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Virulence-related genes are associated with clinical and nutritional outcomes of *Shigella*/Enteroinvasive *Escherichia coli* pathotype infection in children from Brazilian semiarid region: A community case-control study

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ABSTRACT

Shigella/Enteroinvasive *Escherichia coli* (EIEC) pathotype is a major enteropathogen associated with diarrhea and malnutrition in children from developing countries. This study aimed to correlate *Shigella*/EIEC virulence-related genes (VRGs) with clinical symptoms, nutritional status and coenteropathogens in children from the Brazilian semiarid region. We designed a case-control study of community diarrhea in six cities of the Brazil semiarid region with 1200 children aging 2–36 months. Standardized questionnaire was applied for collecting socio-demographic, nutritional status and clinical information of the children. DNA samples were extracted from stools and diagnosed for *Shigella*/EIEC using PCR-based approaches. Positive samples were tested for 28 VRGs using four multiplex PCRs. Intestinal inflammation was determined by measuring fecal myeloperoxidase (MPO). *Shigella*/EIEC pathotype was detected in 5% of the children and was significantly associated with diarrhea. The genes *sen* (encoding *Shigella* enterotoxin 2), *ipgB2*, *ipgB1* (both encoding type 3 secretion system-T3SS effectors that modulate actin filament), and *ospF* (encoding a T3SS effector involved in suppression of host responses) were further associated with diarrhea in *Shigella*/EIEC positive children. Among children presenting diarrhea, *virA* gene (encoding a T3SS effector that promotes microtubule destabilization) was associated with fever, while *virB* (encoding a major transcriptional activator) was associated with low height-for-age z-score. In addition, these VRGs were associated with increased fecal MPO, and coinfection with *Salmonella* spp. was associated with increased abdominal pain. These data reinforce the impact of *Shigella*/EIEC on diarrhea in children from Brazilian semiarid region and highlighted the contributions of specific virulence genes for its pathobiology.

1. Introduction

Bacteria of the *Shigella* genus are a leading cause of infectious diarrhea worldwide, particularly in children from developing countries (Liu et al., 2016). Enteroinvasive *Escherichia coli* (EIEC) is a diarrheagenic *E. coli* (DEC) that shares similar biochemical and pathogenesis features with *Shigella* (Maurelli, 2013). Although these pathogens are classically classified into two different genera based on historical and clinical reasons, several studies that have employed phylogenetic and genomic analyses do not support this distinction (Pupo et al., 2000; Lan et al., 2001; Escobar-Páramo et al., 2003; Lan et al., 2004; Peng et al., 2009; Pettengill et al., 2016).

A recent phylogenomic study using a large number of diverse *Shigella* and EIEC genomes showed that *Shigella* serogroups belong to the same lineage, which contradicts the classification of the *Shigella* genus into species/serogroups (Pettengill et al., 2016). Genomic studies that attempted to identify specific genes for both *Shigella* and EIEC were not able to find any reliable markers. In light of their pathogenesis and genetic similarities, *Shigella* and EIEC have been suggested to be grouped into a single pathovar *Shigella*/EIEC (Kaper et al., 2004; Lan et al., 2004; Pettengill et al., 2016).

The pathogenesis of the *Shigella*/EIEC pathotype is based on its capacity to reach and invade colonic epithelial cells, leading to intracellular multiplication and spreading to adjacent cells with consequent cell death. The major genes that facilitate invasion and spread of *Shigella*/EIEC into human macrophages and enterocytes are encoded by a large virulence plasmid (Belotserkovsky and Sansonetti, 2018). This virulence plasmid contains the conserved 30 kb *mix-spa* locus,

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which encodes the Mxi-Spa type three secretion system (T3SS) and its translocated effectors, such as Vir, Osp, Ipa, Ipg, and ShET2 (Mattock and Blocker, 2017). Several other virulence-related genes (VRGs) are located on the pathogenicity island of the chromosome, and these genes encode proteases (Pic and SigA), factors involved in iron acquisition (IucABDC and IutA), O-antigen conversion, antibiotic resistance, and the *Shigella* enterotoxin ShET1 (Mattock and Blocker, 2017).

There are major gaps in our understanding of the *Shigella*/EIEC infection burden and its virulence factors in the Brazilian semiarid region, mainly because of the paucity of studies. This region is one of the poorest areas of Brazil, with precarious sanitation and low socioeconomic status (Lima et al., 2019 – in under peer review). This study aimed to correlate *Shigella*/EIEC VRGs with clinical symptoms, nutritional status, and coenteropathogens in a case-controlled study of community-acquired diarrhea in children from the Brazilian semiarid region. In order to evaluate virulence determinants of *Shigella*/EIEC infection, we developed a broad multiplex PCR (mPCR) panel that was able to detect 28 VRGs.

2. Material and methods

2.1. Study design and ethical clearance

We conducted a community cross-sectional case-control study (Lima et al., 2019 – under peer review) design in six cities from the Northeast Region of Brazil: Cajazeiras (Paraiba), Crato (Ceará), Ouricuri (Pernambuco), Patos (Paraiba), Picos (Piauí), and Sousa (Paraiba). These cities were randomly selected; all of them present over 50,000 inhabitants and are in the Brazilian semiarid region.

