Epidemiology, species composition and genetic diversity of tetra- and octonucleated Entamoeba spp. in different Brazilian biomes

Deiviane Aparecida Calegar1*, Kerla Joeline Lima Monteiro2, Polyanna Araújo Alves Bacelar1,2, Brenda Bulsara Costa Evangelista1,2, Mayron Morais Almeida1, Jéssica Pereira dos Santos1,2, Márcio Neves Boia3, Beatriz Coronato-Nunes1,4, Lauren Hubert Jaeger1,5† and Filipe Anibal Carvalho-Costa1†

Abstract

Background: Entamoeba species harbored by humans have different degrees of pathogenicity. The present study explores the intra- and interspecific diversity, phylogenetic relationships, prevalence and distribution of tetra- and octonucleated cyst-producing Entamoeba in different Brazilian regions.

Methods: Cross-sectional studies were performed to collect fecal samples (n = 1728) and sociodemographic data in communities located in four Brazilian biomes: Atlantic Forest, Caatinga, Cerrado, and Amazon. Fecal samples were subjected to molecular analysis by partial small subunit ribosomal DNA sequencing (SSU rDNA) and phylogenetic analysis.

Results: Light microscopy analysis revealed that tetranucleated cysts were found in all the studied biomes. The highest positivity rates were observed in the age group 6–10 years (23.21%). For octonucleated cysts, positivity rates ranged from 1 to 55.1%. Sixty SSU rDNA Entamoeba sequences were obtained, and four different species were identified: the octonucleated E. coli, and the tetranucleated E. histolytica, E. dispar, and E. hartmanni. Novel haplotypes (n = 32) were characterized; however, new ribosomal lineages were not identified. The Entamoeba coli ST1 subtype predominated in Atlantic Forest and Caatinga, and the ST2 subtype was predominant in the Amazon biome. E. histolytica was detected only in the Amazon biome. In phylogenetic trees, sequences were grouped in two groups, the first containing uni- and tetranucleated and the second containing uni- and octonucleated cyst-producing Entamoeba species. Molecular diversity indexes revealed a high interspecific diversity for tetra- and octonucleated Entamoeba spp. (H±SD = 0.9625 ± 0.0126). The intraspecific diversity varied according to species or subtype: E. dispar and E. histolytica showed lower diversity than E. coli subtypes ST1 and ST2 and E. hartmanni.

Conclusions: Tetra- and octonucleated cyst-producing Entamoeba are endemic in the studied communities; E. histolytica was found in low proportion and only in the Amazon biome. With regard to E. coli, subtype ST2 was
ST1 and E. coli ST2 [9]. into two subtypes: E. coli has been demonstrated for E. coli, which can be divided Additionally, considerable intraspecific genetic variability in inflammatory activity triggered by this organism [7, 8]. infected children, pointing to the possibility of intestinal have higher levels of stool leucocytes than unin fected. Mexi can be considered a marker of inadequate sanitary con ditions, denoting greater exposure to other fecal patho gens. Recently, however, potential pathogenicity has been attributed to this species. Mexican children infected with E. coli have higher levels of stool leucocytes than uninfected children, pointing to the possibility of intestinal inflammatory activity triggered by this organism [7, 8]. Additionally, considerable intraspecific genetic variability has been demonstrated for E. coli, which can be divided into two subtypes: E. coli ST1 and E. coli ST2 [9].

Typically, the clinical detection of Entamoeba species in fecal samples has been performed through light microscopy. However, the overlap of morphological characteristics between some species and the morphological and size variation in structures are limiting factors for microscopic species-specific diagnosis [10]. The implementation of molecular tools for Entamoeba species characterization has enabled a greater understanding of their taxonomy, phylogenetic relationships and epidemiology [11].

Despite recent advances, the proportion of households without sanitation systems in many regions of Brazil remains high. The country has great socioeconomic, environmental and demographic diversity, and appropriate access to drinking water is restricted in many communities. Similarly, peri-urban and urban communities in large Brazilian cities in more industrialized states frequently have poor sanitation infrastructure. In the present study, we explored the species composition, the inter- and intraspecific genetic diversity and phylogenetic relationships, and the prevalence and distribution of Entamoeba species infecting populations living in different Brazilian biomes.

