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ORIGINAL ARTICLE

Molecular characterization of *Giardia lamblia* in children less than 5 years of age with diarrhoea attending the Bengo General Hospital, Angola

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Background: *Giardia lamblia* is a pathogenic intestinal protozoan with high prevalence in developing countries, especially among children. Molecular characterization has revealed the existence of eight assemblages, with A and B being more commonly described in human infections. Despite its importance, to our knowledge this is the first published molecular analysis of *G. lamblia* assemblages in Angola.

Methods: The present study aimed to identify the assemblages of *G. lamblia* in children with acute diarrhoea presenting at the Bengo General Hospital, Angola. A stool sample was collected and microscopy and immunochromatographic tests were used. DNA was extracted and assemblage determination was performed through amplification of the gene fragment *ssu-rRNA* (175 bp) and *β-giardin* (511 bp) through polymerase chain reaction and DNA sequencing.

Results: Of the 16 stool samples screened, 12 were successfully sequenced. Eleven isolates were assigned to assemblage B and one to assemblage A. Subassemblage determination was not possible for assemblage B, while the single isolate assigned to assemblage A was identified as belonging to subassemblage A3.

Conclusion: This study provides information about *G. lamblia* assemblages in Bengo Province, Angola and may contribute as a first step in understanding the molecular epidemiology of this protozoan in the country.

GenBank accession numbers for the *ssur-RNA* gene: MF479750, MF479751, MF479752, MF479753, MF479754, MF479755, MF479756, MF479757, MF479758, MF479759, MF479760, MF479761.
GenBank accession numbers for the *β-giardin* gene: MF565378, MF565379, MF565380, MF565381.

Keywords: Angola, Children, Diarrhoea, Genotyping, *Giardia lamblia*, Hospital

Introduction

Giardia lamblia is a common intestinal parasite infecting a broad range of vertebrate species, including humans.[1] This parasite has a global distribution and it is estimated that 280 million people are infected worldwide,[2,3] with 200 million people presenting symptomatic giardiasis in the developing countries.[2,4] Children living in developing countries with poor hygiene and

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sanitation conditions[5] are more vulnerable to the clinical consequences of *G. lamblia* infection.[6] The harmful effect of giardiasis on growth and development in children has been observed in several studies and the potential effects of chronic malnutrition on cognition, intelligence and psychomotor development have also been described.[7,8] Several studies have shown that malnutrition in children can be an important consequence of *Giardia* infection.[9–12] However, there are few published studies in recent years exploring the association of the assemblage of *G. lamblia* with infant nutritional status.[12,13]

Currently *G. lamblia* is considered a species complex, comprising eight assemblages (A–H).[6] The majority of these assemblages are host specific, but only assemblages A and B are known to infect humans,[14,15] with assemblage B being described as more common.[3] A recent study reported for the first time assemblage E in humans in Australia.[16] However, the number of molecular epidemiological studies of giardiasis in humans is not enough to determine geographic or socio-economic differences in the distribution of assemblages A and B.[6]

The severity of the disease is determined by the interaction among parasite's virulence, host's nutritional and immunological status, nature of the intestinal microflora and the presence or absence of other pathogenic intestinal agents.[6] Although the different assemblages of *G. lamblia* may eventually produce different toxins or metabolic products that contribute to their pathogenicity[17] or differences in antigenic variation and host specificity,[18–20] studies on the possible association between the genetic groups of *G. lamblia* and their virulence (defined by the probability of causing diarrhoea and other clinical symptoms) continue to show inconsistent results.[6,21,22] Although several studies have shown a correlation between *G. lamblia* infections belonging to assemblage B with more severe symptoms,[23–26] others have correlated the most severe symptoms with assemblage A.[20,22]

An interesting observation raised by Almeida et al.[21] was that there seems to be a relationship between the molecular marker used in the study and the assemblage believed to be more aggressive. These investigators found that almost all genotyping studies based on the *ssu-rRNA* and *tpi* genes support the idea that assemblage A is associated with symptomatic disease, whereas studies based on the *bg* and *gdh* genes associated the symptomatology with assemblage B.[21]

The occurrence and spread of giardiasis in human populations is an emerging public health problem around the world. Molecular studies are essential to clarify the importance of local *Giardia* assemblages.[27]

In Angola, there is limited information available concerning infection with intestinal parasites and only in recent years have some studies been published.[28–31] Two of these studies report *G. lamblia* as being the most prevalent intestinal protozoan in children from the province of Bié and the city of Lubango (Huíla province).[28,31] We have no knowledge of previous studies on molecular characterization of *G. lamblia* carried out in Angola.