Health workers collected fecal samples from children between 2–36 months old in health care units or during active surveillance from November 2009 to February 2012. The study had the following inclusion criteria for diarrhea cases: (1) three or more liquid stools in the last 24 h; (2) not hospitalized at health facilities for more than 12 h; (3) not transferred from another hospital; and (4) had provided written consent. Criteria for inclusion of controls: (1) did not present diarrhea in the last two weeks; and (2) had provided written consent. Demographic and socioeconomic information, as well as breastfeeding status, were collected from each child. A total of 1200 participants (596 cases and 604 controls, age and gender matched) were included in the study.

This research was approved by the National Commission on Ethics in Research of Brazil and the Research Ethics Committee of the Federal University of Ceará (craft no. 550/2006, protocol no. 238/05). Parents or guardians of the children provided written informed consent during fecal specimen collection.

2.2. Clinical outcomes, nutritional status, and intestinal inflammatory biomarker measurement

Clinical information (dehydration, fever, vomiting, inquietude, weakness, abdominal pain, respiratory symptoms, mucous, and bloody stools) of children was collected. This careful evaluation was based on the procedures utilized previously in a large multicenter study of diarrhea etiology in children from developing countries (The MAL-ED Network investigators, 2014). A standardized questionnaire was applied by previously trained clinical staff when interviewing the parents or guardians of the children. In addition, weight, length, and head circumference were collected for calculation of the following anthropometric z-scores: the weight-for-age (WAZ), height-for-age (HAZ), weight-for-length (WLZ), BMI for age (BAZ), and head circumference (HCZ) z-scores (World Health Organization, 2006). Undernourished children were defined as presenting anthropometric z-scores < -2 (World Health Organization, WHO, 2017). Fecal myeloperoxidase (MPO) was chosen as a measure of intestinal inflammation. Stools were tested for MPO by enzyme linked immunosorbent assay (ELISA) using the Immundiagnostik kit (Bensheim, Germany) according to the manufacturer's instructions.

2.3. Fecal DNA extraction and Shigella/EIEC molecular detection

All children had their stools processed for DNA extraction using the QIAamp DNA Stool Mini Kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. DNA quality and quantity were checked by the spectrophotometric method (NanoDrop 2000, ThermoFischer Scientific, Massachusetts, USA). Extracted DNA was stored at -20 °C until further use.

DNA samples were tested for a wide screening of 17 enteropathogens, including virus (rotavirus, norovirus, astrovirus, and sapovirus), bacteria (pathogenic *E. coli*, *Shigella* spp, *Salmonella* spp, *Campylobacter* spp, *Aeromonas* spp, and *Vibrio* spp) and protozoa (*Giardia* spp, *Cryptosporidium* spp, and *Entamoeba hystolitica*), using a PCR-Luminex platform (Bioplex 200 System – Bio-Rad), as previously described (Gondim et al., 2018). After screening for *Shigella*/EIEC, positive samples were confirmed by PCR targeting the invasion plasmid antigen (*ipaH*) gene with the GoTaqGreen kit (Promega, São Paulo, Brazil). PCR conditions were: one cycle for 5 min at 95 °C; 40 cycles for 30 s at 95 °C, 30 s at 60 °C, and 1 min at 72 °C; and a final extension step for 10 min at 72 °C in a thermal cycler (Bio-Rad Laboratories). Bands were visualized and photographed (ChemiDoc XRS; Bio-Rad Laboratories) after electrophoresis using ethidium bromide-stained 2% agarose gel in 1 × Tris-acetate-EDTA-buffer. The primers used are described in Table 1. DNA from the *Shigella flexneri* 2a strain 301 was used as a positive control, and autoclaved Milli-Q water was used as a negative control for the reactions.

2.4. Detection of Shigella/Enteroinvasive E. Coli virulence-related genes

The positive DNA fecal samples for *Shigella*/EIEC were tested for the presence of 28 VRGs. In order to identify these 28 VRGs, four mPCRs were developed for this study, and the other three mPCRs previously described (MEDEIROS et al., 2017) were used. The conditions of the cycles and primers are listed in Table 1.

Specific primers were sought among the nucleotide sequences of *Shigella* and EIEC available in GenBank (NCBI, Bethesda, Maryland, USA). The new primers were designed by Oligo Perfect Designer software (Thermo Fischer Scientific, Massachusetts, USA) and were synthesized by Invitrogen (São Paulo, Brazil), considering similar melting temperatures. Primer quality, including GC content, secondary structure, hetero- and homodimer formation, and off-target identities were analyzed using OligoAnalyzer version 3.1 software (Integrated DNA Technologies, Illinois, USA). In order to confirm the target specificity, a BLAST (Basic Local Alignment Search Tool, NCBI, Bethesda, Maryland, USA) search against the GenBank nucleotide database was performed. Prior to testing and optimization, all primers were evaluated by uniplex PCRs with their respective positive controls.