**Methods**

**Description of study area and population, study design and sampling**

Communities from cities located in four Brazilian biomes were selected: Cachoeiras de Macacu (CAM) in the state of Rio de Janeiro (Atlantic Forest biome), Teresina (TER) and São João do Piauí (SJPI) in Piauí (Cerrado and Caatinga biomes, respectively), and Santa Isabel do Rio Negro (SIRN) in Amazonas and Bagre (BAG) in Pará (Amazon biome) (Fig. 1). In the five municipalities, the studied communities had precarious access to drinking water and poor sanitation systems, to varying degrees and for distinct reasons. Table 1 shows the socio-environmental and demographic characteristics of each municipality and the number of individuals included in each one. These regions differ with respect to climate, proportion of the population living in poverty and human development index, among other parameters. Cross-sectional studies were carried out in the included areas to collect fecal samples and sociodemographic data. Entamoeba spp. fecal samples were identified as positive using parasitological methods (Ritchie’s modified ethyl acetate centrifugation) [12].

**DNA extraction, polymerase chain reaction (PCR) and DNA sequencing**

Fecal samples positive on light microscopy were subjected to molecular analyses for species characterization and phylogenetic studies. In addition, some Entamoeba spp.-negative samples on microscopy were selected, randomly or when another individual from the same household was positive, to be tested by PCR. This was performed in order to improve the number of DNA sequences for genetic analyses. Genomic DNA was extracted from 200 µl of the sedimented fecal material using the ZR Fungal/Bacterial DNA MiniPrep™ extraction kit (Zymo Research, Irvine, CA, USA). PCR was
performed using the Platinum Taq DNA Polymerase kit (Invitrogen, Waltham, MA, USA), with a final volume of 50 μl. The small subunit rRNA gene locus (SSU rDNA) of *Entamoeba* spp. (550 base pairs [bp]) was targeted for amplification, as described in Verweij et al. [13]. Amplification conditions were as follows: each deoxynucleoside triphosphate at 200 mM, 25 pmol of each specific primer, 10X PCR buffer, 1 U of Taq DNA Polymerase, and 5 μl of the DNA sample. The thermal cycling conditions were as follows: initial denaturation of 5 min at 94 °C, 35 cycles of 30 s at 94 °C, 30 s at 55 °C, and 30 s at 72 °C; and final extension of 2 min at 72 °C. The PCR products were purified with polyethylene glycol (PEG) [14] and sequenced using the BigDye Terminator v3.1 kit (Applied Biosystems, Foster City, CA, USA) in an ABI 3730 automated DNA sequencer (Applied Biosystems).

Data analysis

The obtained sequences were edited and analyzed using BioEdit version 7.2.5 software [15]. The Basic Local Alignment Search Tool (BLASTn; NCBI [https://www.ncbi.nlm.nih.gov/]) was used to verify similarity with *Entamoeba* species. The obtained sequences were deposited in GenBank under accession numbers MW026735–MW026794. BioEdit version 7.2.5 [15] was used to align and cut the sequences into equal fragments (542 bp). Phylogenetic inferences were performed using Molecular Evolutionary Genetics Analysis (MEGA) version 7.0.20 software [16]. The maximum likelihood (ML) and neighbor joining (NJ) methods were applied. The substitution model for the data set was chosen using the Bayesian information criterion (BIC) in MEGA version 7.0.20 software [16]. According to the lower BIC score, the Tamura 3-parameter model (T92) was chosen. Branch support was provided by bootstrapping with 1000 replications. *Entamoeba* spp. orthologous sequences (*n* = 46) were used to construct an alignment using the BLASTn tool against the Nucleotide Collection (nr/nt) database (https://www.ncbi.nlm.nih.gov/nucleotide/) (Additional file 1: Table S1). The reference sequences were selected to be representative of the genus. Sequences with degenerate bases were not included.

