpick to avoid agar contamination. DNA was extracted by boiling the colony in 50 μ L of polymerase chain reaction (PCR) or molecular grade water for 5 minutes, followed by centrifugation at 13,000 rpm for 10 minutes; 2 μ L of this lysate was used as a template with a 23 μ L PCR master mix to make a 25 μ L total reaction volume.

Detection of DEC genes. A multiplex real-time PCR⁸ that recognizes eight virulence genes associated with DEC was used: enterotoxigenic (*lt*, *st*), enteropathogenic (*eaeA*), Shiga toxin-producing (*stx1*, *stx2*), enteroinvasive (*ipaH*), enteroaggregative (*aggR*), and diffusely adherent *E. coli* (*daaD*), (Table 1).

Determination of virulence factors. The presence of 18 additional virulence factor encoding genes was analyzed by PCR, using primers and conditions previously described.^{8,9} The virulence factors selected are associated with adhesins, serine protease autotransporters of Enterobacteriaceae (SPATE), dispersin, toxins, biofilm formation, and siderophores (Table 1).

Determination of *E. coli* phylogenetic groups. Phylogenetic groups of *E. coli* (A, B1, B2, and D) were determined using a triplex PCR according to the combination of three genetic markers *chuA*, *yjaA*, and DNA fragment *TspE4.C2*. The primers used for detection of the *chuA* genes were *chuA-F* (5'-GACGAACCAACGGTCAGGAT-3') and *chuA.R* (5'-TGCCGCCAGTACCAAAGACA-3'), *yjaA.1* (5'-TGAAGTGTCAAGAG-ACGCTG-3') and *yjaA.2* (5'-ATGGAGAATGCGTTCCTCAAC-3'), and *tspE4C2.1* (5'-GAGTAATGTCGGGGCATTCA-3') and *tspE4C2.2* (5'-CGCGCCAACAAAGTATTACG-3'), which generate 279-, 211-, and 152-bp fragments, respectively.⁴

Adherence assay. Bacterial cell adhesion assays were performed as previously described.¹⁰ Briefly, monolayers of 10⁵ HeLa cells were grown in Dulbecco's modified Eagle's medium (DMEM) (Gibco, Grand Island, NY) in 35-mm tissue culture dishes (Corning, Charlotte, NC). Bacterial strains were grown statically in 3 mL of tryptic soy broth (Difco, Detroit, MI) for 16–18 hours at 37°C. The monolayers were infected with, approximately 10⁷ bacteria and incubated at 37°C for 3 hours. The infected monolayers were washed twice with sterile phosphate-buffered saline, fixed with methanol, stained with Giemsa, and examined under the light microscope. Strain EPEC 2348/69 was used as a positive LA control adherence, EAEC O42 as an AA positive control and 5019 as control of DA. *Escherichia coli* DH5 α was used as a negative control.

Biofilm assay. Quantitative biofilm formation was evaluated as described by Wakimoto et al.¹¹ Biofilm accumulations were normalized with respect to growth, which yielded the specific biofilm formation (SBF).¹² The SBF was determined with the following formula: $SBF = (DO_{570nm} - DO_{c570nm}) / DO_{c630nm}$, where DO_{570nm} is the amount of biofilm formed, DO_{c570nm} is the amount of crystal violet that adhered to the using microtiter plate, and DO_{c630nm} is the optical density of cells grown in suspended culture. Bacteria from overnight growth were cultured in 24-well plates

containing plastic cover slips with DMEM-HEPES at pH 7.4, supplemented with 0.4% glucose (high-glucose DMEM) and incubated at 37°C in 5% CO₂ incubator for 4 hours. The cover slips with adherent bacteria were washed vigorously, qualitative biofilms were measured for optical density after being stained with 0.5% crystal violet and resuspended with 75% ethanol, and qualitative biofilms were evaluated by microscopy. For quantitative and qualitative biofilm formation at least two replicate experiments were performed for each strain. The prototype strain EAEC O42 was used as a positive control, and nonpathogen *E. coli* DH5 α was used as a negative control.

