Scientific Research Publishing

Advances in Infectious Diseases, 2018, 8, 200-216
http://www.scirp.org/journal/aid
ISSN Online: 2164-2656
ISSN Print: 2164-2648

Brazilian Indigenous Children as Carriers of Diarrheagenic *Escherichia coli* Pathotypes

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How to cite this paper: Coelho, C.V.L., Gomes, T.A.T., Vieira, M.A.M., Rosa, A.C.P., Marinho, D.P., Von Söhsten, B.L., Sanfins, C.S., Périssé, A.R.S. and Regua-Mangia, A.H. (2018) Brazilian Indigenous Children as Carriers of Diarrheagenic *Escherichia coli* Pathotypes. *Advances in Infectious Diseases*, **8**, 200-216.
https://doi.org/10.4236/aid.2018.84017

Received: September 25, 2018
Accepted: November 13, 2018
Published: November 16, 2018

Abstract

Introduction: Diarrheagenic *Escherichia coli* (DEC) is a relevant cause of diarrhea, particularly among infants and young children in developing countries. Methodology: We compared the frequency, antimicrobial resistance, adherence, enterovirulence and genetic diversity of DEC isolates from Guarani indigenous population under five living in distinct villages in Brazil. Results: Of the 314 *E. coli* isolates from 57 children, with and without diarrhea, 15% (48/314) were classified in DEC categories: aEPEC (56%, 27/48), EAEC (35%, 17/48) and ETEC (8%, 4/48). ETEC belonged to plylogroup A, EAEC to groups A, B1, B2 and D, and aEPEC to phylogroups A, B1, and B2. EAEC exhibited the aggregative adherence phenotype while ETEC and aEPEC the aggregative and undefined patterns. Multidrug-resistance was detected in aEPEC, ETEC and EAEC while extensive drug-resistance was found in EAEC and aEPEC. RAPD typing revealed a genetically diverse bacterial population. Conclusion: This is the first report regarding aspects of DEC in an indigenous Brazilian population, showing that Guarani children are DEC carriers and that antimicrobial resistance at high levels is widely disseminated among these enteropathogens.

Keywords

Escherichia coli, Brazilian Indians, Virulence, Antimicrobial Resistance, Diarrheal Disease

1. Introduction

Diarrheal disease remains a relevant cause of morbidity and mortality among

children, especially in nations marked by socioeconomic inequality, poor public health and inadequate sanitation service coverage [1] [2]. Epidemiological studies have pointed to the persistence of large health disparities between Indigenous and non-Indigenous peoples, even in countries, which have developed their own culturally health indicator frameworks [3] [4] [5] [6]. Despite differences in scale, Indigenous communities are also accompanied by poorer infrastructure, health care access and higher social inequity [3] [7]. According to the First National Survey of Indigenous People's Health and Nutrition in Brazil, the overall prevalence of diarrhea was 23.5% among children under five [8]. Higher risk of diarrhea was observed among younger children and those who had less maternal care, lower household socioeconomic status, malnutrition, and occurrence of upper respiratory infection [4] [7] [8].

Several studies have shown the relevance of *Escherichia coli* in the etiology of diarrheal disease worldwide. Intestinal *E. coli* pathotypes (or diarrheagenic *E. coli*, DEC) cause significant morbidity and mortality worldwide in children under 5 years of age, especially in the developing world [9] [10] [11]. DEC comprises important agents of endemic and epidemic diarrhea worldwide, associated with asymptomatic carriage or clinically symptomatic disease related to acute and persistent diarrhea [9] [11] [12] [13].

Diarrheagenic *E. coli* belong to at least six *E. coli* pathotypes that present a characteristic set of virulence factors responsible for different pathomechanism of infections. These pathotypes include: enterotoxigenic *E. coli* (ETEC), enteroinvasive *E. coli* (EIEC), enteroaggregative *E. coli* (EAEC), shiga toxin-producing *E. coli* (STEC), enteropathogenic *E. coli* (EPEC), and diffusely adherent *E. coli* (DAEC) pathotype. Besides the typical forms, which characterize each pathogenic group, antigenic and genetic variants can be found characterizing atypical lineages [12] [13].

Phylogroup characterization of *E. coli* also provides insight into virulence potential and is also useful for identifying human health risks [13]. Commensal isolates mostly group into phylogroups A and B1, extraintestinal *E. coli* strains are derived from group B2 and to a lesser extent from D, whereas *E. coli* strains carrying diarrheagenic markers may be distributed across all phylogroups. Phylogroups differ according to metabolic properties, ecological niches, life-history characteristics and propensity to cause diseases [13].

Assessment of virulence and resistance profiles in clinical and environmental *E. coli* has revealed a high prevalence of antimicrobial resistance and the occurrence of the multidrug-resistant and possible extensively drug-resistant bacteria within phylogroups or among strains carrying diarrheagenic markers [14] [15] [16]. However, such properties among *E. coli* isolates from the Indigenous population is not documented and requires special attention given the vulnerability and risk of exposure of the population being studied [5] [8].

Diarrheagenic *E. coli* (DEC) is a group of microorganisms well known for its pathogenic role and associated severity. The current study describes the genotypic and phenotypic characteristics of *E. coli* isolates from the Indigenous pop-

ulation under five, belonging to the Guarani ethnic group in Brazil.