After optimization, each mPCR tube contained a 25-μL reaction mixture comprising 12.5 μL of master mix (Qiagen Multiplex PCR kit, Qiagen, California, USA), 2.5 μL of Q-solution (Qiagen, California, USA), and 2.0 μL of DNA, in addition to primers and water. All primer concentrations were 0.1 μmol/L, except for *iutA, ipaC,* and *virF* that were 0.2 μmol/L. PCR products were separated by electrophoresis on ethidium-bromide-stained 2% agarose gels and photographed (Chemidoc XRS, Bio-Rad Laboratories, California, USA). The following bacterial reference strains were used as positive controls: *Shigella flexneri* 2a 301 (*ipaH, ipaA-D, set, sen, ial, virB, virF, icsA, icsB, virA, ipgD, iucB, iutA, ospB, ospG, ipaH7.8, ipaH9.8, ipgB1, ipgB2, ospF, ospC1,* and *shiA*), enteroaggregative *Escherichia coli* H223-1 (*sigA, pic*, and *sepA*), and enterohemorrhagic *Escherichia coli* O157:H7 (*stx*). Autoclaved Milli-Q water was used as a negative control.

Table 1
PCR primers, amplifications conditions, and expected PCR products for *Shigella*/EIEC genes.

Target genes description (GenBank accesion number)	Primer sequence (5' – 3')	Amplicon size (pb)	Annealing temperature	Reference
Diagnostic gene				
ipaH – invasion plasmid antigen H (HE616529.1)	TGGAAAAACTCAGTGCCTCT CCAGTCCGTAAATTCATTCT	422	60 ºC	Luscher and Altwegg (1994)
Virulence genes				
Profile A - Multiplex 1				
sen– enterotoxin ShET2 (Z54211.1)	ATCTCCTTGAGGCCAGCAAA GGAAGGAATGGGAGGACGAA	296	58 ºC	Medeiros et al. (2017)
sigA– *Shigella* IgA-like protease (NC_004337)	CCGACTTCTCACTTTCTCCCGCC ATCCAGCTGCATAGTGTTTG	430		Boisen et al. (2009)
pic– protein involved in colonization (U35656.1)	ACTGGATCTTAAGGCTCAGGAT GACTTAATGTCACTGTTCAGCG	570		Restieri et al. (2007)
sepA- *Shigella* extracellular protease (Z48219.1)	GCAGTGGAAATATGATGCGGCTT GTTCAGATCGGAGAAGAACG	794		Restieri et al. (2007)
Profile B - Multiplex 2				
icsA – actin polymerization (AF336770.1)	CCAACCCCTCTCATGCAT ATCACCAGCACCACCATGAC	83	60 ºC	Medeiros et al. (2017)
stx –Shiga toxin (X07903.1)	TTCTGGGAAGCGTGGCATTA CATCAGAATTGCCCCCAGAG	167		Medeiros et al. (2017)
icsB- prevention of autophagic recognition of *icsA* (M86530.1)	GGCCTGCATCAAGTCTTTCG GGCATCGGTACAGCCAAAAA	280		Medeiros et al. (2017)
virB- *ial* regulator (CP001384.1)	CGCGCGAGACAGATTCTCTT TGGTGGATTTGTGCAACGAC	488		Medeiros et al. (2017)
Profile C – Multiplex 2				
ipaC- invasion plasmid antigen C (AF386526.1)	CCTCACCACAAACTAACTCTAGCA AGAAGTTTTATGTTCAGTTGACAGGGATA	93	60 ºC	Boisen et al. (2009)
ipaB– invasion plasmid antigen B (X60777.1)	CAAGCCCTGAATCCGATCAT TGCTGCTGCCTGTTTACCAA	204		Medeiros et al. (2017)
ipaD- invasion plasmid antigen D (AF386526.1)	AAGAAGCCGAGCTTGATGGAG CCTCGCCATTTCCACCTAGA	450		Medeiros et al. (2017)
ipaA- invasion plasmid antigen A (CP000039.1)	CCTGTGTCCCCGAGAAAGAGA TGACGCACAGGCAAAACTTG	628		Medeiros et al. (2017)
Profile D – Multiplex 2				
ipgD- invasion plasmid gene D (AF386526.1)	GAAACCGGAAAGCACAAAGG CTGTCACGCGCAAACAAAAG	149	60 ºC	This study
iucB – aerobactin synthesis (AY277720.1)	CCTCCTGTTCCTGCTTCACC TGACGGCCTTGTAAGCTCAA	270		This study
iutA – complex siderophore-iron receptor (AE005674.2)	AGTATATGCCCCGGGCTCTT CAATGCGCTCATCGTGATCT	528		This study
virA- microtubule desestabilization (AF386526.1)	AAGCCCTTCACTGCTTGGAA ACTCACAAGCCTGCACCAGA	702		This study
Profile E – Multiplex 2				
ospB- outer *Shigella* protein B (AF386526.1)	TTCTTGGGCACGGTAGTCCT ACTTTCAGCAGGGGCATTGT	185	60 ºC	This study
ospG- inhibition of NFκB activation (AF386526.1)	AGTAACGGAGCCCATTCTCG GCAGCATTCGGAGGTACACA	230		This study
ipaH7.8- Macrophage pyroptosis promotion (AF386526.1)	ACCACGGCCCACAGATTTAC AAAGGCCTTCTGATGCCTGA	387		This study
IpaH9.8- inhibition of NFκB activation (AF386526.1)	CTGCCAGCTTTACCCGATTC AAAGGCCTTCTGATGCCTGA	691		This study
Profile F – Multiplex 2				
ipgB2- invasion plasmid gene B2 (AF386526.1)	CCGCTGTACAATGGGGAAAA TTGGTTTACCACCCGGGATA	151	60 ºC	This study
ospF- outer *Shigella* protein F (AF386526.1)	GCATCGAACGTGCCAACTTA CCCACACGAGATTGCTGAGA	284		This study
ipgB1- invasion plasmid gene B2 (AF386526.1)	CCTCGCCCATCATTTGATTC TTTGCTCTGAGGCCAGATGA	404		This study
ospC1- outer protein C1 (AF386526.1)	GATGCACCATCAGACGCTAAAG CAGATCCACAGGGGCAAGAT	675		This study
Profile G – Multiplex 3				
shiA- shiA like inflammation suppressor (AE005674.2)	CTGTGTGGCATGACTTCTCC CAGGTGCTCCTGCTGTCTTA	227	57 ºC	This study
ial- invasion-associated loci (CP001384.1)	CTGGATGGTATGGTGAGG GGAGGCCAACAATTATTTCC	320		Frankel et al. (1989)
set- enterotoxin ShET1 (Z47381.1)	TCCCTTCATACTGGCTCCTG AACACTCTGTGGGGGAACAG	553		Fárfan et al. (2010)
virF- *virB* regulator (AY206433.1)	GCTCAGGCAATGAAACTTTGAC TGGGCTTGATATTCCGATAAGTC	618		Vidal et al. (2005)