A median-joining (MJ) haplotype network was constructed in Network version 10.1.0.0 software [17] (www.}

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![Map of the studied areas in Brazil. The map was created using Google Earth Pro](image)
fluxusengineering.com), with the input file previously prepared in DnaSP (DNA Sequence Polymorphism) version 6 software [18]. Diversity indexes of tetra- and octonucleated Entamoeba populations were determined using the pairwise distance in Arlequin version 3.5.2.2 software [19]. The pairwise $F_{st}$ (fixation index) value was tested in all populations using Arlequin version 3.5.2.2 software [19] to estimate the extent of genetic differentiation among populations with a significance of 1000 random permutations.

### Results

#### Study population

Figure 2 presents the detection rates for tetra- and octonucleated Entamoeba cysts in fecal microscopic examinations in distinct age groups and settings. In the Amazon and Atlantic Forest biomes, tetranucleated cysts were found in fecal samples from children up to 2 years of age, with positivity rates ranging from 3.8 to 10.3%, respectively. In these biomes, one of the highest rates was found in the age group of 11–15 years, reaching 26.5%. In the four studied states, octonucleated cysts were detected through microscopy in 1–20.6% of children up to 2 years of age, and 10.8–49.3% of children aged 6–10 years.

### Molecular epidemiology and genetic diversity of Entamoeba spp.

A total of 60 SSU rDNA Entamoeba spp. sequences (542 bp) were obtained from fecal samples. The BLAST analyses revealed four different Entamoeba species: *E. coli* ($n=32$), *E. dispar* ($n=18$), *E. hartmanni* ($n=8$) and *E. histolytica* ($n=2$) (Table 2). With regard to the *E. histolytica/E. dispar* complex, the Amazon biome presented the greatest species diversity. In Piauí, only *E. dispar* was identified. With respect to *E. coli*, ST1 predominated in Rio de Janeiro and Piauí, and ST2 was predominant in Amazonian states (Amazonas and Pará). Figure 3 displays the ML and NJ trees inferred from uni-,
Fig. 2 The green and brown bars show the number of positive and negative fecal samples for the presence of tetranucleated (a) and octonucleated (b) Entamoeba cysts by light microscopy among subjects studied, by age group, in the four states; red lines depict the proportion of positive samples.
tetra- and octonucleated Entamoeba spp. cysts, with a total of 106 Entamoeba SSU rDNA sequences (n = 60 sequences from the present study plus 46 reference sequences). Two major groups were observed, the first one containing uni- and tetranucleated cyst-producing Entamoeba species, and the second containing Entamoeba species producing uni- and octonucleated cysts. The main differences between the two trees were as follows: (i) in the ML tree, E. moshkovskii was grouped in a single clade (Fig. 3a), while in the NJ tree it was grouped together with other tetranucleated cyst-producing Entamoeba species (Fig. 3b); and (ii) Entamoeba RL5, RL6 and E. insolita were grouped in a single clade on the NJ tree, while on the ML tree they were grouped close to E. hartmanni.

Among 28 samples containing tetranucleated Entamoeba cysts, only two were characterized as the pathogenic E. histolytica. The other 26 were identified as E. hartmanni or E. dispar. Entamoeba histolytica was identified only among isolates from the Amazon biome (Amazonas, n = 2); E. dispar was identified in the Cerrado (Teresina, Piauí, n = 2) and Amazon biomes (Pará, n = 10, and Amazonas, n = 6). E. hartmanni was characterized only in the Amazon biome (Pará and Amazonas in five and three samples, respectively). No Entamoeba moshkovskii or uninucleated cyst-producing Entamoeba species were found.

Entamoeba coli was the species most commonly found (n = 32) and was present in all localities studied. Both E. coli subtypes ST1 (n = 12; 37.5%) and ST2 (n = 20; 62.5%) were identified. Subtype ST1 was described in all regions studied (Piauí, n = 5; Rio de Janeiro, n = 2; Amazonas, n = 3; and Pará, n = 2), while ST2 was found almost exclusively in the Amazon region (Amazonas, n = 9 and Pará, n = 10) (Fig. 4).