The aim of the present study was to perform a genetic characterization of *G. lamblia*, using polymerase chain reaction (PCR) and DNA sequencing, in children with diarrhoea attending the Bengo General Hospital.

Materials and methods

Population and study design

This study is part of a cross-sectional study conducted between September 2012 and December 2013 at the Bengo General Hospital, located in Caxito, the capital city of Bengo Province, 60 km northeast of Luanda, Angola.[32] The study aimed to investigate the aetiology of diarrhoea in children younger than 5 y attending the paediatric emergency service or the hospital outpatient paediatric unit with diarrhoea (three or more loose or liquid stools per day).[32] Children receiving antibiotics or antiparasitic drugs were excluded in order to avoid false negatives. A survey including sociodemographic characterization, information on breastfeeding practices, water source and sanitation conditions was applied by the clinical staff. Symptomatology in the previous 10 days (diarrhoea, vomiting, fever and bloody diarrhoea) was reported by caregivers. Anthropometric measurements (weight and length/height) were assessed to calculate anthropometric indices expressed as a Z-score for each child.[33]

The study protocol was approved by the Ethics and Committee of the Angolan Ministry of Health and the Ethics Committee of the Instituto de Higiene e Medicina Tropical, Portugal. Written informed consent and voluntary consent was obtained from parents or legal guardians prior to the inclusion of each child.

Stool sample collection and initial diagnosis of *G. lamblia*

A single stool sample per child was collected to a sterile container provided by the clinic staff. Once delivered to the laboratory, stool samples were immediately processed for microscopic identification of cysts and/or trophozoites of *G. lamblia* (direct examination with saline and iodine and a concentration method [ParasiTrap system, Biosepar, Mühldorf, Germany]) followed by the detection of *Giardia* antigen through immunochromatographic rapid tests (RIDAQUICK *Cryptosporidium/Giardia* Combi, R-Biopharm, Darmstadt, Germany). Microscopy and immunochromatographic rapid tests were performed in 344 and 338 children, respectively. Positive samples by at least one of the methods applied were preserved at −20°C for DNA extraction (Figure 1).

DNA extraction

DNA was extracted from *G. lamblia*–positive stool samples preserved at −20°C using a QIAamp DNA Stool Mini Kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions, except for the final step where 100 μl of the elution solution were used.

DNA amplification

The DNA of the extracted samples was amplified through PCR, using primers targeting the small subunit ribosomal RNA (ssu-rRNA)[34,35] (Figure 1). The primers used were RH11: 5′-CAT CCG GTC GAT CCT GCC-5′ and RH4: 5′-AGT CGA ACC CTG ATT CTC CGC CCA GG-3′ for the first reaction and GiarF: 5′-GAC GCT CTC CCC AAG GAC-3′ and GiarR: 5′-CTG CGT CAC GCT GCT CG-3′ for the