2. Method

2.1. Indigenous Population

The indigenous population included in this study lives in communities in the State of Rio de Janeiro, and belongs to the Guarani ethnic group. It is distributed in five villages and two municipalities, Angra dos Reis (Sapukai village) and Paraty (Paraty-Mirim, Araponga, Rio Pequeno and Mamanguá villages) (**Figure 1**). The villages have an irregular water supply, besides precarious sewage treatment and waste collection. The Guarani are a semi-nomadic ethnic group, and the intense migration between villages belonging to the same ethnic group is a cultural characteristic that increases their vulnerability to infectious and parasitic diseases [17]. The migration occurs between several Brazilian states, especially those located in the south and southeast regions, and South American countries (Argentina, Paraguay and Uruguay), which enables them to maintain relationships with relatives living in other villages. Organized health services are offered on a permanent basis, but medical care also occurs by spontaneous demand. Villages from the municipality of Paraty have a population of around 180 people, divided into 32 residences. The Sapukai village (Angra dos Reis municipality) is made up of 67 residences, housing around 360 indigenous people.

2.2. Bacterial Samples and Participants

A total of 314 *E. coli* isolates were included in the present study. Clinical samples were recovered during an active search aiming to outline the epidemiological profile of infectious diseases in the Guarani population living in the Araponga, Mamanguá, Rio Pequeno, Sapukai and Paraty-Mirim villages, in Rio de Janeiro state [17]. *E. coli* isolates were obtained from 57 children younger than 5 years old, with (n = 10) and without (n = 47) diarrhea. Children who manifested an increased number of evacuations, with watery feces or with little consistency, showing the presence or not of mucous and/or blood, following observation and reporting by the person responsible at the time of collection of the material were considered to be a case. Diarrhea was considered to be a persistent diarrheic condition if it had a duration of 14 days or more. Children who did not manifest a diarrheic condition within the period that preceded the collection of material by at least 30 days were considered part of the control group. Stool samples were collected and placed in Clair-Blair transport medium and transported in iced boxes within 4 h to the laboratory. Samples were incubated in 3 ml of Tryptic Soy Broth (TSB, Difco) for 18 to 24 h at 37˚C. Following the incubation period, an aliquot of the bacterial growth was streaked onto Eosin Methylene Blue agar (EMB, Difco) and incubated at 37˚C for 18 to 24 h. Characteristics of the colonies were recorded. Up to ten 10 lactose positive and two lactose negative bacterial colonies were selected based on morphological and physiological characteristics suggestive of species. Putative *E. coli* colonies were tested for species-level

