Giardia duodenalis assemblage A: new genotype in non-human primates from the Brazilian Amazon region revealed by high-resolution MLST

Lisiane Lappe dos Reis[1,3,4]/+, Lirna Salvioni Silva de Souza[1], Maria Gabriela Rodrigues Antunes[1], Alessandra Ferreira Dales Nava[2], Ana Carolina Paulo Vicente[3]

[1]Fundação Oswaldo Cruz-Fiocruz, Instituto Leônidas & Maria Deane, Laboratório de Diversidade Microbiana da Amazônia de Importância para a Saúde, Manaus, AM, Brasil
[2]Fundação Oswaldo Cruz-Fiocruz, Instituto Leônidas & Maria Deane, Laboratório de Ecologia de Doenças Transmissíveis na Amazônia, Manaus, AM, Brasil
[3]Fundação Oswaldo Cruz-Fiocruz, Instituto Oswaldo Cruz, Laboratório de Genética Molecular de Microrganismos, Rio de Janeiro, RJ, Brasil
[4]Fundação Oswaldo Cruz-Fiocruz, Instituto Oswaldo Cruz, Programa de Pós-Graduação *Stricto Sensu* em Biologia Parasitária, Rio de Janeiro, RJ, Brasil

BACKGROUND *Giardia duodenalis* is an intestinal protozoan parasite that infects a wide range of vertebrate hosts, including humans, and is the etiological agent of giardiasis. Within this species, assemblage A is further subdivided into three sub-assemblages: AI, AII, and AIII.

OBJECTIVES To apply the high-resolution multilocus sequence typing (MLST) to perform molecular epidemiology of *G. duodenalis* assemblage A in humans and animals in the State of Amazonas, Brazil.

METHODS We performed parasitological analyses of faecal samples from animals and collected *Giardia*-positive samples from humans in the State of Amazonas, Brazil. *Giardia* genotyping was carried out using an MLST scheme based on six genes (*CID1*, *NEK15411*, *DIS3*, *HCMP22547*, *HCMP6372*, *RHP26*), as well as the three conventional loci (*tpi*, *bg* and *gdh*), followed by phylogenetic analysis.

FINDINGS Using an extended MLST scheme for assemblage A, we identified a new genotype infecting two non-human primate (NHP) species from the Amazon region. Additionally, *Giardia* from sub-assemblage AII infecting humans in this region shows a close phylogenetic relationship with isolates from other countries. Furthermore, a sub-assemblage AI isolate from an anteater (*Tamandua tetradactyla*) belongs to a lineage infecting diverse animals and humans, highlighting its zoonotic and cosmopolitan nature.

MAIN CONCLUSIONS These findings reveal a new, genetically distinct lineage within *G. duodenalis* assemblage A, likely representing a new sub-assemblage (AIV) associated with NHPs in the Amazon region, and reinforce the usefulness of high-resolution MLST for detecting fine-scale genetic diversity and potential zoonotic transmission pathways.

Key words: molecular epidemiology - multilocus sequence typing - zoonoses - genetic diversity - giardiasis.

Giardia duodenalis is a protozoan parasite that infects the intestinal tract of a broad range of animals, including humans, causing giardiasis worldwide.[1,2] The *Giardia* genome consists of approximately 12 Mb in size divided among five chromosomes.[3] Within *G. duodenalis*, eight genetically well-defined assemblages (A-H) have been identified, that differing in terms of host specificity and taxonomy. Assemblages A and B infect humans and animals, while the others infect specific hosts.[1,4] Assemblage A is classified into sub-assemblages AI, AII, and AIII.[4] While sub-assemblage AI is mainly zoonotic, most AII transmission occurs among humans, and AIII is found only in animals.[5,6] Furthermore, molecular species delimitation analyses have shown that the sub-assemblages AI and AII in fact represent distinct species of *Giardia* that have been named *G. duodenalis* and *Giardia intestinalis*, respectively.[6,7]

In the Brazilian Amazon, assemblages A and B were identified in humans, domestic animals (such as cats) and wild animals [such as sloths, bats and non-human primates (NHPs), including the species *Lagothrix cana* and *Ateles paniscus*].[8,9,10,11,12] Most studies characterised assemblages based on one, two or three of the most commonly used genes: *β-giardin* (*bg*), *triosephosphate isomerase* (*tpi*), and *glutamate dehydrogenase* (*gdh*).[5]

Until 2018, the classification of *G. duodenalis* into assemblages and sub-assemblages was primarily based on three genes: *bg*, *tpi* and *gdh*. These genes are located

doi: 10.1590/0074-02760250234
Financial support: FAPEAM (Edital No. 002/2021 - Programa Amazônidas - "Mulheres e Meninas na Ciência"), PROEP-LABS/ILMD Fiocruz Amazônia.
+ Corresponding author: lisiane.reis@fiocruz.br | https://orcid.org/0000-0002-7968-3718
Received 28 August 2025
Accepted 16 December 2025