Antimicrobial susceptibility. Strains were analyzed for their antimicrobial susceptibilities by disk diffusion, according to the Clinical and Laboratory Standards Institute guidelines.¹³ The antibiotics analyzed were ampicillin (AMP; 10- μ g disk), gentamicin (GEN; 10- μ g disk), ciprofloxacin (5- μ g disk), aztreonam (5- μ g disk), ceftazidime (CAZ; 30- μ g disk), cefotaxime (CTX; 30- μ g disk), ceftriaxone (30- μ g disk), amoxicillin-clavulanic acid (AMC; 30- μ g disk), and cefepime (FEP; 30- μ g disk). The AMC FEP, CAZ, AZT, and CTX disks were placed on the plate as described previously to detect the presence of extended spectrum β -lactamases (ESBLs).¹⁴

Repetitive extragenic palindromic (REP)-PCR. The REP-PCR was carried out following the method previously described by Gallardo et al.¹⁵ with some modifications. Briefly, the primer 5'-GCGCCGICATGCGGCATT-3' was used under the following conditions: 30 cycles of 1 minute at 94°C, 1 minute at 40°C, and 1 minute at 65°C, with a final extension of 16 minutes at 65°C. The reaction was prepared with 5 μ L of boiled bacterial suspension, 1 μ L of 5 mM primer, and PCR beads (Pharmacia A.B., Uppsala, Sweden). Fifteen microliters of the PCR products were separated on 1.5% agarose gels, containing ethidium bromide 0.5 mg/L.

RESULTS

Detection of DEC. Of the 70 *E. coli* strains isolated from children under 5 years, seven strains (10%) were identified as belonging to DEC pathotypes, including: one *aggR*-positive (EAEC), one *eaeA*-positive (EPEC), one *st*-positive (ETEC), one *daaD*-positive (DAEC), and three strain were positive to *aggR* and *daaD* at the same time (EAEC/DAEC). All these strains were found exclusively in children less than 1 year of age.

Determination of virulence factors genes. Genes associated with fimbriae type adhesins were found in all strains except EPEC. Aggregative adherence-fimbria III (AAFIII) was present in DAEC and the three EAEC/DAEC strains. The presence of the biofilm-associated *shf* gene was identified in the ETEC strain whereas all strains had Ag43, except EPEC (Table 2). Dispersin associated genes were found in EAEC and the three EAEC/DAEC strains. All isolates had the siderophore encoding gene *fyuA* with the exception of the ETEC strain. Only EPEC and ETEC strains did not presented biofilm formation (qualitative and quantitative). No toxin and SPATE genes were found, except for *sat*, present in one DAEC strain (Figure 1).

***E. coli* phylogenetic group.** The isolates belongs to the phylogenetic groups, A (EAEC and EPEC), B1 (ETEC), B2 (DAEC and EAEC/DAEC), and D (two EAEC/DAEC).

Antimicrobial resistance. We did not find the presence of ESBL-producing *E. coli*. The isolates were susceptible to the tested antibiotics except to AMP and GEN (Table 2).