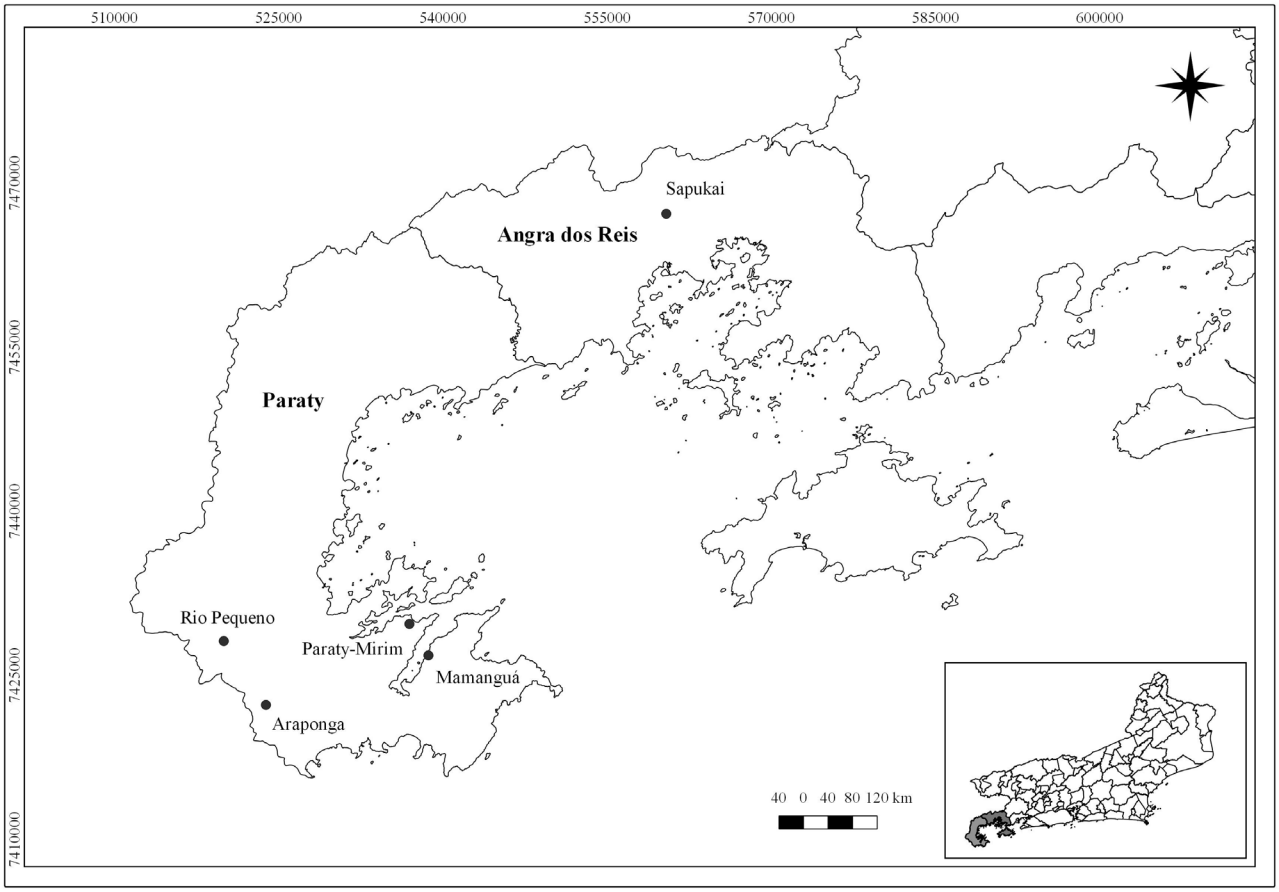

Figure 1. Location map of Araponga, Rio Pequeno, Mamanguá, Sapukai and Paraty-Mirim indigenous villages in the municipalities of Angra dos Reis and Paraty.

identification by traditional biochemical assays. Isolates identified as presumptive *E. coli* were assayed for the detection of the *E. coli*-specific *uidA* gene. *E. coli* isolates were kept frozen and screened for adherence phenotype, antimicrobial resistance, phylogrouping, genetic diversity and diarrheagenic potential. This study was approved by the Escola Nacional de Saúde Pública Sergio Arouca Ethics Committee on Human Research (CEP/ENSP), (reference number 0056.0.031.000-09), Fundação Oswaldo Cruz, Rio de Janeiro, Brazil.

2.3. Antimicrobial Susceptibility

Antimicrobial resistance was assessed using a standard disk diffusion method according to the guidelines published by the Clinical and Laboratory Standards Institute (CLSI) [18]. Bacterial suspension was adjusted to a 0.5 McFarland standard and tested against the following antimicrobials: amikacin (AMI, 30 μg), ampicillin (AMP,10 μg), cephalotin (CFL, 30 μg), cefepime (CPM, 30 μg), ceftriaxone (CRO, 30 μg), cefoxitin (CFO, 30 μg), ciprofloxacin (CIP, 5μg), gentamicin (GEN, 10 μg), nalidixic acid (NAL, 30 μg), nitrofurantoin (NIT, 300 μg), norfloxacin (NOR, 10 μg), trimethoprim-sulfamethoxazole (STU, 25 μg). Reference *E. coli* strains ATCC 25922 and ATCC 35218 were used as controls. The

isolates were scored as susceptible, intermediate or resistant to a given antimicrobial according to the inhibition zone diameter around the disk. Intermediate and resistant *E. coli* isolates were subsequently grouped in the same resistant class and categorized as multidrug-resistant (MDR), extensively drug-resistant (XDR) or pandrug-resistant (PDR) [19].

2.4. Cell Adherence

E. coli isolates carrying diarrheagenic genetic markers were tested for adherence to epithelial cells as described [20]. Adhesion assays were performed with HEp-2 cells cultivated in 24-well tissue culture plates (50% to 70% confluence) containing Dulbecco's Modified Eagle Medium (DMEM, Gibco), supplemented with 50 μg/mL gentamicin, 2.5 μg/mL fungizone and 5% fetal calf serum (FCS) (Gibco). After two washes with phosphate buffered saline (PBS), 1.0 mL of fresh DMEM (supplemented with 1% D-mannose) was added to each well. *E. coli* strains were grown overnight in Luria Bertani (LB) broth under static conditions and an aliquot of the overnight culture (20 μL,) was added to the media contained on the microplates for 3 h/6h at 37˚C in 5% CO_2. The infected monolayers were washed with sterile phosphate-buffered saline, fixed with 70% methanol, stained with 10% Giemsa stain, and examined for adherence patterns under light microscopy. Weakly adherent or non-adherent isolates were retested using the same procedure with an additional incubation period of 3 h (6 h assay). ATCC EAEC 042 (AA+), EPEC E2348/69 (LA+) and H1/1 (DA+) and non-adherent *E. coli* K12 (DH5-*α*) were used as controls.

2.5. Hybridization Assays for Diarrheagenic *E. coli*

All *E. coli* isolates were screened by colony DNA hybridization assays using specific radiolabelled DNA probes to identify the following DEC virulence sequences: *st*, *lt*, *eae*, *bfpA*, *stx*1, *stx*2, *att* and *ipaC* [21]. *E. coli* isolates were classified as typical EPEC if they lacked *stx* DNA probe sequences, and carried the *eae* and *bfpA* genes, and as atypical EPEC if they carried *eae* only. *E. coli* isolates that carried *st* and/or *lt* genes were classified as ETEC, *att* gene as EAEC, *ipaC* gene as EIEC and *stx*1 and/or *stx*2 genes as STEC. *E. coli* HB101 (pBR 322) and *E. coli* K12 carrying recombinant plasmids were used as controls.