How to cite: dos Reis LP, de Souza LSS, Antunes MGR, Nava AFD, Vicente ACP. *Giardia duodenalis* assemblage A: new genotype in non-human primates from the Brazilian Amazon region revealed by high-resolution MLST. Mem Inst Oswaldo Cruz. 2026; 121: e250234.
Handling editor: Geovane Dias-Lopes | https://orcid.org/0000-0003-4448-1398

on chromosomes 4 (*bg* and *gdh*) and chromosome 5 (*tpi*).[13] While these markers allowed differentiation of the main assemblages (A-H), they offered limited resolution for intra-assemblage variability.[5] In 2018, Ankarklev et al. proposed a more sophisticated multilocus sequence typing (MLST) scheme for typing assemblage A, based on six genes distributed across four chromosomes.[13] In contrast, the genes in the previous scheme were located on only two chromosomes.[13]

The MLST approach using six genes confirmed the genetic groups already identified within assemblage A (sub-assemblages AI, AII and AIII) with significantly improved sensitivity. It can therefore be used as a robust tool to assess the zoonotic potential, investigate outbreaks and identify new genotypes.[5,13] Here, we used this robust MLST approach based on six genetic loci (*CID1*, *NEK15411*, *DIS3*, *HCMP22547*, *HCMP6372* and *RHP26*) to genotype assemblage A and perform a molecular epidemiological survey of *Giardia* assemblage A infecting humans and animals in the Brazilian Amazon region looking for zoonotic evidence.

MATERIALS AND METHODS

Study area and collection of faecal samples - We extracted sequences from this set of genes from all the available *G. duodenalis* assemblage A genomes in GenBank (accessed 20 April 2025) in order to perform a phylogenetic analysis. We also included sequences from eight *Giardia* isolates from this study: four from humans and four from animals (two capuchin monkeys, one spider monkey and one anteater) (Fig. 1). These samples were collected in Manaus (3º3′58.48″S, 59º59′48.85″W), Amazonas State, Brazil. The capuchin monkeys were of the species *Sapajus apella* and were assigned as C46 (a young male found in a neighbourhood adjacent to a forest reserve in Manaus) and C60 (a young male found on the street exhibiting symptoms of diarrhoea). The spider monkey (*A. paniscus*) was assigned as 700S and the anteater (*Tamandua tetradactyla*) was designated C62. Faecal samples from animals were collected at the Wild Animal Screening and Rehabilitation Centre (CETAS) by convenience sampling. Human faecal samples positive for *Giardia* were obtained by convenience sampling from patients diagnosed with giardiasis at local health units in Manaus, Amazonas State, Brazil.

Laboratory processing of faecal samples - Faecal samples from animals were examined by spontaneous sedimentation[14] and zinc sulphate centrifugal-flotation.[15] The presence of *Giardia* cysts was confirmed through the observation by an optical microscope at 40x magnification. *Giardia* cysts from both animal and human samples were partially purified by the sucrose density gradient method.[16]

Molecular analysis - Genomic DNA was extracted using QIAamp Stool Mini kit following five freeze-thaw cycles.[17] Polymerase chain reaction (PCR) was carried out targeting the *tpi*[18] *bg*[19,20] and *gdh*[21] encoding genes, as well as the MLST scheme[13] [Supplementary data (Table I)]. The amplicons were purified using PureLink Quick PCR Purification Kit (Invitrogen, Lithuania), according to the manufacturer's instructions. The fragments were Sanger sequenced using a BigDye Terminator Cycle Sequencing Ready Reaction Kit. Sequencing was performed on the ILMD/Fiocruz Amazônia and Instituto Oswaldo Cruz/Fiocruz-RJ sequencing platforms.

We assembled a dataset based on a concatenated sequence alignment of the six loci (*CID1*, *NEK15411*, *DIS3*, *HCMP22547*, *HCMP6372* and *RHP26*) of the MLST scheme, as well as the three conventional loci (*tpi*, *bg* and *gdh*). Our study only included samples containing sequences of these nine genes.

We then performed phylogenetic analyses using datasets processed in Phylosuite:[22,23] IQ-Tree v1.6.8[24] was used for maximum likelihood (ML) inference, and ModelFinder v2.2.0[25] was applied to select the best-fit partition model (Edge-unlinked) using AICc criterion. For the phylogenetic tree based on three concatenated loci, the best-fit models were GTR+F+I+G4 (*bg*+*gdh*), GTR+F+G4 (*tpi*). For the phylogenetic tree based on six concatenated loci, the best-fit models were K2P+I+G4 (CID1), K2P+I+G4 (DIS3), GTR+F+I+G4 (HCMP22547), SYM+I+G4 (HCMP6372), HKY+F+I+G4 (NEK15411), HKY+F+I+G4 (RHP26). Clade support was estimated using 5,000 replicates of both ultrafast bootstrap (UFBoot) and the Shimodaira-Hasegawa approximate likelihood ratio test (SH-aLRT), with support values displayed at the nodes as SH-aLRT / UFBoot (%). The final phylogenetic trees were visualised in Figtree v1.4.0 and subsequently edited in Inkscape.