The mPCRs conditions for the amplification of the VRGs were optimized for: 95 °C for 15 min; 35 cycles of 94 °C for 45 s, annealing temperature for 45 s (variable) and 72 °C for 1 min; and 72 °C for 10 min.

2.5. Statistical analysis

Data were typed into an Excel spreadsheet v. 4.0 (Microsoft Corp., Seattle, WA) by two independent data entry workers and then verified by comparing these worksheets. Statistical analyses were performed using the Statistical Package for Social Science v. 20.0 (SPSS, Inc., Chicago, IL). For the qualitative variables, the chi-square test or Fisher's test was used, while the Mann-Whitney test was employed for quantitative variables. Graph Pad Prism software version 6.00 for Windows (San Diego, California, USA) was used for complementary statistical

analysis, table formatting, and figures. Z-scores were calculated by EpiInfo software v. 6.0 (Center for Disease Control, Atlanta, GA) using the World Health Organization Multi-Country Growth Reference Study (World Health Organization, 2006).

In order to investigate the association of clinical manifestations with combinations of coenteropathogens or VRGs, Classification and Regression Tree Analysis (CART, Salford Systems, USA) was employed. This approach involves the construction of models in a stepwise fashion that show the combinations of factors that are most strongly associated with symptoms. The odds ratios (ORs) with 95% confidence intervals (95% CI) are shown to indicate the risk found between a variable and the outcome analyzed. A significance level of < 0.05 was used in the statistical analysis.

3. Results

3.1. Molecular detection of the Shigella/EIEC pathotype

The *Shigella*/EIEC pathotype was detected in 5% (60/1200) of the total population. Among cases, *Shigella*/EIEC was found in 7.05% (42/596), while the detection rate was of 2.98% (18/604) in the control group. The presence of *Shigella*/EIEC was associated with the diarrhea case definition (p = 0.0013; OR = 2.47 and 95% CI = 1.4–4.3).

3.2. Characterization of Shigella/EIEC-positive population

Among the 60 *Shigella*/EIEC-positive children, the majority were male (61.3%, 37/60), older than 12 months (76.6%, 46/60) (mean = 18.88 months and median = 19 months), and had mixed breastfeeding (91.6%, 55/60). The families of these children had a monthly average income of US$ 506.90 (equivalent to R$ 1090.00 Brazilian currency according to the mean exchange rate at the time of the study). Almost all of their mothers had some degree of education (94.9%, 57/60), but none had completed graduation. Regarding sanitation conditions, 56.7% (34/60) of the children's households had suitable sanitation and 88.3% (53/60) had a sanitary latrine at their household (Table 2).

Regarding the clinical signals and symptoms evaluation, each of these were observed in less than 50% of the children analyzed. The highest prevalence was observed for dehydration (47.8%, 20/42), followed by vomiting and seizure (both 35.7%, 15/42). The lowest prevalence was observed for bloody stools (4.76%, 2/42) (Table 3). For the nutritional status analysis, no significant differences between cases and controls were observed.

Table 2
Characteristics of *Shigella*/EIEC positive children from this study.

Parameters	Total N = 60 (%)
Gender	
Male	37 (61.3%)
Female	23 (38.3%)
Age (months)	
> 2 < 6	2 (3.33%)
> 6 < 12	12 (20%)
> 12 < 24	25 (41.6%)
> 24 < 36	21 (35%)
Breastfeeding status	
Exclusive breastfeeding	0%
Mixed breastfeeding	27 (45%)
No breastfeeding	5 (8.33%)
Family monthly income (of minimum wages)	
≤ ½ ≤ 2	54 (90%)
2 ≤ 5	5 (8.33%)
≥ 5	1 (1.67%)
Maternal education degree	
None degree	3 (5%)
Some degree	57 (94.9%)
Graduated	0 (0%)
Sanitation conditions	
Suitable sanitation	34 (56.7%)
Public sewage	20 (33.3%)
No sewage	6 (10%)
Presence of sanitary latrine	
Yes	53 (88.3%)
No	7 (11.7%)

Table 3
Clinical signals and symptoms reported from children infected with *Shigella*/EIEC.