2.6. PCR-Multiplex for Diarrheagenic *E. coli*

The *E. coli* isolate was assigned to a particular pathotype as described [22]. All strains were screened for the following target genes: *escV*, *bfpB*, *stx*1, *stx*2, *elt*, *estIa*, *estIb*, *invE*, *astA*, *aggR*, *pic* and *uidA* (*E. coli marker*). *E. coli* isolates were classified as tEPEC (*escV* positive, *bfp* positive, *stx* negative), aEPEC (*escV* positive, *bfp* negative, *stx* negative), STEC (*escV* positive/negative, *bfp* negative, *stx* positive), ETEC (*elt* positive and/or *estIa* positive, *estIb* positive), EIEC (*invE* positive) or EAEC (*astA* positive and/or *aggR* positive and/or *pic* positive). PCR-amplified fragments were separated on 2.0% (wt/vol) agarose gels, visua-

lized under UV light and photographed using a digital image capture system (Silver UVIPro, Cambridge, UK). To estimate the size of the fragments, a 100 bp DNA ladder standard (Invitrogen) was used. Each PCR analysis included a negative DNA control for enterovirulence genes and a nontemplate control. Clinical *E. coli* isolates were used as positive controls for diarrheagenic genetic markers: 243IV (tEPEC, *escV*+ *bfpB*+), 36IV (ETEC, *lt*+), 245I (ETEC, *st*+), E30138 (STEC, stx_2+), E40705 (STEC, stx_1+), 103V (EAEC, *aggR*+) and 129III (EIEC, *inv*+). Isolates lacking one or more of the genes defining a given set were considered nonenteropathogenic.

2.7. Random Amplification of Polymorphic DNA

RAPD-PCR was performed as previously described with the primers A04 (AATCGGGCTG), 1254 (CCGCAGCCAA), and M13 (GAGGGTGGCGCTTCT) [23]. RAPD profiles were inspected visually and the genetic patterns were defined according to the presence or absence and intensity of polymorphic bands. A 1Kb DNA ladder was used as a molecular weight marker (Invitrogen, Rio de Janeiro, Brazil). Semiautomated analysis used the UVI Soft Image Acquisition and Analysis Software, program UVIPro Bandmap, version 11.9 (Uvitec, Cambridge). Cluster analysis was performed using the Unweighted Pair Group Method with Arithmetic averages (UPGMA) of the Image Analysis System. The percentages of similarity were estimated by Dice coefficient. The reproducibility of RAPD amplifications was assessed using the selected primers with different DNA samples isolated independently from the same strain and amplified at different times.

2.8. PCR-Triplex for *E. coli* Phylotyping

E. coli A, B1, B2 and D phylogroups were determined according to previously defined criteria in a triplex amplification of the *chuA* and *yjaA* genes and the DNA fragment (TSPE4.C2) [24]. The characterization into phylotypes was based on the different combinations of these phylogroup genetic markers. Amplification products were visually inspected under UV light and photographed using a digital image capture system (silver UVIPro, Cambridge, UK). To estimate the size of the fragments, a 100 bp DNA ladder standard (Invitrogen) was used. Molecular assays included a nontemplate reaction and the clinical UPEC strain (L75A, *chuA*+ *yjaA*+ TSPE4.C2+) as the positive control for the phylogenetic markers.

3. Result

Enterovirulence assays revealed that 136 (43%) of the 314 *E. coli* isolates were carriers of, at least one of the genetic sequences investigated, with the *astA* gene being the most frequent (26%, 83/314) followed by *escV* (8%, 24/314), *aggR* (5%, 17/314), *pic* (2%, 5/314), *estIb* and *elt* (1%, 3/314). All the isolates were carriers of the *uidA* gene. The following genetic profiles were detected: *escV*, *aggR*/*astA*,

aggR/*pic*, *estIb*/*elt*/*astA*, *astA*, *pic*, *aggR*/*pic*. 15% (48/314) of the isolates were classified in the diarrheagenic categories: aEPEC (56%, 27/48), EAEC (35%, 17/48) and ETEC (8%, 4/48).

Distribution by indigenous village showed that 83% of the *E. coli* isolates from the Araponga village (n = 6) were carriers of the *escV* gene (aEPEC pathotype). Among the isolates from the Rio Pequeno village (n = 18), the genetic markers for enteropathogenicity were detected in 44% (8/18) of isolates, with the *aggR* gene found in 50% (4/8) (EAEC pathotype) and the *astA* gene in 25% (2/8). Among the isolates from the Mamanguá village (n = 22), genes for enterovirulence were detected in 45% (10/22), with only 30% (3/10) being carriers for the *escV* gene (aEPEC pathotype) and 50% (5/10) of isolates were carriers of the *astA* gene. Among the *E. coli* isolates from Sapukai (n = 135), the genetic markers for enterovirulence were detected in 44% of isolates (n = 56): *astA* (33/56), *escV* (aEPEC, 14/56), *aggR* (EAEC, 2/56), and *pic* (2/56). Among the isolates obtained from the Paraty Mirim village (n = 133), the genes for enterovirulence were detected in 43% of the samples (n = 57): *astA* (38/57), *aggR* (EAEC, 6/57), *estlb*/*elt*/*astA* (ETEC, 3/57), *aggR*/*pic* (EAEC, 3/57), *aggR*/*astA* (EAEC, 2/57), *escV* (aEPEC, 2/57). The hybridization assays detected the *eae* gene (aEPEC, 16/314) among the isolates from the Araponga and Sapukai villages, the *att* gene (EAEC, 13/314) in the Sapukai, Paraty Mirim and Rio Pequeno villages and the lt gene (ETEC, 1/314) in the isolates from the Sapukai village. From all the *E. coli* isolates identified as potential DEC strains (n = 48), 47 were identified by the amplification assays, and 30 by the DNA hybridization assays. Of these isolates, 29 showed results, which agreed with both the molecular tests. Results of phenotypic and genotypic characteristics of *E. coli* isolates carrying DEC markers are shown in Table 1. aEPEC (n = 27) was isolated from 10 children without diarrhea of up to six months of age, EAEC (n = 12) from 7 children, with and without diarrhea, with 6 to 60 months of age, and ETEC (n = 4) from two children with 36 months without diarrhea.