The reference sequences of the *G. duodenalis* genomes and genes used in the phylogenetic analyses are in the [Supplementary data (Table II)].

Ethical approval - This study was approved by SISBIO (General Licence for the Collection of Animals) under licence number 67153-3, by CEUA-UFAM (the State Ethics Committee for the Use of Animals at the Federal University of Amazonas) under licence number 017/2020 and by CEP/FMT-HVD under licence number CAAE 86170325.3.0000.0005.

Fig. 1: animals positive for the new *Giardia duodenalis* sub-assemblage A genotype: (A) *Sapajus apella* and (B) *Ateles paniscus*. The animals were housed individually at the Wild Animal Screening and Rehabilitation Centre (CETAS) facility during treatment and rehabilitation.

RESULTS AND DISCUSSION

The novel MLST scheme for *G. duodenalis* assemblage A[13] has demonstrated good resolution in revealing diversity among isolates from this assemblage, allowing robust epidemiological analyses.[26,27] Here, we successfully amplified the six genes, as well as three genes (*tpi*, *bg* and *gdh*) from four positive human samples (4/33) and four positive animal samples (4/14) for *G. duodenalis*.

When we applied the conventional scheme of three genes (*tpi*, *bg* and *gdh*), the phylogenetic analysis showed seven clusters corresponding to assemblages A-G (Fig. 2). All *Giardia* isolates from our study were grouped in the assemblage A cluster. However, when we applied the six-gene MLST scheme to assemblage A, using the same set of animal and human isolates from the Amazon region, the phylogenetic tree (Fig. 3), based on 3,343 bp from the six genes, revealed four major clusters. Cluster 1 (yellow) comprises all the human isolates worldwide, including those from the Amazon region (from this study), which are classified as sub-assemblage AII. Indeed, studies performed worldwide using different approaches, such as complete *Giardia* genome sequencing or genotyping based on one to six genes, have shown that sub-assemblage AII occurs in several countries, including Brazil, Canada, the USA, Sweden and Germany.[6,7,27] In Brazil, sub-assemblage AII has been identified in humans in the states of Amazonas, Piauí, Ceará, Rio de Janeiro, São Paulo and Mato Grosso, as determined by genetic sequencing of the *tpi*, *bg* and/or *gdh*.[8,12,28-30] Cluster 2 (green) contains both animal and human sequences from various geographical regions, including C62/anteater from *T. tetradactyla* (this study), which also belongs to sub-assemblage AI. Interestingly, a previous study reported the presence of *G. duodenalis* assemblage B in a *T. tetradactyla* living in a Polish zoo.[31] Notably, this cluster contains sets of sequences of both human and animal origin from several countries, including Canada, the USA, Italy and the Czech Republic,[26,32,33,34] which is likely to reflect the zoonotic nature of sub-assemblage AI, as previously demonstrated.[7,13,26] Cluster 3 (grey) comprises a single sub-assemblage AIII sequence derived from a cat and, so far, has been reported exclusively in animals.[6] Cluster 4 (pink) comprises the three NHP sequences obtained in this study. These NHPs belong to the species *S. apella* and *A. paniscus*, which are both widely distributed in South America.[35,36]