TABLE 1
Primers and PCR product size of genes analyzed

Primer	Orientation	Sequence (5'-3')	Target	Size (bp)	T _m (°C)	Ref
<i>AggR</i>	F	CGAAAAAGAGATTATAAAATTAAC	<i>aggR</i>	100	77.07 ± 0.68	8
<i>st1</i>	R	GCCTCTCTCTTTTGTTAT	<i>stla</i>	159	81.45 ± 0.27	8
<i>st2</i>	F	TTTCCCCTCTTTTAGTCAGTCAA	<i>stlb</i>	138	81.45 ± 0.27	8
<i>Lt</i>	R	GCAGGATTACACACAAATTCACAGCAG	<i>Lt</i>	322	85.88 ± 0.34	8
<i>eaeA</i>	F	TGCTAAACCAGTAGAGCTTTCAAAA	<i>eaeA</i>	248	83.93 ± 0.31	8
<i>stx1</i>	R	GCAGGATTACACACAAATTCACAGCAG	<i>stx1</i>	150	87.37 ± 0.32	8
<i>stx2</i>	F	TCCTATGTGCATACGGAGC	<i>stx2</i>	255	89.65 ± 0.33	8
<i>ipaH</i>	R	CCATACTGATTGCCGCAAT	<i>ipaH</i>	619	91.54 ± 0.26	8
<i>daaD</i>	F	ATGCTTAGTGTGGTTTAGG	<i>daaD</i>	444	93.81 ± 0.4	8
<i>aggC</i>	R	GCTTTCATCATTTTCGCTTC	<i>aggC</i>	538	45	9
<i>aafC</i>	F	CTGGATTAAATGTCGCATAGTG	<i>aafC</i>	1,144	48	9
<i>agg3C</i>	R	AGAACGCCCACTGAGATCATC	<i>agg3C</i>	485	50	9
<i>agg4C</i>	F	GGCACTGTCTGAAACTGCTCC	<i>agg4C (hdaC)</i>	700	55	9
<i>agn43</i>	R	TCGCCAGTTATCTGACATTCTG	<i>agn43</i>	600	55	9
<i>Sat</i>	F	GTTCTTGACCGCTTTCCGATACCGTC	<i>Sat</i>	387	56	9
<i>sepA</i>	R	GCCGGTCAGCCACCCTCTGAGAGTAC	<i>sepA</i>	794	56	9
<i>Pet</i>	F	TGAACGGGAGTAAAGGAAGATG	<i>Pet</i>	832	58	9
<i>sigA</i>	R	GTCCGCCATCACATCAAAA	<i>sigA</i>	430	58	9
<i>Pic</i>	F	TATTAACCATGGTAGCG	<i>Pic</i>	572	58	9
<i>Shf</i>	R	GCCAAGATCCGAGATTGA	<i>Shf</i>	613	50	9
<i>set1A</i>	F	TGCAGACTGATAATGCTC	<i>set1 A (ShET1 A)</i>	309	55	9
<i>set1B</i>	R	GTTATAGCTCGACATATTG	<i>set1 B (ShET1 B)</i>	147	56	9
<i>Sen</i>	F	GTTTGAACCGGGAATTACATTG	<i>sen (ShET2)</i>	799	55	9
<i>astA</i>	R	ATACTTTAGATACCCCTCAGAGTAC	<i>astA (EAST-1)</i>	110	55	9
	F	GGGGAGGGAATAGATATGG				
	R	CTCATCAGAGCCACACTCA				
	F	ACGCACAACCATCAATAAA				
	R	CCGCCTCCGATCTGAATGC				
	F	ACTGGCGGACTCATTGCTGT				
	R	AACCTGTGAAGAACTGAGC				
	F	GCAGTGGAAATATGATCGGGC				
	R	TTGTTGAGATCGGAGAAAGACG				
	F	ACTGGCGGACTCATTGCTGT				
	R	GCGTTTTTCCGTTCCCTATT				
	F	CCGACTTCTCACITTTCTCCCG				
	R	CCATCCAGCTGCATAGTGTG				
	F	ACTGGATCTTAAGGCTCAGGAT				
	R	GACTTAATGTCACTGTTTCAGCG				
	F	ACTTTCTCCCGAGACATTC				
	R	CTTTAGCGGGAGCATTCAT				
	F	TCACGCTACCATCAAGA				
	R	TATCCCCCTTTGGTGGTA				
	F	GTGAACCTGCTGCCGATATC				
	R	ATTTGTGGATAAAAAATGACG				
	F	ATGTGCTGCTATTATTAT				
	R	CATAATAATAAGCGGTCAGC				
	F	ATGCCATCAACACAGTATAT				
	R	GCGAGTGACGGCTTTGTAGT				

(continued)

TABLE 1
Continued

Primer	Orientation	Sequence (5'-3')	Target	Size (bp)	T _m (°C)	Ref
<i>aatA</i>	F	CTGGCGAAAGACTGTATCAT	<i>aatA</i> (CVD432 probe)	629	55	9
	R	AATGTATAGAAATCCGCTGTT				
<i>Aap</i>	F	CTTGGGTATCAGCCTGAATG	<i>aap</i> (<i>aspU</i>)	310	55	9
	R	AACCCATTCGGTTAGAGCAC				
<i>fyuA</i>	F	TGATTAAACCCCGCGACGGGAA	<i>fyuA</i>	650	55	9
	R	CGCAGTAGGCACGATGTTGTA				

F = forward; R = reverse; Ref = References; T_m = melting temperature; in the first nine genes (real time PCR) is reported the mean ± SD, whereas the remaining temperatures are annealing temperature of the conventional PCR.