The MJ haplotype network based on tetra- and octonucleated cyst-producing Entamoeba showed a similar topology to the ML tree discriminating species or subtypes (Fig. 5). A total of 60 SSU rDNA sequences from the present study plus 29 reference sequences were distributed in 55 haplotypes. Thirty-six different haplotypes were identified in our sequences, and several new haplotypes were found. In E. coli ST1 and ST2, five and 13 new haplotypes were characterized, respectively. Entamoeba

Table 2  Molecular diversity indexes of tetra- and octonucleated cyst-producing Entamoeba based on SSU rDNA locus (542 bp, n = 89)

| Species (N) | Region (N) | Statistics | No. of haplotypes | No. of polymorphic sites | No. of substitutions | No. of transitions | No. of transversions |
|------------|------------|------------|-------------------|-------------------------|---------------------|-------------------|---------------------|
|            |            | n ± SD     |                   |                         |                     |                   |                     |
| E. coli (38) | All ST1 (16) | 0.816 ± 0.095 | 9                          | 45                      | 44                  | 27                 | 17                  |
|            | Brazil (12)  | 0.772 ± 0.127 | 7                          | 44                      | 44                  | 27                 | 17                  |
|            | BAG + SIRN (5) | 0.709 ± 0.218 | 3                          | 5                       | 5                   | 3                  | 2                   |
|            | TER (2)       | 1.000 ± 0.5000 | 2                         | 1                       | 1                   | 1                  | 0                   |
|            | SIRN (3)      | 0.6667 ± 0.3143 | 2                        | 4                       | 4                   | 2                  | 2                   |
|            | CAM (2)       | 1.000 ± 0.5000 | 2                         | 7                       | 7                   | 3                  | 4                   |
|            | All ST2 (22)  | 0.952 ± 0.029 | 15                        | 52                      | 57                  | 26                 | 31                  |
|            | Brazil (20)   | 0.957 ± 0.028 | 14                        | 52                      | 57                  | 26                 | 31                  |
|            | BAG + SIRN (19) | 0.959 ± 0.030 | 14                       | 52                      | 57                  | 26                 | 31                  |
| Entamoeba dispar (21) | All (21) | 0.500 ± 0.132 | 7                         | 26                      | 22                  | 16                 | 6                   |
|            | Asia (3)      | 0.000 ± 0.000 | 1                         | 0                       | 0                   | 0                  | 0                   |
|            | Brazil (18)   | 0.568 ± 0.137 | 7                         | 26                      | 22                  | 16                 | 6                   |
|            | TER (2)       | 0.625 ± 0.139 | 7                         | 26                      | 22                  | 16                 | 6                   |
|            | BAG + SIRN (16) | 0.000 ± 0.000 | 1                         | 0                       | 0                   | 0                  | 0                   |
| E. hartmanni (10) | All (10) | 0.977 ± 0.054 | 9                         | 28                      | 24                  | 16                 | 8                   |
|            | Brazil (8)    | 0.964 ± 0.077 | 7                         | 28                      | 24                  | 16                 | 8                   |
| E. histolytica (6) | All (6) | 0.533 ± 0.172 | 2                         | 4                       | 2                   | 1                  | 1                   |
|            | North America (3) | 0.000 ± 0.000 | 1                         | 0                       | 0                   | 0                  | 0                   |
|            | Brazil (2)    | 0.000 ± 0.000 | 1                         | 0                       | 0                   | 0                  | 0                   |
| All* (89) | 0.9625 ± 0.0126 | 55               | 353                      | 272                     | 203                 | 199                |                     |

H ± SD: gene diversity ± standard deviation. All*: E. dispar, E. histolytica, E. hartmanni, E. coli, Entamoeba sp., E. moshkovskii, E. ecuadoriensis, E. bangladeshi, E. nuttalli, E. muri, E. terrapinae, E. insolita, E. invadens, Entamoeba RL7, Entamoeba RL10, Entamoeba RL5, Entamoeba RL6. Further details for reference strains can be found in Additional file 1: Table S1. Bold indicates sequences obtained in this study (Brazil). BAG Bagre, CAM Cachoeiras de Macacu, SIRN Santa Isabel do Rio Negro, SJPI São João do Piauí, TER Teresina
hartmanni, E. dispar and E. histolytica had seven, six and one new haplotype, respectively. Entamoeba dispar and E. coli subtype ST1 presented a star-shaped haplotype network (Fig. 5), with a central and dominant haplotype. Interestingly, most of our samples belong to this ancestral haplotype.