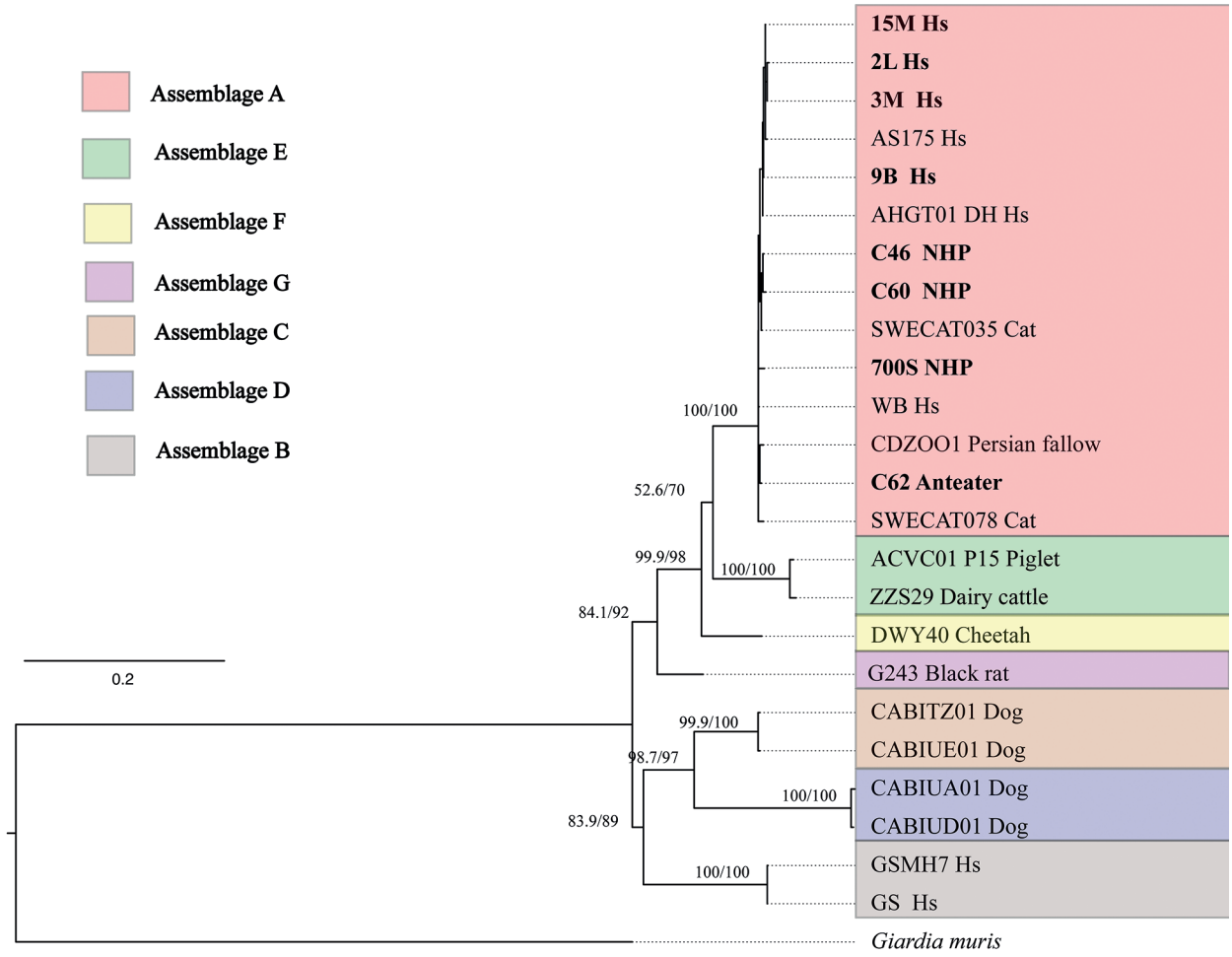

Fig. 2: phylogenetic tree based on concatenated *bg*, *tpi* and *gdh* genes (1,752 bp). In bold, animal and human sequences from this study. Hs: *Homo sapiens*; NHP: non-human primate. Branch support values ≥ 50% [maximum likelihood (ML)] are indicated above the branches.