Clinical manifestation	Cases N = 42 (%)
Dehydration	20 (47.6)
Fever	16 (38.1)
Vomiting	15 (35.7)
Inquietude	15 (35.7)
Weakness	14 (33.3)
Abdominal pain	11 (26.2)
Cough or other respiratory symptoms	10 (23.8)
Mucous stools	8 (19)
Bloody stools	2 (4.76)

3.3. Shigella/EIEC virulence-related genes distribution and correlation with clinical measures and inflammation

After optimization, these mPCRs were used for detection of *Shigella*/EIEC VRGs in the fecal DNA samples positive for *Shigella*/EIEC. Individual frequencies of each gene are shown in Table 4. The results from amplification reactions of chromosomal and virulence plasmid-encoded genes showed that the chromosomal gene *iucB* that encodes for the siderophore aerobactin was the most frequently detected (90%, 54/60), followed by *iutA,* which encodes for siderophore-iron receptor (83.3%, 50/60). Low prevalence rates were observed for plasmid-borne genes encoding TS33 effectors *ipgD* (26.6%, 16/60) and *ipaC* (21.6%, 13/60). Only one DNA fecal sample was negative for all 28 VRGs analyzed.

Considering VRG distribution between cases and controls, the genes *sen* (p = 0.0267; OR = 4.25; 95% CI = 1.27–14.15), *ipgB2* (p = 0.0267; OR = 4.25; 95% CI = 1.27–14.15), *ospF* (p = 0.0354; OR = 3.66; 95% CI = 1.12–11.9), and *ipgB1* (p = 0.0354; OR = 3.66; 95% CI = 1.12–11.9) were significantly associated with diarrhea by univariable analysis (Table 4). Further, the gene *sen* (p = 0.033; OR = 3.96; 95% CI = 1.12–13.98) was also associated with diarrhea when analyzed between the other genes that encoded enterotoxin factors by multivariable regression logistic analysis (Table 4).

When analyzing whether VRGs were correlated with clinical symptoms associated with diarrhea, we found that fever was strongly associated with infection by the *Shigella*/EIEC pathotype harboring the *virA* gene (p = 0.0005; OR = 33; 95% CI = 1.79–608). Indeed, all children who reported fever were infected with the *virA*-positive pathotype (100%, 16/16). Further investigation using CART analysis of VRG combinations that could be associated with clinical symptoms did not show any significant findings. The *virB* gene was associated with the lower 25th percentile of HAZ values (p = 0.0275; OR = 7.11, 95% CI = 1.422–36.7) regardless of the presence of diarrhea, and this finding was not significant when analyzing only children without diarrhea. In addition, the mean of the HAZ values for children that were positive for the *virB* gene was -2.51 (reflecting undernutrition), and this was -1.26 for children that were negative for the *virB* gene.

Further, children infected with strains harboring the VRGs associated with clinical outcomes were assessed regarding the levels of the intestinal inflammatory biomarker MPO. Consistently, MPO levels were increased in children infected with strains harboring *ipgB1* (p = 0.0003), *ipgB2* (p = 0.0003), *ospF* (p = 0.0003), *sen* (p = 0.0003), *virA* (p = 0.0197), and *virB* (p = 0.0018), when compared with children negative for each of these VRGs, respectively.

Table 4
Distribution of *Shigella*/EIEC virulence-related genes (VRG) in DNA fecal samples from case and control children and its variable analysis.