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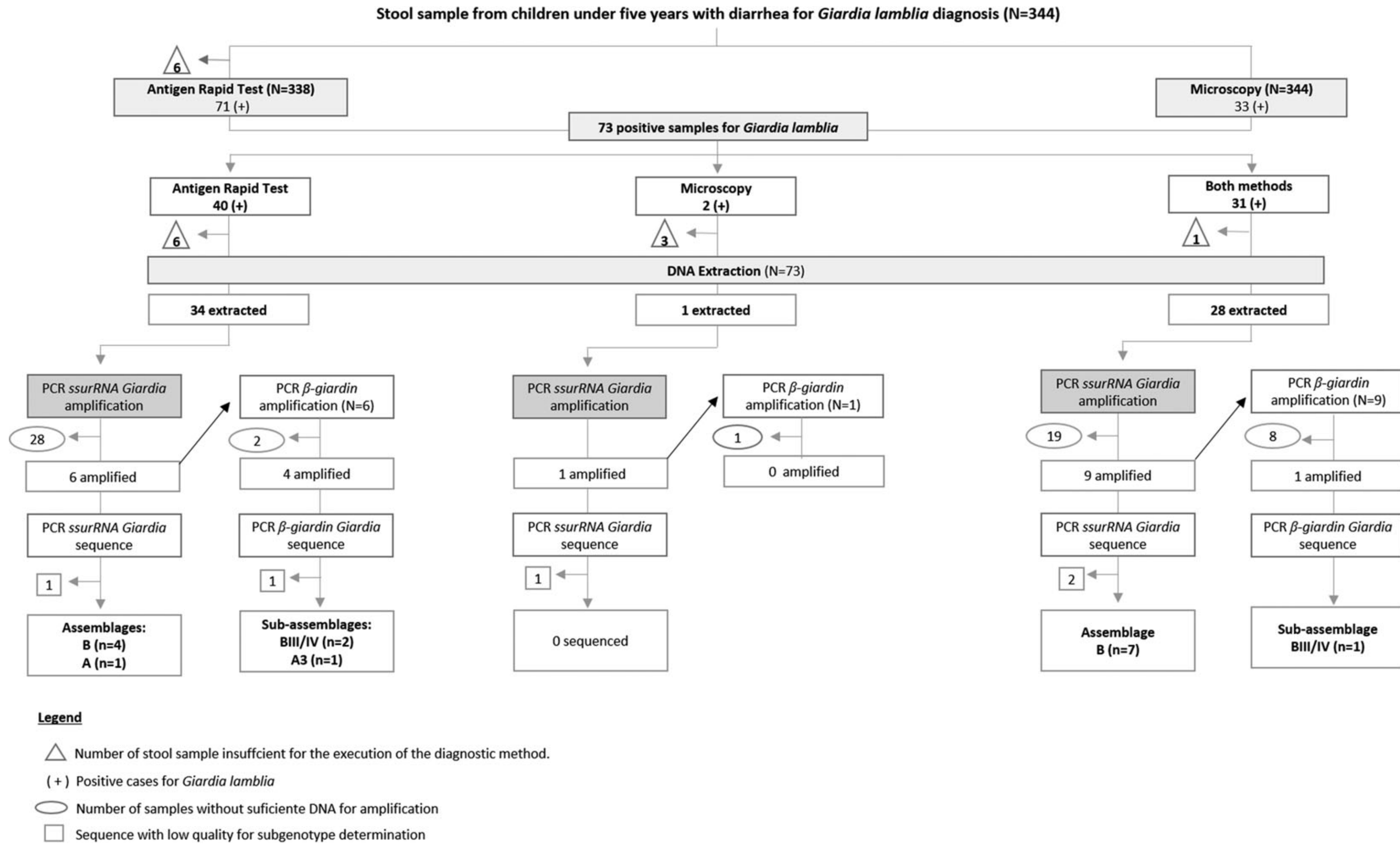

Figure 1. Molecular characterization of *Giardia lamblia* detected by microscopy and/or rapid antigen test in stool samples from children with diarrhoea attending the Bengo General Hospital, Angola.

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secondary PCR.[34,35] All samples successfully amplified for ssu-rRNA were posteriorly amplified for the β-giardin (bg) loci (Figure 1). The primers used were G7: 5′-AAG CCC GAC GAC CTC ACC CGC AGT GC-3′ and G759: 5′G AG GCC GCC CTG GAT CTT CGA GAC GAC-3′ for the first reaction and G8: 5′-GAA CGA ACG AGA TCG AGG TCC G-3′ and G9: 5′-CTC GAC GAG CTT CGT GTT-3′.[36,37]

Amplification reactions were performed using 2 μl of DNA template in a final volume of 25 μl, using illustra PuReTaq Ready-To-Go PCR beads (GE Healthcare, Little Chalfont, UK). Both positive (DNA isolated from the Portland-1 strain; ATCC 30888D, ATCC-LGC Promochem, Manassas, VA, USA) and negative controls (no template added) were included in each series of PCRs. PCR products were visualized on 2% agarose gels stained with ethidium bromide.

DNA sequence analysis

For sequence analysis, PCR products of amplified samples were purified using illustra GFX PCR DNA and the Gel Band Purification Kit (GE HealthCare) according to the manufacturer's instructions. DNA sequencing reactions were carried out in both directions for ssu-rRNA (175 bp) and β-giardin (511 bp) PCR-generated fragments.