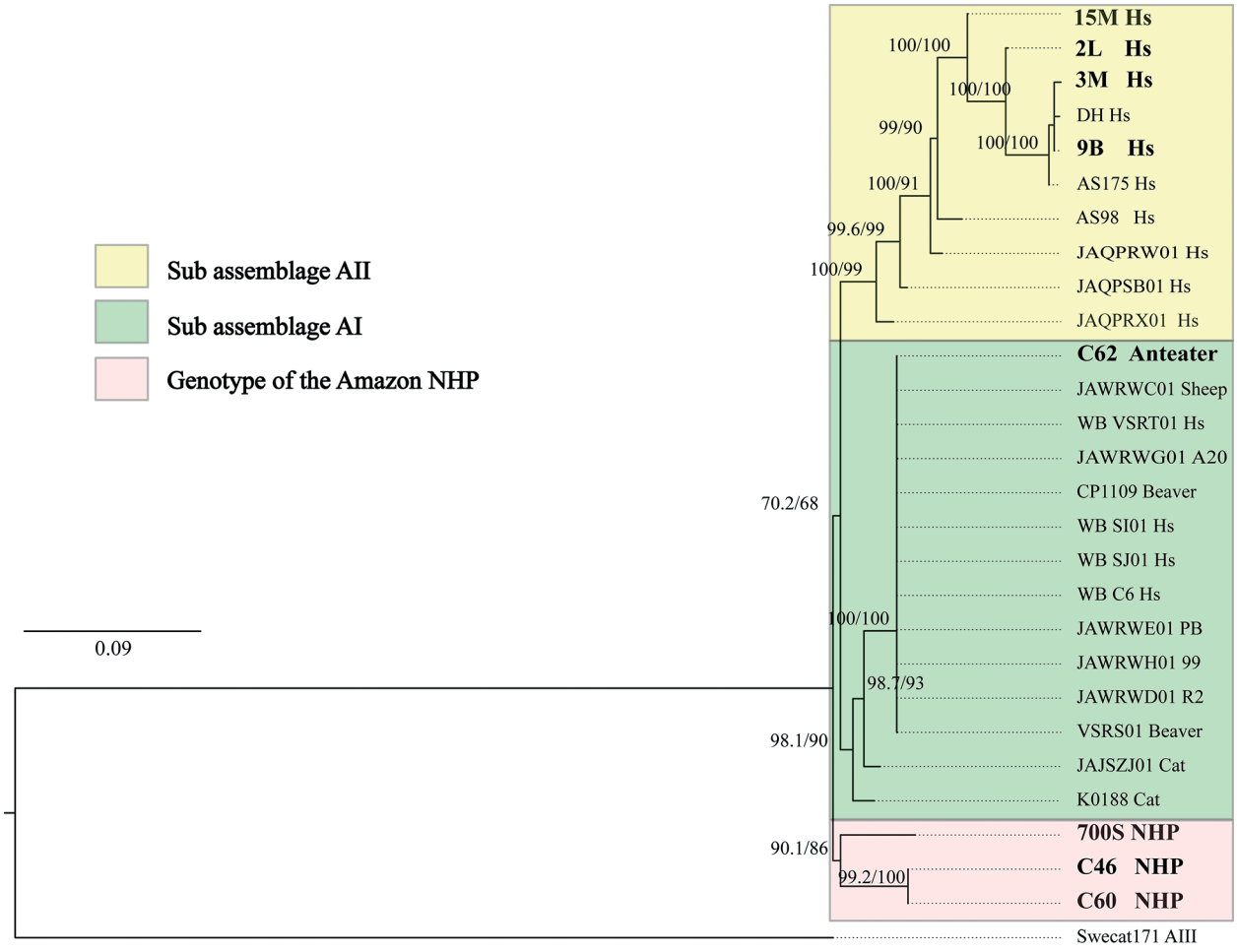

Fig. 3: phylogenetic tree based on concatenated *HCMP22547*, *RHP26*, *CID1*, *NEK15411*, *HCMP6372* and *DIS3* genes (3,343 bp). In bold, animal and human sequences from this study. Hs: *Homo sapiens*; NHP: non-human primate. Branch support values ≥ 70% [maximum likelihood (ML)] are indicated above the branches.

We had previously identified *Giardia* assemblage A in isolate 700S from *A. paniscus* in the Brazilian State of Amazonas, using the *tpi* gene.[8] Notably, when the novel six-gene MLST scheme specific to assemblage A was applied, isolate 700S and the two additional NHP isolates (C46 and C60) from *S. apella* clustered separately from the AI, AII and AIII sub-assemblages. To date, global genetic and genomic studies of assemblage A have reported only sub-assemblages/genotypes AI, AII, and AIII, which differ in host preferences, with AI occurring in both humans and animals, AII exclusively in humans, and AIII almost exclusively in wild ruminants.[6] However, the tree topology (Fig. 3) revealed a new, independent cluster containing *Giardia* sequences from two NHPs species. This cluster may represent a new sub-assemblage/genotype of assemblage A (AIV), which is genetically distinct from the known AI, AII, and AIII sub-assemblages within *G. duodenalis* assemblage A.

Interestingly, one of the animals of the species *S. apella* exhibited diarrhoea at the time of capture. However, parasitological analysis revealed the presence of *Giardia* trophozoites and cysts, as well as hookworm eggs and larvae. Therefore, no direct association can be established between the diarrhoea and the new *Giardia* genotype. These NHP species infected by this new *G. duodenalis* assemblage A genotype have a wide geographic distribution in South America. Although they are wild animals, they are not uncommonly in close contact with humans. Consequently, this new *Giardia* sub-assemblage A genotype could be circulating among other animals and humans in the Amazon region, and potentially being transmitted to them.

One limitation of this study is the current lack of multilocus datasets worldwide that employ the same six gene targets. As a result, only a small number of reference sequences are available in public databases, which restricts broader comparative analyses. Another limitation is the absence of complementary analyses, such as whole-genome sequencing of the newly identified genotypes. Such analyses would provide a more comprehensive understanding of their genetic profile and taxonomic status.

AUTHORS' CONTRIBUTION

LLR, AFDN and ACPV - conceptualisation; LLR, ACPV, LSSS and MGRA - methodology; LLR and ACPV - writing - original draft, writing - review and editing; LSSS, AFDN and

MGRA - writing - review and editing. All authors read and approved the final manuscript. The founders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript. The authors declare that they have no competing interests.

DATA AVAILABILITY

All data generated or analysed during this study are included in this published article (and its Supplementary data). The GenBank accession numbers of the novel sequences generated in this study are: PV769412 - PV769459.

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OPEN PEER REVIEW

Memórias do IOC thanks the anonymous reviewers for their contribution to the peer review of this work.

FIRST REVIEW ROUND

REVIEWERS' COMMENTS

REVIEWER #1

The manuscript by Lappe dos Reis et al. entitled "Giardia duodenalis Assemblage A Zoonotic Potential and a Novel Genotype in Non-Human Primates from the Brazilian Amazon Revealed by High-Resolution MLST" describes a putatively new Giardia duodenalis assemblage A genotype as identified by the application of the MLST scheme developed by Ankarklev et al. 2018 (PMID: 29438742). The observation is worth publishing, but I suggest to clarify some parts of the manuscript.

Throughout the manuscript: Please check the English language (at best by a native speaker).

Line 1: The title is long and not very specific. Please reconsider to change the title. Maybe: "Giardia duodenalis assemblage A: Identification of a novel genotype in non-human primates from the Brazilian Amazon region"

Line 29: Please correct language, "to applied"……"to performed" does not make sense.