VRGs	Encoded factors	Cases N = 42 (%)	Controls N = 18 (%)	Total N = 60 (%)	Univariable P value/OR/95%IC	Multivariable P value/OR/95%IC
sen	Enterotoxins	34 (80.9%)	9 (50%)	43 (71.6%)	0.0267*/4.25/1.27-14.15	0.033**/3.96/1.12-13,98
stx		0 (0%)	1 (5.5%)	1 (1.67%)	0.3000/0.13/0.005 – 3.55	1,000/0/0-0
set		24 (57.1%)	6 (33.3%)	30 (50%)	0.1581/2.66/0.84 – 8.46	0,164/2,465/0,691-8,795
sigA	Proteases	31 (73.8%)	11 (61.1%)	42 (70%)	0.3669/1.79/0.55 – 5.87	0,285/2,008/0,559-7,209
pic		21 (50%)	9 (50%)	30 (50%)	1.0000/1.00/0.33 – 3.01	0,552/0,665/0,173-2,557
sepA		19 (45.2%)	7 (38.8%)	26 (43.3%)	0.7785/1.30/0.42 – 4.00	0,577/1,437/0,402-5,136
iucB	Iron absorption	40 (95.2%)	14 (77.7%)	54 (90%)	0.0602/5.71/0.94 – 34.7	0,600/2,000/0,150-26,734
iutA		38 (90.5%)	12 (66.6%)	50 (83.3%)	0.0524/4.75/1.14 – 19.7	0,274/3,167/0,402-24,961
shiA	Immunomodulators	21 (50%)	7 (38.8%)	28 (46.6%)	0.5738/1.57/0.51 – 4.83	NR
ipaA	T3SS effectors	16 (38.1%)	2 (11.1%)	18 (30%)	0.0629/4.92/0.99 – 24.3	0,337/3,920/0,242-63,617
ipaB		26 (61.9%)	8 (44.4%)	34 (56.7%)	0.2614/2.03/0.66 – 6.22	0,848/1,617/0,012-222,074
ipaC		12 (28.6%)	1 (5.5%)	13 (21.6%)	0.0841/6.80/0.81 – 56.9	0,796/1,579/0,049-50,607
ipaD		17 (40.5%)	6 (33.3%)	23 (38.3%)	0.7734/1.36/0.43 – 4.33	0,521/0,449/,039-5,172
ipgD		12 (28.6%)	4 (22.2%)	16 (26.6%)	0.7549/1.40/0.38 – 5.12	1,000/0,316/0-0
virA		29 (69%)	8 (44.4%)	37 (61.6%)	0.0887/2.78/0.89 – 8.69	0,230/7,268/0,285-185,499
icsA		29 (69%)	10 (55.5%)	39 (65%)	1.0000/1.09/0.34 – 2.96	1,000/0/0-0
icsB		31 (73.8%)	11 (61.1%)	42 (70%)	0.3669/1.79/0.55 – 5.87	1,000/0/0-0
ospB		26 (61.9%)	8 (44.4%)	34 (56.6%)	0.2614/2.03/0.66 – 6.22	0,999/0/0-0
ospG		27 (64.3%)	10 (55.5%)	37 (61.6%)	0.5712/1.44/0.46 – 4.43	0,999/0/0-0
ospF		33 (78.6%)	9 (50%)	42 (70%)	0.0354*/3.66/1.12-11.9	1,000/1,031/0-0
ospC1		34 (80.9%)	10 (55.5%)	44 (73.3%)	0.0580/3.40/1.01 – 11.4	1,000/0/0-0
ipaH7.8		34 (81%)	10 (55.5%)	44 (73.3%)	0.0580/3.40/1.01 – 11.3	1,000/ - /0-0
ipaH9.8		28 (66.6%)	8 (44.4%)	36 (60%)	0.1517/2.50/0.80 – 7.73	1,000/ - /0-0
ipgB1		33 (78.6%)	9 (50%)	42 (70%)	0.0354*/3.66/1.12-11.9	1,000/2,642/0-0
ipgB2		34 (80.9%)	9 (50%)	43 (71.6%)	0.0267*/4.25/1.27-14.1	1,000/0/0-0
ial		33 (78.6%)	9 (50%)	42 (70%)	0.4047/1.73/0.58 – 5.13	0,729/0,546/0,018-16,758
virB	Regulators	31 (73.8%)	11 (61.1%)	42 (70%)	0.3669/1.79/0.55 – 5.87	0,877/0,886/0,191-4,099
virF		31 (73.8%)	9 (50%)	40 (66.6%)	0.1335/2.81/0.89 – 8.92	0,141/1,268/0,692-13,282

*Associated with cases, P < 0.05 by Fischer's exact test when compared to controls. ** Associated with cases, P < 0.05 by Hosmer and Lemeshow Test when compared to controls.

3.4. *Shigella*/EIEC and coenteropathogen subclinical and clinical enteric infection

From the total of *Shigella*/EIEC-positive samples, 91.6% (55/60) were positive for at least one other coenteropathogen (Table 5). The highest prevalence was observed for the coinfection with enteroaggregative *Escherichia coli* (EAEC) (43.3%, 26/60), followed by *Salmonella* spp. (40%, 24/60). The lowest prevalence was observed for coinfections with enterohemorrhagic *Escherichia coli* (EHEC), adenovirus, and norovirus (all 1.66%, 1/60). No coinfection was observed with *Entamoeba* spp. Regarding the distribution of coinfections between cases and controls, both had high prevalence rates of coinfections: 92.8% (39/42) and 88% (16/18) were positive for other coenteropathogens in cases and controls, respectively.

When evaluating whether any coinfection could be associated with clinical signals or symptoms, we found that abdominal pain was associated with coinfection with *Salmonella* spp (p = 0.0262; OR = 5.75; 95% CI = 1.362–20.73). No single coenteropathogen was significantly associated with diarrhea or any other clinical signals or symptoms evaluated. Further investigation using CART analysis did not show any coenteropathogen combinations that were significantly associated with clinical symptoms.

Table 5
Prevalence of coinfections in children *Shigella*/EIEC positive.

Coenteropathogens	Cases N = 42 (%)	Controls N = 18 (%)	Total N = 60 (%)	P value
EAEC	20 (47.6)	6 (33.3)	26 (43.3)	0.3979
EHEC	1 (2.38)	0 (0)	1 (1.66)	0.9999
EPEC	13 (30.9)	6 (33.3)	19 (31.6)	0.9999
ETEC	6 (14.3)	3 (16.6)	9 (15)	0.9999
Adenovírus	1 (2.38)	0 (0)	1 (1.66)	0.9999
Astrovírus	2 (4.76)	1 (5.55)	3 (5)	0.9999
Norovírus	1 (2.38)	0 (0)	1 (1.66)	0.9999
Rotavírus	2 (4.76)	0 (0)	2 (3.33)	0.9999
Sapovírus	1 (2.38)	1 (5.55)	2 (3.33)	0.5136
Salmonella spp.	14 (33.3)	10 (55.5)	24 (40)	0.1517
Campylobacter spp.	5 (11.9)	3 (16.6)	8 (13.3)	0.6863
Aeromonas spp.	2 (4.76)	3 (16.6)	5 (8.33)	0.1537
Vibrio cholera	1 (2.38)	2 (11.1)	3 (5)	0.2116
Entamoeba spp.	0 (0)	0 (0)	0 (0)	0.9999
Cryptosporidium spp	2 (4.76)	3 (16.6)	5 (8.33)	0.1537
Giardia lamblia	16 (38.1)	4 (22.2)	20 (33.3)	0.3705

EPEC - enteropathogenic *E coli*; EAEC - enteroaggregative *Escherichia coli*; EIEC - enteroinvasive *E coli*; ETEC - enterotoxigenic *E coli*; STEC - Shiga-toxin-producing *E coli*.