Sequences obtained (75.0% [12/16] for ssu-rRNA and 80.0% [4/5] for β-giardin) were aligned with previously published sequences of *G. lamblia* isolates available in the GenBank database (http://blast.ncbi.nlm.nih.gov/Blast.cgi), using BLAST for assemblage determination and Clustal Omega (http://www.ebi.ac.uk/Tools/msa/clustalo/) for subassemblage determination.

Results

Microscopic identification and antigen detection of *G. lamblia* was performed in a total of 338 children, of whom 73 (21.6%) were positive: 40 (54.8%) were only detected through immunochromatographic tests, 2 (2.7%) only by microscopy and 31 (41.2%) through both methods (Figure 1). DNA extraction was performed in 63 positive samples, of which 16 were amplified for the *ssu-rRNA* gene fragment and 12 were successfully sequenced (Figure 1). All analysed samples belonged to assemblage B (11/12), except one that belonged to assemblage A (1/12) (GenBank accession numbers: MF479750, MF479751, MF479752, MF479753, MF479754, MF479755, MF479756, MF479757, MF479758, MF479759, MF479760, MF479761) (Table 1). These samples were also amplified for the *bg* gene fragment, for subassemblage determination, but only four sequences had enough quality for sequencing, three belonging to assemblage B and one to assemblage A (GenBank accession numbers: MF565378, MF565379, MF565380, MF565381) (Table 1). It was not possible to determine the subassemblages belonging to assemblage B due to the high polymorphism observed in the chromatogram. However, the isolate belonging to assemblage A was found to belong to the A3 subassemblage (Table 1).

Discussion

To our knowledge this is the first study on the molecular characterization of *G. lamblia* in Angola. Despite the small number of the samples studied, in the present study assemblage B was clearly predominant (93.8%), with only one sample assigned to assemblage A. The prevalences of assemblages A and B are differently distributed among and within countries, although there is no solid evidence that explains this geographic variation around the world.[22] Studies conducted in Mexico, Brazil and Colombia have identified high frequencies of assemblage A, while studies in Nicaragua and Argentina have shown that assemblage B predominates in these countries.[38–40] In South and Southeast Asia, including India, assemblage A seems to predominate, just as in Europe.[41–43] Reports from Africa have also shown high prevalences of assemblage B in countries such as Egypt (80%), Rwanda (85.9%) and Morocco (81.8%).[12,44,45]

It has been suggested that assemblage A is associated with zoonotic transmission, since it is commonly found in a wide range of animals, including in a large number of cattle.[6,37,46] Assemblage B was associated with a higher rate of cyst shedding compared with assemblage A in a study conducted in Brazilian children, which may contribute to a greater spread and consequently a higher incidence of assemblage B.[47] In this study, assemblage B was more common in the studied children and this could indicate that the major transmission route was more likely to occur between humans. Indeed, in the studied population about 20% of the households did not have a latrine, which may contribute to *G. lamblia* faecal–oral transmission between humans.[32] Furthermore, there are no clear geographic differences or socio-economic factors responsible for the distribution of both assemblages.[6,37,48]

Assemblage B is considered genetically diverse and the isolates present a high substitution rate that makes the real subassemblage patterns difficult to determine.[49] The difficulty in defining subassemblages in assemblage B is described in several studies.[3,39,50,51]

This study has some limitations. This is a hospital-based study that included children less than 5 years of age with diarrhoea, thus the results on the prevalence of *G. lamblia* and respective assemblages cannot be generalized to the entire population. To overcome this limitation, since infection by this protozoan is mainly asymptomatic,[52,53] a case–control design would be interesting in order to compare the prevalence of *G. lamblia* and its assemblages with other enteric pathogens in participants with diarrheal and non-diarrheal stools. Moreover, in diarrheal samples the non-resistant forms of this pathogen (trophozoites) are predominant, which can impair the microscopic diagnosis. The use of immunochromatographic tests was able to prevent this problem. It was not possible to assemblage some stool samples, probably due to freezing and thawing of the samples since their initial collection, which may contribute to DNA degradation. The limited number of samples studied, with only one assemblage A being detected, precludes investigating the differences between assemblage A and B in relation to symptomatology, malnutrition and sociodemographic conditions.

Despite the limitations mentioned above, we believe that an important contribution was made through the identification of *G. lamblia* assemblages circulating in children attending the Bengo General Hospital, Angola.