Line 36/37: I suggest to change "we identified a novel genotype" by " …we identified a new genotype…."

Line 40: should read "animals" instead of "animal".

Line 42: key words are very cryptic.

Line 52: Headline "Introduction" is missing

Line 53/54: the statement "..a neglected disease by the WHO (1).." is not correct anymore. G. duodenalis is currently not in the list. Please omit.

Line 56/57: The sentence should end with "… and host specificity." Then a new sentence explaining what you mean by "..and supported by distinct genetic marker" should be included.

Line 58/59: please clarify the statement: " …since individual markers are unstable and show conflicting results when examined in isolation". It is not clear what the authors intended to say.

Line 68-70: Clarify that the MLST scheme is only for typing of assemblage A.

Line 101: Did the authors sequence bi-directionally? How did the authors check quality?

Line 127-129: I am not sure whether these statements are necessary. It is not clear where the zoo animal got the infection from and when the animal it was captured.

Line 133: I don't understand this statement. Ankarklev et al. 2018 showed typing of AIII with the new MLST scheme. Clarify.

Primer table: There are still some Spanish terms.

REVIEWER #2

The article is very interesting and presents updated information on the zoonotic parasite Giardia duodenalis. However, I suggest the following modifications:

Introduction

Expand the introduction by including, in one paragraph, what has already been conducted with non-human and human primates in Brazil—particularly in Amazonian states—in terms of molecular biology applied to Giardia duodenalis, highlighting the target genes used and the main findings reported in previous studies.

Materials and Methods

I recommend reorganizing this section into subsections.

1. Sampling

Clarify the type of sampling used — was it a convenience sampling?

2. Collection and laboratory processing of fecal samples

Describe in detail the sampling process, the total number of samples obtained, and the laboratory procedures, including the microscopic techniques applied to both animal and human samples. Indicate the type of microscope used and the magnification applied during slide reading.

3. Molecular analysis

Specify whether DNA extraction strictly followed the manufacturer's instructions. If so, it is unnecessary to detail parameters such as "lysis buffer temperature to 95 °C for 15 min, and 200 µL of elution buffer for 10 min at room temperature."

Clarify the volume of DNA applied during purification and whether electrophoresis was performed to verify the integrity and presence of the expected bands.

Indicate the sequencing platform used and whether the samples were sequenced in both directions (forward and reverse strands).

In line 117, add the acronym MLST.

Results and Discussion

• Line 123: Specify in which countries subtype AII has already been reported and indicate whether previous records exist in Brazil.

Line 130: Apply the same clarification to the expression "several countries."

• Line 146: The finding of subtype AVI is relevant and, being unprecedented, deserves comparison with other parasites detected in non-human primates in Brazil, such as Toxoplasma gondii (atypical isolates) and Balantioides coli (atypical isolate in Brachyteles arachnoides). Emphasize that these genetic divergences may reflect regional particularities and propose possible explanations for this pattern.

• Clarify whether the electrophoresis run showed any difference related to the molecular weight of the detected subtype band.

Figures and Phylogenetic Analyses

Standardize the phylogenetic trees in terms of formatting — remove underscores, harmonize fonts, and unify host names (either all in scientific names, italicized, or all in common names).

Include Brazilian sequences from other studies for comparison with your results.

I also recommend removing the animal photographs, as they do not add relevant scientific information.

Final Considerations

Include the study's limitations in the discussion, highlighting aspects such as sample size, lack of complementary analyses, or methodological constraints.

AUTHORS' RESPONSE TO THE REVIEWERS

Reviewer: 1

The manuscript by Lappe dos Reis et al. entitled "Giardia duodenalis Assemblage A Zoonotic Potential and a Novel Genotype in Non-Human Primates from the Brazilian Amazon Revealed by High-Resolution MLST" describes a putatively new Giardia duodenalis assemblage A genotype as identified by the application of the MLST scheme developed by Ankarklev et al. 2018 (PMID: 29438742). The observation is worth publishing, but I suggest to clarify some parts of the manuscript.

Reviewer comment: Throughout the manuscript: Please check the English language (at best by a native speaker).

Response: We check the English language.

Reviewer comment: Line 1: The title is long and not very specific. Please reconsider to change the title. Maybe: "Giardia duodenalis assemblage A: Identification of a novel genotype in non-human primates from the Brazilian Amazon region"

Response: We thank the reviewer for the comment and have modified the title of the manuscript to "Giardia duodenalis assemblage A: new genotype in non-human primates from the Brazilian Amazon region revealed by High-Resolution MLST". We believe it is important to include 'by high resolution MLST' in the title, as few Giardia genotyping studies to date have used this six-gene scheme with higher resolution to identify sub-assemblages of assemblage A with such a high level of resolution. Our study is only the fifth in the world to use this more sophisticated scheme, as suggested by Ankarklev et al (2018). We have changed "...we identified a novel genotype" to " …we identified a new genotype…." as suggested by reviewer.