4. Discussion

This study evaluated *Shigella*/EIEC VRG association with clinical symptoms, nutritional status, and coenteropathogens in children. We used a case-controlled design of community diarrhea with children ages 2–36 months in the Brazilian semiarid region. This is the first time the burden of *Shigella*/EIEC infection was investigated in this region, which has the lowest income and highest social inequity (IPEA (Institute of Economic and Applied Research), 2015) and certainly accounts for the larger proportion of enteric infections burden in Brazil (Mendes et al., 2013). This study examined children with community diarrhea who did not require hospitalization. Classical studies have evaluated more severe infections that lead to hospitalizations, but the burden of community diarrhea on children's health was recently highlighted in a large cohort of children from eight different countries (MAL-ED network) (Platts-Mills et al., 2015).

Studies have supported the recognition of *Shigella*/EIEC as a single pathovar based on genomic analysis (Lan et al., 2004; Kaper et al., 2004; Pettengill et al., 2016). Although taxonomic revisions may be difficult, Pettengill and colleagues support that this change would improve outbreak characterizations and communication in the long-term (Pettengill et al., 2016). Accordingly, the use of *ipaH*-gene positive criteria for detecting *Shigella*/EIEC pathogens has already been

indicated in some recent important studies of enteric infection etiology (Platts-Mills et al., 2017; Liu et al., 2016). Further, metagenomic sequencing supported this approach, indicating that genetic sequences from these samples are similar to those classically identified as *Shigella* (Liu et al., 2018).

The *Shigella*/EIEC pathotype was associated with diarrhea in a large multicenter case-control study of moderate to severe diarrhea in Africa and Asia (Liu et al., 2016). Corroborating this finding, the present study showed an association between *Shigella*/EIEC diagnosis and diarrhea in the Brazilian semiarid region. Altogether, these data reinforce the importance of the *Shigella*/EIEC pathovar for causing diarrhea in children and support molecular diagnosis, as well as alerts for the need for vaccine development (Kotloff et al., 2017). Of note, the complete characterization of diarrhea etiology in the study population has been addressed separately (Lima et al., 2019 – in under peer review).

Several attempts to understand *Shigella* pathobiology using virulence gene detection in clinical samples have been made (Casabonne et al., 2016; Nave et al., 2016; Medeiros et al., 2017). In Brazil, some studies were performed, but just a few VRGs were evaluated (Cruz et al., 2014; Silva et al., 2008; Sousa et al., 2013). Recently, we applied a 16 VRG diagnostic panel for *Shigella* spp. isolated from children with moderate to severe diarrhea in Fortaleza, a city in northeastern Brazil (Medeiros et al., 2017). In this study, we expanded the detection panel to 28 VRGs and applied it in a large case-controlled study of community diarrhea in the Brazilian semiarid region. Remarkably, some VRGs investigated here had not been previously detected in clinical surveys, such as *ipgB1, ipgB2, ospF, iutA, ospB, ospG, ipaH7.8, ipaH9.8, ospC1* that encodes for T3SS effectors, and *shiA* that encodes for immunomodulatory factors (Mattock and Blocker, 2017). While genomic approaches are the gold standard, the use of broader PCR panels of genetic markers that cover diverse microbial virulence strategies should contribute to a better understanding of the pathobiology in the *Shigella*/EIEC infection, especially in low income countries, because of their low cost, rapidity, and simple-to-operate protocols.

Some VRGs in this study were associated with diarrhea: *sen*, *ipgB1*, *ipgB2*, and *ospF*, which reflected specific biological functions. The gene *sen* (or *ospD3*), which encodes *Shigella* enterotoxin 2 – ShET2, has been previously associated with bloody diarrhea in children from Brazil and Iran (Cruz et al., 2014; Yaghoubi et al., 2017). Other studies have shown high prevalence rates of *sen* in *Shigella* isolates with diarrhea (Zhang et al., 2014). Thus, our findings reinforce *sen* as a major contributor to severe *Shigella*/EIEC infections. In addition, using multivariate analysis within the enterotoxins encoding genes, the major contribution of ShET2 to diarrhea among other enterotoxins was further reinforced. In our previous study that evaluated only isolated *Shigella* spp. from children with moderate to severe diarrhea, the gene *sen* was present in all samples, reinforcing the role of *sen* in diarrhea and reflecting differences in populations between the studies (Medeiros et al., 2017).