Adherence assay and biofilm formation. All strains harboring the *aggR* gene presented the AA pattern. The strains harboring only the *daaD* gene, without the *aggR* gene, presents the DA pattern, and the strains harboring the *eae* gene presents the LA pattern. All strains, except those belonging to EPEC and ETEC genotypes, were able to form biofilm (Table 2); the quantitative biofilm formation was determined by the SBF index (Figure 2). Three strains (M4, M5, and M6) had a SBF index higher than the EAEC O42 control strain.

REP-PCR. The fingerprinting with REP1 primer generated identical patterns for the EAEC/DAEC strains (M4, M5 and M6), showing they belong to a same clone. All other strains have a different pattern (Figure 3).

DISCUSSION

In the present study, DEC genes were found in the 10% of bacteremic *E. coli* strains in children. The presence of genes associated with diarrhea in these strains might reveal their fecal origin and acquisition of virulence factors that enhanced their ability to cause systemic infection. The original niche of commensal *E. coli* is the mucous layer of the mammalian colon. Several highly adapted *E. coli* clones that have acquired specific virulence attributes which confer an increased ability to adapt to new niches may cause a broad spectrum of diseases. Three general clinical syndromes can result from infection with one of these pathotypes: enteric/diarrhea disease, UTIs, and sepsis/meningitis.³ Abe et al.⁶ studied the role of diarrheagenic strains in urinary tract infections whereas Herzog et al.¹⁶ reported a case of an immunocompromised patient who presented a mixed EPEC and EAEC infection in relation with diarrhea, UTI, and sepsis, which highlights the potential of EAEC to cause severe ExPEC infections.

It is not clear the role played by particular virulence factors on their pathogenic capability. In fact, reports on the association between the risk of bacteremia/septicemia and the presence of *E. coli* virulence factors are contradictory. Thus, whereas some studies propose that P fimbriae and α -hemolysin are associated with the risk of bacteremia,¹⁷ others indicate that there are no such association and that *E. coli* virulence factors do not differ between those isolated from bacteremia and isolates from pyelonephritis.¹⁸

The AAF/I, AAF/II, and AAF/III fimbriae are encoded by an apparently well conserved high molecular weight plasmid (pAA), which also encodes several virulence factors that include *aap* (dispersin protein), a segment of the CVD432 gene (*aat* gene) that encodes the dispersin transporter protein complex and finally the best studied virulence gene of EAEC,¹⁹ the transcriptional activator (*aggR*). Some of these genes have been widely used to identify EAEC strains.²⁰ Our strains probably could be consider to belong to the EAEC group1, because of the presence of these genes,²¹ and could had then gained the *daaD* gene.

In this study, all strains encoding DEC genes had some type of adhesion genes, including antigen 43 (Ag43), which promotes biofilm formation²² and cell-to-cell aggregation, or fimbria, except EPEC. However, EPEC strain have several other adherence factors (i.e., such as the bundle-forming pilus responsible of forming compact bacterial clusters in tissue cultured cells that characterize the LA pattern). We have not studied these additional adherence factors or different Ag43 alleles.²²

TABLE 2
Genotypic and phenotypic characterization of *E. coli* strains isolated from bacteremia harboring genes of diarrheagenic *E. coli*