Future investigations are needed to clarify the current genetic diversity and better understand the importance of local *G. lamblia* assemblages. Studies with a larger number of samples, also including information about the seasonality of *G.*

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Table 1. *Giardia lamblia* assemblages identified in children with diarrhoea attending the Bengo General Hospital, Angola (N=12) and respective descriptions of sample collection (month/year), gender (male/female), age in months, reported symptomatology (vomiting, fever and lethargy), type of enteric infection identified according to the enteric pathogens detected (simple: the child was infected only by *G. lamblia*; multiple: the child was infected by *G. lamblia* and other enteric pathogens), laboratory results of microscopy and rapid test detection methods performed.

Identification		Sociodemographic characterization		Symptomatology			Enteric infection	Diagnosis of *Giardia lamblia*					
Number	Collection date (month, year)	Gender	Age (months)	Vomiting	Fever	Lethargy	Type	Microscopy	Rapid test	Molecular assemblages			
										ssu	GenBank accession nos.	bg	GenBank accession nos.
1	February 2013	Male	11.4	Yes	Yes	No	Multiple	Negative	Positive	B	MF479750	n.a.	
2	April 2013	Female	10.5	Yes	Yes	Yes	Simple	Negative	Positive	A	MF479751	A3	MF565378
3	May 2013	Female	8.6	No	Yes	Yes	Multiple	Negative	Positive	B	MF479752	B*	MF565379
4	May 2013	Male	11.3	Yes	Yes	No	Multiple	Negative	Positive	B	MF479753	B*	MF565380
5	May 2013	Female	13.1	No	Yes	No	Multiple	Negative	Positive	B	MF479754	n.a.	
6	June 2013	Female	12.8	No	Yes	Yes	Multiple	Positive	Positive	B	MF479755	n.a.	
7	August 2013	Male	9.3	No	Yes	No	Multiple	Positive	Positive	B	MF479756	n.a.	
8	August 2013	Female	27.4	No	No	No	Simple	Positive	Positive	B	MF479757	B*	MF565381
9	August 2013	Female	14.2	No	No	No	Multiple	Positive	Positive	B	MF479758	n.a.	
10	August 2013	Male	27.3	Yes	Yes	No	Multiple	Positive	Positive	B	MF479759	n.a.	
11	October 2013	Female	30.9	No	Yes	Yes	Simple	Positive	Positive	B	MF479760	n.a.	
12	December 2013	Male	17.3	No	Yes	No	Multiple	Positive	Positive	B	MF479761	n.a.	

*Subassemblage determination not possible.
n.a.: not applicable.

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lamblia prevalence and the zoonotic potential of this protozoan, would aid our understanding of the epidemiology of giardiasis in Angola, contributing to better definition of key priorities in its control.

Authors' contributions: CG participated throughout the entire sampling, carried out parasitological analysis (microscopy and immunochromatographic rapid tests), contributed to both data analysis and interpretation, drafted the initial manuscript, critically reviewed the manuscript and approved the final submitted manuscript. FSF carried out molecular analysis, contributed to both data analysis and interpretation, drafted the initial manuscript, critically reviewed the manuscript and approved the final submitted manuscript. ACM coordinated and supervised the clinical staff, critically reviewed the manuscript and approved the final manuscript as submitted. MCM carried out parasitological analysis (microscopy and immunochromatographic rapid tests), drafted the initial manuscript, critically reviewed the manuscript and approved the final manuscript as submitted. SVN conceptualized and designed the study, coordinated the planning phase of the study, contributed to the analysis and interpretation of data, critically reviewed the manuscript and approved the final manuscript as submitted. ASR carried out parasitological analysis (microscopy and immunochromatographic rapid tests) and DNA extraction, contributed to both data analysis and interpretation, drafted the initial manuscript, critically reviewed the manuscript and approved the final submitted manuscript. DPC carried out molecular analysis, critically reviewed the manuscript and approved the final submitted manuscript. MB conceptualized and designed the study, coordinated and supervised data collection, contributed to the analysis and interpretation of data, drafted the initial manuscript and critically reviewed and approved the final submitted manuscript.

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Competing interests: None declared.

Ethical approval: The study protocol was approved by the Ethics Committee of the Angolan Ministry of Health and the Ethical Committee of the Instituto de Higiene e Medicina Tropical, Portugal.

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