Table 1. Distribution of *E. coli* isolates carrying diarrheagenic genetic and phenotypic markers according to the indigenous villages.

Indigenous village	DEC pathotype	Isolates	Genotypic markers				Phenotypic markers			
			Pathotype marker	RAPD profiles			Phylogroup	Adherence pattern	Multidrug-resistance	
				Primer 1254	Primer A04	Primer M13			MDR	Possible XDR/PDR
Sapukai	ETEC	8IV	*lt*/*estlb*	1	1	1	A	U	–	–
	EAEC	18VII	*aggR*/*att*	3	3	3	B1	AA	+	–
		18VIII	*aggR*/*att*	4	4	3	B1	AA	+	–
	aEPEC	2VI	*escV*	9	14	9	B1	U	–	–
		4I	*escV*/*eae*	10	15	10	A	U	+	–
		4III	*escV*/*eae*	10	15	11	B1	U	–	+
		4IV	*escV*/*eae*	10	15	11	B1	U	+	–
		4V	*escV*/*eae*	10	15	11	B1	U	–	+

Continued

		4VI	*escV*/*eae*	10	15	11	A	U	+	−
		7I	*escV*	11	16	12	A	U	−	−
		7II	*escV*	12	16	12	A	U	+	−
		7III	*escV*	12	16	12	A	U	−	−
		7V	*escV*	12	16	12	A	U	−	+
		12V	*escV*	13	17	13	A	U	−	−
		12VII	*eae*	14	18	14	B2	AA	+	−
		13II	*eae*	15	19	12	A	U	−	−
		20II	*escV*/*eae*	10	20	13	A	U	+	−
		20III	*escV*/*eae*	16	20	14	A	U	+	−
		20V	*eae*	17	20	13	A	U	+	−
		20VII	*escV*/*eae*	18	20	13	A	U	−	−
Paraty Mirim	ETEC	24III	*lt*/*estIb*/*astA*	2	2	2	A	U	+	−
		24IV	*lt*/*estIb*/*astA*	2	2	2	A	U	+	−
		24V	*lt*/*estIb*/*astA*	2	2	2	A	U	−	−
	EAEC	30I	*aggR*/*pic*/*att*	4	5	4	D	AA	−	−
		30II	*aggR*/*pic*/*att*	4	6	4	D	AA	+	−
		30III	*aggR*/*pic*/*att*	4	7	4	D	AA	−	−
		30IV	*aggR*	5	6	4	D	AA	−	−
		30V	*aggR*	3	8	5	B1	AA	−	+
		37I	*aggR*/*astA*	7	10	6	A	AA	+	−
		46II	*aggR*/*att*	6	11	7	B1	AA	+	−
		46III	*aggR*/*att*	6	12	7	B2	AA	+	−
		46V	*aggR*/*att*	6	12	7	B2	AA	+	−
		46VI	*aggR*/*att*	6	11	7	B2	AA	+	−
		54IV	*aggR*/*astA*	6	13	8	A	AA	−	−
	aEPEC	42II	*escV*	22	24	18	A	AA	−	−
		55VI	*escV*	23	25	25	B1	U	−	−
Rio Pequeno	EAEC	32VI	*aggR*/*att*	5	6	4	B1	AA	−	+
		32VII	*aggR*/*att*	6	8	5	B1	AA	+	−
		33VI	*aggR*	6	9	5	A	AA	+	−
		33VII	*aggR*	6	9	5	A	AA	−	+
Mamanguá	aEPEC	26II	*escV*	19	21	15	A	AA	−	−
		26III	*escV*	19	21	15	A	U	−	−
		26V	*escV*	19	22	16	A	U	−	−
Araponga	aEPEC	34I	*escV*/*eae*	20	23	16	A	U	+	−
		34II	*escV*/*eae*	20	23	16	B1	U	−	−
		34III	*escV*/*eae*	20	23	16	B1	U	+	−
		34IV	*escV*/*eae*	20	23	16	A	U	−	−
		34VI	*escV*/*eae*	21	23	17	A	U	−	+

DEC Diarrheagenic *E. coli*; *ETEC* enterotoxigenic *E. coli*; *EAEC* enteroaggregative *E. coli*; *aEPEC* atypical enteropathogenic *E. coli*; *MDR* multidrug-resistant; *XDR* extensively drug-resistant; *PDR* pandrug-resistant; *RAPD* random amplification of polymorphic DNA; *AA* aggregative adherence; *U* undefined adherence pattern.

Most of the *E. coli* isolates were identified as phylogroup A (56%), followed by B1 (26%), B2 and D (9%, each). In the phylogroup A the following genetic variants were detected: A^1 –,–,– (40%, 19/48) and A^2 –,+,– (16%, 8/48), in the phylogroup B2 the genotypes $B2^1$ +,+,– (2%, 1/48) and $B2^2$ +,+,+ (7%, 3/48) and in the phylogroups B1 and D the phylotypes –,+,+ 26% (13/48) and +,-,- 9% (4/48), respectively. ETEC were classified in the phylogroup A, EAEC in A, B1, B2 and D and the pathotype aEPEC in the phylogroups A, B1, and B2. Considering the distributions of the phylotypes according to indigenous village, the following occurrences were detected: Araponga village with 60% (3/5) of the DEC belonging to phylotype A^1 and 40% (2/5) to group B1; the Rio Pequeno village, 50% (2/4) belonged to the phylotype A^1 and 50% (2/4) to B1; the Mamanguá village, phylotypes A^1 (33%, 1/3) and A^2 (67%, 2/3); Sapukai village, the phylotypes A^1 (60%, 12/20), B1 (30% (3/20), A^2 (5%, 1/20) and $B2^1$ (5%, 1/20) and in the Paraty Mirim village, phylotypes A^1 (6%, 1/16), A^2 (31%, 5/16), B1 (19%, 3/16), $B2^2$ (19%, 3/16) and D (25%, 4/16).