Reviewer comment: Line 29: Please correct language, "to applied"……"to performed" does not make sense.

Response: We have made changes to the text (Line 30/31).

Reviewer comment: Line 36/37: I suggest to change "we identified a novel genotype" by " …we identified a new genotype…."

Response: We have made the suggested modifications to the text (Line 38).

Reviewer comment: Line 40: should read "animals" instead of "animal".

Response: The spelling of a word in the text was corrected by us (Line 40).

Reviewer comment: Line 42: key words are very cryptic.

Response: We have included other keywords that are listed in the MeSH Terms.

Reviewer comment: Line 52: Headline "Introduction" is missing

Response: We included the term 'Introduction' in the text.

Reviewer comment: Line 53/54: the statement "..a neglected disease by the WHO (1).." is not correct anymore. G. duodenalis is currently not in the list. Please omit.

Response: This statement was omitted from the text.

Reviewer comment: Line 56/57: The sentence should end with "… and host specificity." Then a new sentence explaining what you mean by "..and supported by distinct genetic marker" should be included.

Response: We have made the following modification to the text (lines 57/ 64):

The sentence on lines 56 and 57, '...and supported by distinct genetic markers', has been deleted, as this is already explained on lines 64/71.

Reviewer comment: Line 58/59: please clarify the statement: " …since individual markers are unstable and show conflicting results when examined in isolation". It is not clear what the authors intended to say.

Response: We would like to point out that there are situations in which the different genetic markers do not agree, which may be due to their low discriminatory power. For example, only a single nucleotide difference discriminates between the AI and AII allele subtypes within the bg amplicon, whereas two differences are required for the tpi subtypes. Although classification into three distinct sub-assemblages was supported, individual markers were less stable and produced conflicting results when analyzed in isolation. For example, Yang et al. (2025) classified the Giardia duodenalis isolate from ferret as assemblage AII at the tpi locus, whereas at the gdh and bg loci, it was classified as sub-assemblage AI. Thus, for Giardia, the use of MLST is essential for the identification of sub-assemblages of assemblage A, particularly the six-gene scheme proposed by Ankarklev. The six genes are located on four of the five chromosomes of Giardia duodenalis, providing a more robust phylogenetic identification of the sub-assemblages A, and similar to phylogenomics analysis.

Therefore, we have deleted the sentence on lines 58/59, "...and supported by distinct genetic marker", as this has already been explained on lines 64/71.

Reviewer comment: Line 68-70: Clarify that the MLST scheme is only for typing of assemblage A.

Response: We clarify in the text that the MLST scheme is only for typing of assemblage A. (Line 72).

Reviewer comment: Line 101: Did the authors sequence bi-directionally? How did the authors check quality?

Response: Yes, the samples were sequenced bi-directionally. Both forward and reverse sequences were obtained for each amplicon using the same primers applied in PCR amplification.

Sequence quality was checked using Geneious Prime (Biomatters Ltd., New Zealand). Raw chromatograms were visually inspected, and low-quality regions were trimmed using the "Trim Ends" function based on Phred quality scores (error probability limit = 0.01, equivalent to Q20). The forward and reverse reads were then mapped to the reference sequences to generate consensus sequences. All base discrepancies were manually verified by inspecting the chromatograms.

Reviewer comment: Line 127-129: I am not sure whether these statements are necessary. It is not clear where the zoo animal got the infection from and when the animal it was captured.

Response: We have removed that sentence.

Reviewer comment: Line 133: I don't understand this statement. Ankarklev et al. 2018 showed typing of AIII with the new MLST scheme. Clarify.

Response: We agree with the reviewer, and we have modified the sentence (Line 132).

Reviewer comment: Primer table: There are still some Spanish terms.

Response: We translated the terms into English.

Reviewer:2

The article is very interesting and presents updated information on the zoonotic parasite Giardia duodenalis.

However, I suggest the following modifications:

Reviewer comment: Introduction

Expand the introduction by including, in one paragraph, what has already been conducted with non-human and human primates in Brazil—particularly in Amazonian states—in terms of molecular biology applied to Giardia duodenalis, highlighting the target genes used and the main findings reported in previous studies.

Response: We inserted one paragraph with the suggestion into the text.

Reviewer comment: Materials and Methods

I recommend reorganizing this section into subsections.

1. Sampling

Clarify the type of sampling used — was it a convenience sampling?

Response: The animal and human samples were obtained by convenience sampling. This information was then incorporated into the text.

Reviewer comment: 2. Collection and laboratory processing of fecal samples

Describe in detail the sampling process, the total number of samples obtained, and the laboratory procedures, including the microscopic techniques applied to both animal and human samples. Indicate the type of microscope used and the magnification applied during slide reading.

Response: We have incorporated the reviewer's suggestions into the text. Animal fecal samples (n = 322) collected between March 2023 and November 2024 were analyzed at the Fiocruz Amazônia laboratory in Manaus, Brazil. The positivity rate for Giardia in the animal samples was 4.3% (14/322). However, the parasitological analysis of human fecal samples was carried out at the district laboratory of the Manaus Health Department. Consequently, only human samples positive for Giardia were collected at the district laboratory (n = 33).