Analysis of molecular diversity indexes revealed high interspecific diversity for tetra- and octonucleated Entamoeba spp., with $H \pm SD = 0.9625 \pm 0.0126$ and 353 polymorphic sites (Table 2). The intraspecific diversity varied according to species or subtype. Entamoeba dispar and E. histolytica showed lower intraspecific variability ($H \pm SD = 0.500 \pm 0.132$ and 0.533 ± 0.172, respectively) than E. coli subtypes ST1 and ST2 and E. hartmanni ($H \pm SD = 0.816 \pm 0.095, 0.952 \pm 0.029$, and 0.977 ± 0.054, respectively) (Table 2). The intraspecific variability of these two species was similar to the interspecific variability for the species. The $F_{st}$ results corroborate the diversity analysis results (Additional file 2: Table S2). The E. dispar sequences from different Brazilian biomes showed low $F_{st}$ values, indicating no isolation between populations. For E. coli ST1 and ST2 sequences, even with high genetic variability, there was no evidence of significant
isolation between populations (Additional file 2: Table S2 and Fig. 5).

Tetra- and octonucleated cyst co-infections were identified by microscopy in 18.6% (72/387) of fecal samples. Of these, 39 samples were subjected to PCR. Overlapping peaks were not observed in the sequences, indicating failure to detect co-infections. Table 3 shows the results obtained by microscopy and PCR. All fecal samples collected ($n = 1728$) were subjected to the traditional parasitological technique (Ritchie’s modified ethyl acetate centrifugation) and microscopy. It was not possible to perform PCR on all samples due to technical or logistical limitations. Two hundred and fifty-three samples were subjected to PCR. Of these, 65.3% (77/118) were positive for both techniques and 34.7% (41/118) were PCR-positive and microscopy-negative (Table 3). Finally, 60 Entamoeba spp. sequences were successfully obtained (50.8% of PCR amplifications), and many sequences from other organisms were identified, including fungi, bacteria, plants and other intestinal protozoa (Endolimax, Iodamoeba and Blastocystis genera) (data not shown).

**Discussion**

In the present study, we describe high positivity rates for infection by Entamoeba spp. in economically vulnerable communities with poor health infrastructure in different regions of Brazil. Amebiasis is a water- and food-borne disease with strong socio-environmental determinants, and its distribution is poverty-related and heterogeneous among human populations [20–22] and remains endemic in many Brazilian regions.

We characterized species and subtypes of Entamoeba spp. circulating in different communities. The interspecific variability of Entamoeba spp. based in SSU rDNA
locus was high, $H$ close to 1. Our results draw attention to the characterization of new haplotypes for all *Entamoeba* species. Of the 36 haplotypes found, only four had already been described. These four haplotypes were in a central region of the haplotype network, indicating their ancestral nature, as in *E. dispar* and in *E. coli* ST1 and ST2. In addition, 14 new haplotypes described here originated from these ancestral haplotypes. Despite this, no new ribosomal lineages were identified. Among the members of the family Entamoebidae, the genus

![Fig. 5 Haplotype network based on SSU rDNA locus of tetra- and octonucleated cysts of Entamoeba (542 bp, n = 89). Area of the circle is proportional to number of sequences.](image)