Adherence tests revealed that 100% (17/17) of the EAEC exhibited the typical aggregative pattern (AA) in the 3 and 6 hour assays. ETEC exhibited the AA phenotype (AA) in the 3 hour assay (75%, 3/4) and one isolate (25%, 1/4) was adherent, however, without exhibiting a defined pattern. aEPEC exhibited the AA pattern (11%, 3/27) in 3-hour assays and the other isolates showed a discrete and moderate adhesion. *E. coli* isolates which were only carriers of the *pic* gene showed the typical aggregative pattern, while the isolates, which were only carriers of the *ast* gene exhibited the phenotypes of localized, aggregative and undefined adherence patterns in 6-hour assays.

Antimicrobial susceptibility tests revealed that 88% (276/314) of the *E. coli* isolates were resistant to, at least one of the 11 antimicrobials tested characterizing 72 distinct profiles (I to LXXII). Eight single resistance profiles (I to VIII) were observed for AMI, AMP, CFL, COM, GEN, NIT, NOR and SUT. The percentage of resistance for each antimicrobial was 69% (216/314) AMP, 63% (197/314) CFL, 54% (171/314) SUT, 18% (58/314) GEN, 12% (39/314) AMI, 9% (28/314) CRO, 6% (19/314) NAL, 6% (20/314) CFO, 6% (19/314) NIT, 4% (11/314) CIP, 3% (14/314) NOR and 2% (5/314) CPM. The intermediary resistance phenotypes were found in 65% of the isolates (203/314). The multiresistance classified 26% of the isolates as MDR (80/314) and 11% as possible XDR/PDR (36/314). The distribution of resistant isolates by indigenous village showed that 91% (20/22) from the Mamanguá village, 88% (121/135) from Sapukai, 87% (116/133) from Paraty Mirim and 83% in the Rio Pequeno (15/18) and Araponga (5/6) villages were resistant. In the group of *E. coli* isolates that carried diarrheagenic genetic markers (n = 48), 24 profiles with resistance to up to 9 antimicrobials were detected, with the profile XXIV shared by the ETEC, aEPEC and EAEC pathotypes and, types III, XII and XXXVIII by the EAEC and aEPEC pathotypes. The MDR phenotype was observed in 23 isolates from the Paraty Mirim, Sapukai, and Araponga villages, belonging to the EAEC, aEPEC

and ETEC pathotypes. Two EAEC isolates obtained from diarrheal children exhibited the MDR phenotype. Possible XDR/PDR isolates were observed among the EAEC and aEPEC isolated from Paraty Mirim, Rio Pequeno, Sapukai and Araponga villages.

RAPD typing based on 1254, A04 and M13 primers revealed a genetically diverse bacterial population. The amplification reactions generated polymorphic, stable, and reproducible profiles, composed of 2 to 6 bands (primer 1254), 2 to 8 bands (primer A04) and 5 to 10 bands (primer M13), ranging from 500 - 2000 bp, 500 - 3000 bp, 500 - 2000 bp, respectively. RAPD clustering showed a bacterial population arranged into separate branches or organized in small clonal groups, exhibiting Dice similarity indices ranging from 10% to 100% (1254), 2% to 100% (A04) and 5% to 100% (M13). The genetic relationship of the *E. coli* isolates belonging to the same pathotype was in agreement in terms of the primers used and higher indices of similarity were observed among the isolates from the same child and indigenous village.

4. Discussion

Studies carried out around the world report that the pathogenic groups of *Escherichia coli* associated with human diseases and in animals are markedly diversified, being able to cause intestinal or extra-intestinal infections according to their repertoire of virulence traits [9] [12] [13]. *E. coli* as an etiological agent of intestinal infections can be associated with manifestations with broad clinical spectrum, ranging from asymptomatic and subclinical forms to clinical conditions of great severity potentially leading to death. Epidemiological surveys of indigenous populations conducted in countries on the American continent, have shown that diarrhea contributes significantly to rates of morbi-mortality and hospitalization of indigenous populations [3] [4] [5] [6] [7]. In Brazil, these populations generally live in villages with inadequate sewage treatment and in conditions of poverty [4] [7]. Studies on the etiology of diarrhea in indigenous populations are very limited and there are no reports regarding the occurrence of diarrheic *E. coli* in indigenous children of the Guarani ethnicity. Our findings revealed the circulation of the diarrheagenic pathotypes among indigenous children younger than 5 years of age, with and without diarrhea.