We successfully amplified six genes suggested by Ankarklev et al. (2018), as well as three conventional loci (tpi, bg and gdh), from four positive human samples (4/14) and four positive animal samples (4/33) for Giardia. Consequently, our study only included samples of the Giardia assemblage A with sequences of these nine genes.

In all positive samples, we purified the cysts using sucrose gradient flotation. The samples were examined using a Zeiss Primostar trinocular microscope with transmitted LED light for bright field and objectives of 4x, 10x, 40x and 100x magnification, as well as a colour digital microscopy camera with 5 MB resolution. We examined the slides at 40x magnification.

Reviewer comment: 3. Molecular analysis

Specify whether DNA extraction strictly followed the manufacturer's instructions. If so, it is unnecessary to detail parameters such as "lysis buffer temperature to 95 °C for 15 min, and 200 µL of elution buffer for 10 min at room temperature."

Clarify the volume of DNA applied during purification and whether electrophoresis was performed to verify the integrity and presence of the expected bands.

Indicate the sequencing platform used and whether the samples were sequenced in both directions (forward and reverse strands).

In line 117, add the acronym MLST.

Response: We have made the necessary modifications to the text.

Electrophoresis was performed using 4 µL of the amplified product to verify sample integrity and the presence of the expected and non-specific bands. The remaining PCR product, totalling 46 µL, was purified after gel electrophoresis.

Sequencing was performed on the ILMD/Fiocruz Amazônia and Instituto Oswaldo Cruz/Fiocruz RJ sequencing platforms. The samples were sequenced in both directions (forward and reverse strands).

We added the acronym MLST as suggested.

Results and Discussion

Reviewer comment: • Line 123: Specify in which countries subtype AII has already been reported and indicate whether previous records exist in Brazil.

Response: We have included the revisor's suggestions in the text.

Reviewer comment: Line 130: Apply the same clarification to the expression "several countries."

Response: As suggested by the reviewer, we have added the countries to the text.

Reviewer comment: • Line 146: The finding of subtype AVI is relevant and, being unprecedented, deserves comparison with other parasites detected in non-human primates in Brazil, such as Toxoplasma gondii (atypical isolates) and Balantioides coli (atypical isolate in Brachyteles arachnoides). Emphasize that these genetic divergences may reflect regional particularities and propose possible explanations for this pattern.

Response: Our work focuses on showing the presence of a new Giardia genotype in NHPs. The nucleotide variability presented by the six genes suggested by Ankarklev et al (2018) allowed us to identify a new genotype of G. duodenalis assemblage A. Therefore, we are not conducting any evolutionary analyses on genetic divergence. Thus, we are unable to comment on genetic divergence. The presence of a new genotype in non-human primates in the Amazon is to be expected since this biodiversity hotspot has not yet been extensively explored.

Reviewer comment: • Clarify whether the electrophoresis run showed any difference related to the molecular weight of the detected subtype band.

Response: We performed nested PCR for each of the genetic markers included in the study. As expected for this protocol, the electrophoresis profiles showed a single amplicon band for each target, corresponding to the expected molecular size.

Reviewer comment: Figures and Phylogenetic Analyses

Standardize the phylogenetic trees in terms of formatting — remove underscores, harmonize fonts, and unify host names (either all in scientific names, italicized, or all in common names).

Response: We have made the suggested changes to the figures.

Reviewer comment: Include Brazilian sequences from other studies for comparison with your results.

Response: Unfortunately, there are no studies conducted in Brazil that have sequenced these six Giardia genes of the MLST scheme. Our study is the fifth in the world to use this new six-gene MLST scheme. Thus, in order to conduct a more robust analysis of the six gene sequences suggested by Ankarklev et al. (2018), we extracted these sequences from assemblage A genomes, which are available at NCBI.

Reviewer comment: I also recommend removing the animal photographs, as they do not add relevant scientific information.

Response: The animal photograph is a guideline from the MIOC journal. Therefore, we will not remove it.

Final Considerations

Reviewer comment: Include the study's limitations in the discussion, highlighting aspects such as sample size, lack of complementary analyses, or methodological constraints.

Response: As suggested by the reviewer, we have added the limitations of the study to the text.

The limitations of the study have been included in the manuscript. These limitations comprise the small number of studies conducted worldwide to date that have used the six genes, resulting in only a limited number of sequences for these genes being available on the NCBI database. Another limitation is the absence of complementary analysis of the whole-genome the newly identified genotypes, which is important for this study.

SECOND REVIEW ROUND

REVIEWERS' COMMENTS

REVIEWER #1

I am satisfied with the responses of the authors and have no further comments. Thank you.

REVIEWER #2

No other comments.

DECISION: ACCEPT SUBMISSION | RECEIVED 28 AUGUST 2025 | ACCEPTED 16 DECEMBER 2025