### Table 3 *Entamoeba* spp. microscopy and PCR results in the present study

| Biome/locality       | Samples | Result                                    |
|----------------------|---------|-------------------------------------------|
|                      | Microscopy-positive $N$ (%) | PCR-positive* $N$ (%) | Microscopy-positive + PCR-positive $N$ (%) | Microscopy-negative + PCR-positive $N$ (%) | *Entamoeba* spp. sequences obtained** $N$ (%) |
| Amazon               |         |                                           |                                     |                                           |                                      |
| Santa Isabel do Rio Negro (AM) | 392     | 160/392 (40.8)                             | 38/98 (38.7)                         | 16/38 (42.1)                             | 22/38 (57.9)                          | 23/38 (60.5)                          |
| Bagre (PA)           | 362     | 65/362 (17.9)                              | 36/59 (61)                           | 30/36 (83.3)                             | 6/36 (16.7)                           | 27/36 (75)                            |
| Caatinga and Cerrado|         |                                           |                                     |                                           |                                      |                                      |
| Teresina (PI)        | 298     | 59/298 (19.7)                              | 14/26 (53.8)                         | 10/14 (71.4)                             | 4/14 (28.6)                           | 4/14 (28.5)                           |
| São João do Piauí (PI)| 131    | 31/131 (23.6)                              | 16/33 (48.4)                         | 13/16 (81.3)                             | 3/16 (18.7)                           | 4/16 (25)                             |
| Atlantic Forest      |         |                                           |                                     |                                           |                                      |                                      |
| Cachoeiras de Macacu (RJ)| 545  | 72/545 (13.2)                              | 14/37 (37.8)                         | 8/14 (57.1)                              | 6/14 (42.9)                           | 2/14 (14.2)                           |
| Total                | 1728    | 387/1728 (22.3)                            | 118/253 (46.6)                       | 77/118 (65.3)                            | 41/118 (34.7)                         | 60/118 (50.8)                         |

Only fecal samples positive on light microscopy were subjected to molecular analyses for species characterization and phylogenetic studies. Some *Entamoeba* spp. negative samples on microscopy were selected, randomly or when another individual from the same household was positive, to be tested on PCR. AM Amazonas, PA Pará, PI Piauí, RJ Rio de Janeiro. $N$: absolute number, (%): percentage. *Includes microscopy positive and negative results. **Sequences from other organisms were also obtained, including fungi, bacteria, plants and other intestinal protozoa (*Endolimax* genus)
Entamoeba is the most well studied due to the pathogenic potential of certain species. In the phylogenetic trees generated in the present study, sequences were grouped into two groups for the Entamoeba genus, as in Stensvold et al. [9] and Jacob et al. [23]. However, some differences could be seen. Another limitation of the study is the fact that we obtained sequences of 542 bp, which corresponds to one third the size of the gene chosen for the analyses. This generated relatively low bootstrap values in some branches of the phylogenetic trees.

The most evident difference was that the uninucleated E. polecki species was grouped in the same group as the octonucleated E. coli, unlike Stensvold et al. [9], where it was grouped with the uni- and tetranucleated species. Our result is similar to the study by Jacob et al. [23], in which E. polecki shared a common ancestor with the octonucleated E. coli. Another evident difference was E. moshkovskii, which shared a common ancestor with uni- and tetranucleated in our ML tree. In contrast, in the NJ tree, this species shared a common ancestor with the tetranucleated species E. eucadoriensis, E. dispar, E. histolytica, E. nuttalli and E. bangladesshi, as in Stensvold et al. [9] and Jacob et al. [23]. Additionally, E. hartmanni shared a common ancestor with E. insolita, E. terrapinae and Entamoeba RL5 and RL6 in the ML tree. In the NJ tree this species shared a common ancestor with E. terrapinae, as seen in Stensvold et al. [9]. Other small differences were seen when compared with Stensvold et al. [9] and Jacob et al. [23]. The differences observed between our study and previous studies can be explained by the fact that we did not use all the sequences available in GenBank, since we selected reference sequences without degenerate bases. Moreover, these results make us wonder whether the SSU rDNA locus or the "number of nuclei in the mature cyst" morphological character are suitable for the taxonomic classification of the Entamoeba genus. Apparently much still needs to be studied to understand the genetic complexity of members of this genus.