aEPEC was the most prevalent pathotype recovered from children without diarrhea residing in Sapukai, Mamaguá, Araponga and Paraty Mirim villages. aEPEC has not yet been described in indigenous populations, however this pathotype is gaining greater significance in non-indigenous populations associated with symptomatic and asymptomatic intestinal disorders [12] [13] [25]. Recent studies have shown that infections by aEPEC seem to surpass those caused by tEPEC, both in developed and developing countries, mainly associated with human persistent diarrhea [9] [12] [13] [26]. In Brazil, aEPEC has been implicated as the cause of diarrhea in different urban areas [9] [12] [27]. aEPEC has also been isolated from animal sources, including dogs, rabbits, monkeys and sheep,

which has led to suppose the role of these animals as environmental reservoirs of these enteropathogens [9] [12]. The precarious sanitation infrastructure where Guarani communities reside and the close coexistence with domestic and forest animals, are unfavorable environmental conditions which may have contributed to the DEC spread and contamination of the indigenous children [8] [17] [28] [29]. As well as via direct contact, transmission of the microorganism can also take place in an indirect manner through ingesting contaminated water and food, representing an additional risk of exposure for the health of the indigenous community. Our data from the adherence assays (3 and 6 hours) showed that aEPEC isolates were adherent but did not express a defined pattern and 11% (3/27) exhibited the aggregative phenotype (AA). These findings are in agreement with previous observations, which underline that adherence is also a variable characteristic of the pathotype, even among epidemiologically related bacterial isolates [12] [27]. Phylogrouping categorized the pathotype mainly in groups A and B1. In general, A and B1 strains appear to be found within guts of a range of vertebrates and also are more prevalent in freshwater samples than other strains [24]. So, we assume that the aEPEC isolates are microorganisms possibly derived from non-human sources or other environmental compartments [9] [14] [15] [24]. These findings reinforce the role of the environment as a reservoir and source of contamination of these pathogens. aEPEC isolates were also categorized in B1, B2, and D phylogroups, which shows the concept of the diverse structure of the category and its different potential risks to human health as well as their high adaptability to environments or diverse niches [29]. Results from the antimicrobial resistance showed that 81% of aEPEC isolates (22/27) were resistant to at least one of the 11 antibiotics tested and that the MDR and possible XDR/PDR phenotypes were observed for 37% and 11% of aEPEC isolates, respectively. Our findings are in accordance with previous studies carried out in Brazil and in other geographic regions, that report the emergence of resistance among aEPEC clinical isolates from non-indigenous children [9] [14] [16] [27]. This phenomenon alerts us to the inappropriate use of antimicrobials with humans and animals, which ends up favoring the selection of resistant bacteria in the environment. The impact of the spread of resistance at high levels can assume even greater proportions, especially if we consider that their genetic determinants can move quickly between different bacterial species and genera [12] [13] [29] [30].

EAEC was the second most frequent pathotype isolated from children both with and without diarrhea, resident in Sapukai, Paraty Mirim and Rio Pequeno villages. The diarrheic children associated with EAEC isolates manifested acute diarrhea, liquid feces with an absence of fever, and vomiting or abdominal pain. Epidemiological studies have associated EAEC infection with acute cases but especially with persistent diarrhea, particularly in developing countries [9] [11] [12] [16] [25] [26]. The pathogenic properties of EAEC require more detailed study, especially seeking to investigate its clinical significance through long term

studies, given that the pathotype is also isolated from asymptomatic adult and child carriers [12] [13]. EAEC is a pathotype of recognized variability, exhibiting elevated genetic and antigenic diversity. Though a great diversity of adhesins, toxins and proteins are possibly involved in the pathogenesis of EAEC, the prevalence of these associated factors or genes is highly variable and is not found in all the isolates, which has hampered diagnostic accuracy for this pathotype [9] [12] [13] [31]. The virulence properties of the pathotype that are associated with symptoms of diarrhea include the production of biofilm and of diverse enterotoxins, such as thermo-stable enteroaggregative toxin (EAST-1), the Pet cytotoxin and the anti-aggregative protein known as dispersin [9] [12] [13] [32] [33] [34]. EAST-1 is codified by the *astA* gene, located in a transposon, and has high homology with the amino-acid sequence of the thermo-stable entertoxin (ST) of ETEC. In our study, the *astA* gene was the most frequent genetic marker in EAEC isolates, obtained from acute diarrhea, suggesting the possible role of EAST-1 in the manifestation of the indigenous clinical status. As well as toxins, EAEC strains may exhibit diverse adhesins such as the Pic protein, associated or not, with other virulence factors of the pathotype [33]. In our study, the *pic* gene was only detected among EAEC obtained from asymptomatic children. In these *E. coli* isolates, no other EAEC virulence associated markers were observed suggesting that colonization can require additional factors for the efficiency of the infection. Despite the genotypic differences detected in EAEC, all the isolates originating both from asymptomatic and diarrheal indigenous children were classified as typical (tEAEC) due to presence of the *aggR* gene, which codes for the transcriptional activator AggR [13] [33] [34]. Typical EAEC has closely associated with the expression of the typical aggregative pattern, corroborating with the adherence phenotype observed during the 3 and 6-hour assays [13] [19]. Variations in this adherence pattern have been observed and seem to involve adhesins of a diverse nature. Molecular methods for diagnosis purposes based on hybridization tests and amplification assays are being widely used as alternative tools to the gold-standard test with epithelial cells in culture [13] [14]. In the present study, molecular assays were used as the EAEC diagnostic method, and subsequently, the isolates were tested on HEp-2 cells for the observation of the expression of the adhesion phenotypes. Our results showed the importance of molecular diagnosis for tEAEC, given that all the EAEC isolates exhibited the typical aggregative adherence (AA) pattern. However, considering that not all the EAEC strains were found to harbor the pAA plasmid, the diagnosis of the pathotype in the present study could have been underestimated in the indigenous population. Results from phylogrouping and genome typing agree with previous reports that describe the elevated genetic diversity of this pathotype observed both in clinical and environmental isolates [26] [34]. EAEC typing revealed that EAEC isolates belong to the main four phylogroups, suggesting distinct pathogenic potentials for these isolates [13] [19] [20] [21]. The absence of a correlation with phylogenetic markers or specific virulence patterns with a