Code	Hospital	Date of isolation	Age	DEC type	DEC gene	Clinical data	Samples						
							M1	M2	M3	M4	M5	M6	M7
							HNERM May 2008 10 months EPEC eaeA	HNERM November 2008 3 months ETEC St	HNCH January 2008 10 months EAE aggR	HMA August 2009 1 day DAEC daaD	INSN August 2010 24 months EAE/DAEC aggR + daaD	HEP May 2010 11 months EAE/DAEC aggR + daaD	HNDAC August 2009 12 months EAE/DAEC aggR + daaD
							N/A No Pielonefritis	N/A No N/A	None No N/A	N/A No N/A	Hypertension No UTI	Anemia/Pneumonia No Pielonefritis	None No Pneumonia
						Virulence factor							
						AAF/I (aagC)	-	+	+	-	-	-	-
						AAF/II (aafC)	-	-	-	-	-	-	-
						AAF/III (agg3C)	-	-	-	-	-	-	-
						AAF/IV (agg4C)	-	+	+	+	+	+	+
						ag43	-	-	-	-	-	-	-
						sat	-	-	-	-	-	-	-
						sepA	-	-	-	-	-	-	-
						pet	-	-	-	-	-	-	-
						sigA	-	-	-	-	-	-	-
						pic	-	-	-	-	-	-	-
						shf	-	+	-	-	-	-	-
						set1A	-	-	-	-	-	-	-
						set1B	-	-	-	-	-	-	-
						sen	-	-	-	-	-	-	-
						astA	-	-	-	-	-	-	-
						CVD432	-	-	+	-	+	+	+
						aap	-	-	+	+	+	+	+
						fyuA	+	-	+	+	+	+	+
						AMP	R	S	S	R	R	R	R
						GEN	S	S	S	S	S	S	S
						CIP	S	S	S	S	S	S	S
						AZI	S	S	S	S	S	S	S
						CAZ	S	S	S	S	S	S	S
						CTX	S	S	S	S	S	S	S
						CRO	S	S	S	S	S	S	S
						AMC	S	S	S	S	S	S	S
						FEP	S	S	S	S	S	S	S
						Phylogenetic groups	A	B1	A	B2	B2	D	D
						Adherence pattern	LA	NA	AA	DA	AA	AA	AA
						Qualitative biofilm	-	-	+	+	+	+	+
						REP-PCR	1	2	3	4	5	5	5

AA = aggregative adherent; AMC = moxifloxacin/clavulanic acid; AMP = ampicillin; ATM = aztreonam; CAZ = ceftazidime; CIP = ciprofloxacin; CTX = cefotaxime; CRO = ceftriaxone; DA = diffuse adherent; FEP = gentamicin; GEN = gentamicin; HEP = Hospital de Emergencias Pediátricas; HNCH = Hospital Nacional Cayetano Heredia; HNDAC = Hospital Nacional Daniel Alcides Carrión; HNER = Hospital Nacional Edgardo Rebagliati Marín; HMA = Hospital María Auxiliadora; INSN = Instituto Nacional de Salud del Niño; LA = localized adherent; NA = nonadherent; N/A = nonavailable; R = resistance; S = susceptible; UTI = urinary tract infection.

FIGURE 1. Virulence factors harboring strains isolated from bacteremia: L = ladder (Molecular weight, bp). 1. *aatA* - *aap* (positive control), 2. M6, 3. Negative control, 4. *sat* (positive control), 5. M4, 6. Negative control, 7. *ag43* (positive control), 8. M7, 9. Negative control, 10. *fyuA* (positive control), 11. M1, 12. Negative control, 13. *Shf* (positive control), 14. M2, 15. negative control, 16. *agg3C* (positive control), 17. M3, 18. Negative control, 19. *aggC* (positive control), 20. M2, 21. Negative control.

The DAEC and EAEC strains present characteristics adherence patterns in HeLa/HEp-2 cell lines.¹⁰ In this study, among the strains harboring both *daaD* and *aggR* genes, we found the AA phenotype only; probably, this is a “stronger” phenotype that overcome the DA pattern; moreover, all strains presented the gene *Agg3c*. This gene encodes the AAF-III adhesion, a fimbrial adhesin involved in the AA phenotype.²³

The ability to form biofilms is a trait that is closely associated with bacterial persistence and virulence; many persistent and chronic bacterial infections have this characteristic. In this study, all EAEC and DAEC strains were capable of forming biofilm. However, they did not have the *shf* gene, involved in biofilm formation in the EAEC O42 reference strain.²⁴ In the present study, this gene was identified in the ETEC strain. This pathotype had the CS1, the prototype of a class of pili of ETEC associated with diarrheal disease in humans. The genes encoding this pilus are carried on a large plasmid, pCoo and the region *e* of pCoo is 95% identical to the *shf* operon of the *Shigella flexneri* virulence plasmid pINV.²⁵ The *AggR* factor has been more frequently found among isolates forming biofilm; its relationship with both clinical and microbiological characteristics could play a role in the pathogenicity of infectious diseases such as bacteremia.²⁶

Likewise, the *fyuA-irp* gene cluster contributes to the virulence of highly pathogenic *Yersinia*.²⁷ The cluster encodes an iron uptake system mediated by the siderophore yersinia-bactin and reveals features of a pathogenicity island (HPI). The HPI belonging to *Yersinia pestis* evolutionary group can be disseminated among species of the family *Enterobacteriaceae* such as EAEC and in *E. coli* blood culture isolates,²⁸ but also is rarely found in EPEC, EIEC, and ETEC. In our study, all isolates had the siderophore *fyuA* with the exception of the ETEC strain. The presence of this iron-acquisition system might increase the invasiveness of DEC isolates, favoring a bacteremia infection.