The main finding regarding tetranucleated cyst-producing Entamoeba species was the low proportion of E. histolytica found in the obtained sequences. Most sequences were characterized as E. dispar or E. hartmanni, considered nonpathogenic species. It is speculated that E. dispers and E. hartmanni are responsible for most infections that were previously considered to be associated with E. histolytica [3, 24, 25]. In fact, molecular epidemiological studies show that E. dispers is the species most commonly found among the tetranucleated cysts [21, 26–29]. Our results revealed that E. dispers presented a wider geographic distribution, whereas E. histolytica was identified only in the Amazon biome. Our results corroborate other studies conducted in Brazil, which have suggested that E. histolytica is more common in northern and northeastern Brazil and is less frequently detected in other regions [26, 30, 31]. However, we cannot rule out a sample bias and limitations in the PCR technique that could favor the amplification of one species over another.

In this study, we found only one haplotype for E. histolytica, and it was distinguished from the haplotype previously described in North America, Africa and Asia (X65163, E. histolytica HM-1:IMSS strain) [32–34]. In an overview of the diversity of Entamoeba histolytica by Zermeño and colleagues [35], they argue that although many haplotypes are found in only a single country, there are no lineages within the networks that may be related to a particular geographic region or infection outcome. These positive subjects in the present study had no symptoms. We must consider that most E. histolytica infections can be asymptomatic and that only 10% of those infected have symptoms [36–38].

The intraspecific variability of E. hartmanni was as high as the interspecific variability of the Entamoeba genus. In addition, among the eight sequences obtained from E. hartmanni, seven different haplotypes were found. Moreover, and all sequences were obtained in the Amazon biome. Only one haplotype obtained has been previously described in humans and nonhuman primates (100% similarity to KX618191 and AF149907, respectively) [39, 40]. Previous studies have suggested low variability for E. hartmanni based on restriction fragment length polymorphism and SSU rDNA sequencing [9, 41]. However, the real epidemiological significance of the high diversity found in the present study remains to be clarified.

The phylogenetic trees and MJ network revealed two main clusters for E. coli corresponding to the previously described subtypes ST1 and ST2 in Stensvold and colleagues [9]. In the present study, Entamoeba coli ST1 had a wider geographic distribution, being identified in all studied biomes, with the presence of five new haplotypes. Two previously described haplotypes were found, one isolated from humans in Nigeria (FR686364) [9] and the other from humans in the USA and nonhuman primates in Germany (AF149915 and FR686410, respectively) [9, 39]. Entamoeba coli ST2 subtype was identified in the Caatinga and Amazon biomes, being the predominant subtype in the Amazon. Twenty sequences were obtained and 13 new haplotypes were described. Only one haplotype has been previously described, isolated from humans in England (AF149914) [41]. It is hypothesized that the ST1 subtype is more common than ST2 in humans [42], and ST2 was recently identified in wild nonhuman primates in Asia and Africa [43, 44]. It is intriguing to observe a large number of new E. coli ST2 subtypes in humans living in the Amazon rainforest, the habitat of great diversity of neotropical primates.
We observed that 65.3% of the samples had a positive result with both techniques used (microscopy and PCR). Moreover, despite a reasonable number of PCR-positive samples (n = 118), we were only successful in sequencing and obtaining Entamoeba spp. SSU rDNA sequences in 50.8% of them. This makes us reflect on the limitations of the techniques used as well as of the fecal samples. Many factors can influence the success of the analysis, including low parasitic load in the sample and randomness in pipetting, unspecific PCR amplifications (multiple bands on the agarose gel), limitations in the PCR technique (primer binding, reaction), intrinsic limitations of the fecal samples (presence of microbiota and in different counts), and differences in the sensitivity of the techniques used. In addition, non-Entamoeba spp. sequences were obtained in this study, such as fungi or bacteria, which makes the analysis difficult and time-consuming.

The microscopy identified co-infections with octo- and tetranucleated cysts. In contrast, by direct nucleotide sequencing it was not possible to verify the presence of more than one species, and only a small amount of “background” was present in chromatograms. The species identified in the sequencing were related to the number of cysts observed under microscopy, in which more cysts represent more DNA available for PCR. The morphological similarity between E. histolytica and E. dispar, and the unusual large cysts of E. hartmanni, can also lead to misidentification [26], making microscopic analysis problematic for the diagnosis of non-symptomatic amebiasis.