diarrhea condition is also observed in other studies with non-indigenous populations [23] [25] [26]. These results underline the need to investigate pathogenic specific characteristics, especially when considering the potential aggressiveness of the diverse virulence factors already described and the existence of cases of significant severity associated with the pathotype. 94% of EAEC isolates (16/17) were resistant to at least one of the 11 antimicrobials tested and the multidrug-resistance was also a phenomenon observed for EAEC, characterizing the MDR and possible XDR/PDR phenotypes for 65% and 12% of the isolates, respectively. Resistance to antimicrobials is also reported worldwide in clinical EAEC strains among the isolates from hospitals and communities [9] [10] [12] [16] [25] [31]. However the occurrence of MDR and possible XDR/PDR phenotypes among EAEC is not reported and reinforces the need for environmental monitoring aiming to minimize the exposure of human populations and the limitation of treatment options.

ETEC is one of the classic *E. coli* pathotypes mainly in non-indigenous children younger than five years of age and in travelers coming from endemic regions [9] [12]. Studies with indigenous populations report that ETEC is a diarrheagenic pathotype common in children who live in rural communities in Guatemala, associated both with acute and persistent diarrhea as well as among control children [5]. In our study, ETEC presented low prevalence and was only isolated from children without diarrhea, suggesting that this pathotype does not represent a relevant etiological agent for diarrhea in the indigenous Guarani population. The virulence genotype detected, codifying for both classes of enterotoxin, has been detected in ETEC isolates from non-indigenous children without diarrhea, which is in accordance with our findings [9] [11] [12]. ETEC clinical isolates frequently exhibit diverse adhesins capable of mediating the bacterial adherence with the surface of intestinal cells, contributing in this way to the full expression of the enteroinfection [9] [12]. Given that in our study, these adhesins were not investigated, it was not possible to define a more complete ETEC virulence profile, and it was consequently difficult to establish an association with the clinical status. In addition to enterotoxins, the *astA* gene is recognized as an accessory gene for pathogenic *E. coli* populations, including diarrheagenic pathotypes such as ETEC and extra-intestinal lineages [9] [15] [32] [34] [35]. However, in our study the detection of this gene among bacterial isolates obtained from asymptomatic individuals may be explained as a consequence of a continuous process of genetic exchange, between pathogenic and non-pathogenic *E. coli* populations and/or other bacterial genes which share the same ecological niche [28] [30] [36]. Phylogrouping assays classified the ETEC isolates as belonging to group A. These results are in accordance with previous findings, which revealed that phylogroup A is associated with clinical isolates of ETEC obtained from asymptomatic individuals, commensals of environmental origin or even of low virulence potential [9] [12] [14] [15] [37]. Our results from adherence assays revealed that the ETEC isolates were adherent, exhibiting the aggregative phenotype, typically observed in EAEC, and undefined patterns.

ETEC isolates of environmental and asymptomatic origin commonly exhibit elevated antigenic heterogeneity, influencing in this manner, the expression of its specific virulence factors [36]. Recent studies that correlate the dynamics of microbial populations and evolution of bacterial resistance have revealed that increased resistance is associated in most cases, either directly or indirectly, with decreased virulence [30] [37]. In our study, the MDR phenotypes and multi-drug-resistance to up to four antimicrobials were observed among ETEC isolates originated from asymptomatic children, which reinforces this observation. RAPD typing confirms the high variability among ETEC isolates thus characterizing this bacterial population as being epidemiologically unrelated [15] [37].

Studies on the molecular epidemiology of diarrheagenic *E. coli* in indigenous populations is very limited worldwide and non-existent in Brazil. Our results revealed that the aEPEC pathotype was predominant in the indigenous Guarani children, followed by EAEC and ETEC. This data draws our attention considering the association of these pathotypes with malnutrition and infant development. The precarious sanitation conditions and the life style of the population studied, which routinely maintains a close relationship with diverse environmental reservoirs, create conditions of greater vulnerability for the establishment of antimicrobial resistance and bacterial virulence, thus facilitating the rapid dissemination of these properties.

5. Conclusion

This is the first report regarding the circulation and characterization of diarrheagenic *E. coli* among indigenous Guarani children residing in villages located in the south of the state of Rio de Janeiro. The variability of genotypes and resistance to a wide range of antibiotics reflects the inherent genomic plasticity of *E. coli*, which involves a dynamic genetic exchange between commensal and pathogenic microorganisms. This epidemiological context could lead to serious problems in public health through the emergence of highly virulent new lineages and to the spread of resistance in the environment. These findings alert us to the need for strategic actions in the prevention and health surveillance of this indigenous population, especially considering that the child population is exposed to adverse environmental conditions and inadequate coverage of the health services, which makes it even more vulnerable to infections caused by these microorganisms and serious complications.

Acknowledgements

The authors would like to thank Rose Mary Pimentel Bezerra for technical assistance in the bacteriological procedures.

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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