Autotransporter proteins are found in all *E. coli* pathotypes and are often associated with virulence. Thus, the secreted autotransporter toxin, Sat, which belongs to the subfamily of SPATE, acts as a virulence factor in ExPEC and intestinal pathogenic *E. coli*.²⁹ The *sat* gene has been frequently found in DAEC isolates from stools of children from but also in EAEC,^{30,31} causing intestinal damage in animal model assays.³² In accordance, we detected this gene in the DAEC isolate, but none of the EAEC/DAEC isolates possess it.

Currently, it is assumed that the differences between pathogenic and commensal *E. coli* correlate with four main

FIGURE 2. Quantitative biofilm formation among bacteremia *Escherichia coli* strains harboring diarrhoeagenic *E. coli* genes. SBF = specific biofilm formation; O42 = positive biofilm control; DH5α = negative biofilm control; M1-7 = bacteremia *E. coli* strains.

FIGURE 3. REP-PCR for EAEC/DAEC strains isolated from bacteremia: L = ladder, 1. strain M5 from INSN, 2. strain M6 from HEP, and 3. strain M7 from HNDAC. GeneRuler™ 100 bp Plus DNA Ladder (Thermo Scientific, Lithuania). INSN = Instituto Nacional de Salud del Niño; HEP = Hospital de Emergencias Pediátricas; HNDAC = Hospital Nacional Daniel Alcides Carrión.

phylogenetic groups (A, B1, B2, and D). Thus, it is considered that extraintestinal virulent strains belong mainly to group B2 and to a lesser extent, to group D, while most commensal strains/resistant and less virulent belong to groups A or B1.⁴ In this study, four strains belong to phylogenetic B2 and D groups although the limited number of samples makes the analysis difficult; the data seem to be in accordance with the aforementioned virulence/resistance distribution.

The pathogenicity has been correlated with the presence of genes encoding virulence factors organized on large blocks called pathogenicity islands. It has been shown, further, that pathogenicity islands can disseminate horizontally between distinct *E. coli* strains whether they are located on plasmids, bacteriophages, or even the bacterial chromosome.³³ These results suggest virulence evolution and regulation in *E. coli* that can combine several of these virulence factors allowing the microorganisms to induce different diseases. Indeed, one of the lessons is the recent EAEC/EHEC Germany outbreak, which highlights the enormous *E. coli* genome plasticity, which leads to the emergence of a both highly virulent and multidrug-resistant strain.^{6,34}

The main limitation of the present study was the limited sample size. Nonetheless, 10% of the recovered isolates were classified as DEC, showing the potential of these pathogens to cause bloodstream infections. Moreover, it is needed to highlight that no children infected by a DEC isolate reported previous diarrhea but three out of seven (42.8%) shows a urinary focus. This reported focus and lack of association between previous diarrhea and presence of DEC as a cause of bacteremia suggests the relevance of asymptomatic presence of DEC in feces, which has been largely described in the area,³⁵ as a bloodstream infection risk factor as well as the possible DEC route from asymptomatic gut presence to cause a UTI and therefore leading to bacteremia opening the door to develop further studies to determine these findings. Moreover, the presence of clonal-related EAEC/DAEC in three out of seven (42.8%) cases suggests the

emergence risk of a new pathogenic clone with enhanced facility to lead towards bloodstream infections being needed to develop studies to further characterize.

In summary, the present study showed the potential of diarrheagenic *E. coli* to produce an invasive illness. This fact may be of special concern in low- and middle-income countries, in which sanitary conditions and low socioeconomic environment may result in high frequency of diarrheic episodes that may favor bacteremia with these strains.

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