Of note, three other genes (*ipgB1*, *ipgB2*, and *ospF)* were associated with diarrhea for the first time. IpgB1 and IpgB2 are both effectors secreted by T3SS that promote cell invasion by modulating actin filament structures (Mattock and Blocker, 2017). Interestingly, it has been suggested that isolates harboring invasive genes are associated with the ability to cause diarrhea (Fan et al., 2017). OspF promotes suppression of the innate immune response in epithelial cells (Arbibe et al., 2007). Our data suggest that these mechanisms might play major roles in diarrhea. Importantly, the presence of these VRGs was correlated with increased MPO, a major marker of intestinal inflammation (Guerrant et al., 2016; Kosek, 2017).

We further investigated whether specific VRGs could correlate with clinical severity of diarrhea, defined by the presence of fever, abdominal pain, vomiting, and bloody stools. The only gene significantly associated with a clinical outcome such as fever was *virA*. VirA is an effector which degrades microtubules by means of cysteine protease-like activity, being critical for intra and intercellular spreading (Yoshida et al., 2006). Zhang et al. (2014) detected the *virA* gene in 100% of highly virulent strains and suggested its essential role in pathogenesis. Al-Talib et al. (2014) used the *virA* gene for detection of *Shigella* spp by mPCR assay, and all of these strains were positive. High prevalence rates of the *virA* gene in children with diarrhea were reported (Lluque et al., 2015). Our results further corroborate *virA* as a major virulence factor associated with clinical severity of *Shigella*/EIEC diarrhea.

In addition to assessing VRG associations with diarrhea with the *Shigella*/EIEC pathotype, we evaluated the correlation of genes with anthropometric z-scores. *Shigella* has been shown to be associated with decreased linear growth in children from rural Bangladesh (Lee et al., 2014). Our findings showed an association between children infected with *Shigella*/EIEC harboring *virB* and undernutrition in the total *Shigella*/EIEC positive population, but not within the non-diarrhea subgroup. The gene *virB* is a major transcriptional activator, which regulates expression of *ipa*, *mxi*, and *spa* virulence operons and consequently genes of the entry region (Mattock and Blocker, 2017). This is the first study that associates a specific *Shigella*/EIEC genetic marker with undernutrition. Of note, *virB* was found in almost 100% of the samples from our previous study that evaluated only isolated Shigella from children with moderate to severe diarrhea (Medeiros et al., 2017). Interestingly, we observed high rates of VRGs in children without diarrhea, albeit less than in diarrheal cases for all VRGs. A previous study in children from India showed similar findings (Ghosh et al., 2014). Subclinical *Shigella*/EIEC infections have recently been shown to lead to stunted growth and intestinal inflammation in children from developing countries, as reported in a multicenter longitudinal birth cohort (Kosek, 2017; Rogaswki et al., 2018). Further studies are necessary to investigate whether specific microbial virulence factors might play a role in these silent subclinical enteric infection consequences.

The high rates of copathogens of *Shigella*/EIEC infections in children from our study population is in agreement with high environmental contamination reported in these settings (Lima et al., 2019 – in under peer review). In addition, the potential harmful consequences of coinfections have been suggested in several studies (Valentini et al., 2013; Liu et al., 2016; Lima et al., 2017). However, little is known about which coinfection combinations may be more clinically important (Liu et al., 2016; Lima et al., 2017). EAEC was the most prevalent copathogen in this study, which corroborates previous studies on the etiology of enteric infections in children (Platts-Mills et al., 2015; Lima et al., 2017). In our data, the presence of *Salmonella* spp. was associated with *Shigella*/EIEC-infected children presenting abdominal pain. *Salmonella* spp. is also one of the most important causes of bacterial diarrhea worldwide (Eng et al., 2015). These pathogens share similar pathogenesis mechanisms (Dekker and Frank, 2015; Mama and Alemu, 2016) and might act in synergism for causing disease. This potential interaction could contribute to more severe diarrheal cases and should be analyzed in the future.

This study has some limitations. The use of bacterial isolates would provide results that are more precise. Some virulence genes might be shared by other Enterobacteriacea and cannot be interpreted from single bacteria. However, the clear polymicrobial nature of the infections presented by these children indicates that diagnosing these genes from isolated bacteria would still not reflect the reality of enteric infections in children from developing countries. Further, in recognition of the greater prevalence of *Shigella*/EIEC due to molecular techniques, detecting virulence genes from fecal DNA can provide useful information about *Shigella*/EIEC virulence strategies and their consequences to children. Moreover, the small sample size hampered the possibility of more robust analysis. However, the data collected was still able to provide significant, clinically relevant findings, especially due to the previous lack of investigations of microbial virulence genes from the stools of children from developing settings.

5. Conclusions

We developed a broad mPCR panel of VRGs that can be further applied in other settings and help with the understanding of *Shigella*/EIEC pathobiology. This study further corroborates *Shigella*/EIEC as a major enteric pathotype associated with diarrhea in children from the Brazilian semiarid region, and suggests the T3SS effectors ShET2, IpgB1, IpgB2, and OspF as major contributors to this outcome Moreover, the effector VirA was associated with diarrhea-associated fever, and the effector VirB was associated with lower values of the z-score HAZ. Coinfection with *Salmonella* spp. may be an indicator of more severe *Shigella*/EIEC infections. Altogether, these data provide a better understanding of *Shigella*/EIEC pathobiology, identifying specific virulence strategies of clinical relevance.

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