As mentioned above, the communities included in the present study are situated in four different biomes with very distinct climates. Human populations in the Amazon, Caatinga and Cerrado regions have a higher prevalence of intestinal parasites, a higher incidence of diarrheal diseases and a lower proportion of the population with access to adequate sanitation and safe drinking water [45–48]. Although there are great differences in both the supply of drinking water and the water resource management strategies in these regions, in both there is a shortage of clean water for human consumption [47, 49].

In Brazil, intestinal parasitism control strategies target soil-transmitted helminths through mass albendazole administration. Moreover, the precarious conditions of sanitation and water management systems in some regions contributes to the current trend in the etiological profile of intestinal parasitism, characterized by the permanence of protozoan infections, with a reduction in the prevalence of soil-transmitted helminths [50–52]. Even though intestinal infections with soil-transmitted helminths are a major problem, affecting mainly children worldwide, estimates from the Global Burden of Disease Study indicate that other agents, including intestinal protozoa, are responsible for more than 6 million disability-adjusted life years (DALYs) [51].

Conclusion
Tetra- and octonucleated cyst-producing Entamoeba species are endemic in the studied communities, which represent low-income regions with nonexistent or insufficient sanitation systems. The pathogenic E. histolytica was found in a low proportion and only in the Amazon biome. Additionally, other tetranucleated species were commonly found in the studied regions. The distribution of Entamoeba species in Brazil is clinically important information, since many E. histolytica-positive parasitological examinations in fact represent infections with non-pathogenic species within the E. histolytica/E. dispar complex or E. hartmanni. Entamoeba coli subtypes present a geographically uneven distribution, with the ST2 subtype—commonly found in nonhuman primates—being predominant in the Amazon biome.

Abbreviations
ST1: Subtype 1; ST2: Subtype 2; CAM: Cachoeiras de Macacu; TER: Teresina; SJIPI: São João do Piauí; SIRN: Santa Isabel do Rio Negro; BAG: Bagre; PCR: Polymerase chain reaction; SSU rDNA: Small subunit rRNA gene; ML: Maximum likelihood; NJ: Neighbor-joining; BIC: Bayesian information criterion; MJ: Median joining.

Supplementary Information
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Authors’ contributions
FACC, DAC and LHJ conceived and designed the study and critically revised the manuscript. DAC AND LHJ performed the experiment, analyzed the data and drafted the manuscript. DAC, LHJ, FACC and BCN helped in data analysis and manuscript revision. DAC, LHJ, KILM, BCN, PAA, BBCE, MMA, MNB AND JPS helped in study implementation. All authors read and approved the final manuscript.

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Availability of data and materials
The datasets analyzed during the present study are available from the corresponding author upon reasonable request. All sequence files are available from the GenBank database under accession numbers MW026735–MW026794.

Declarations

Ethics approval and consent to participate
The study was approved by the Research Ethics Committee (license CAAE 12125713.5.0000.5248) of the Oswaldo Cruz Institute, Fiocruz. All subjects included in this study provided written informed consent, and the parent or legal guardian of all children included in this study provided written informed consent on their behalf.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

Author details
1 Laboratório de Epidemiologia e Sistêmática Molecular, Instituto Oswaldo Cruz, Fundação Oswaldo Cruz, Rio de Janeiro, RJ, Brazil. 2 Centro/Norte, Escritório Técnico Regional - Fundação Oswaldo Cruz, Piauí, Rua Magalhães Filho, 519, Teresina, Piauí, Brazil. 3 Laboratório de Biologia e Parasitologia de Mammíferos Silvestres Reservatórios, Instituto Oswaldo Cruz, Fundação Oswaldo Cruz, Rio de Janeiro, RJ, Brazil. 4 Faculdade de Medicina de Petrópolis (FMP)/Faculdade Arthur Sá Earp Neto (FASE), Rua Machado Fagundes, 326, Cascatinha, Petrópolis, Rio de Janeiro, Brazil. 5 Faculdade de Farmácia, Universidade Federal de Juiz de Fora, Rua José Lourenço Kelmer, s/n – Campus Universitário, Bairro São Pedro, Juiz de Fora, Minas Gerais, Brazil.

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