Article

High Diversity of *Giardia duodenalis* Assemblages and Sub-Assemblages in Asymptomatic School Children in Ibadan, Nigeria

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Abstract: *Giardia duodenalis* is a significant contributor to the burden of diarrheal disease in sub-Saharan Africa. This study assesses the occurrence and molecular diversity of *G. duodenalis* and other intestinal parasites in apparently healthy children (*n* = 311) in Ibadan, Nigeria. Microscopy was used as a screening method and PCR and Sanger sequencing as confirmatory and genotyping methods, respectively. Haplotype analyses were performed to examine associations between genetic variants and epidemiological variables. At microscopy examination, *G. duodenalis* was the most prevalent parasite found (29.3%, 91/311; 95% CI: 24.3–34.7), followed by *Entamoeba* spp. (18.7%, 58/311; 14.5–23.4), *Ascaris lumbricoides* (1.3%, 4/311; 0.4–3.3), and *Taenia* sp. (0.3%, 1/311; 0.01–1.8). qPCR confirmed the presence of *G. duodenalis* in 76.9% (70/91) of the microscopy-positive samples. Of them, 65.9% (60/91) were successfully genotyped. Assemblage B (68.3%, 41/60) was more prevalent than assemblage A (28.3%, 17/60). Mixed A + B infections were identified in two samples (3.3%, 2/60). These facts, together with the absence of animal-adapted assemblages, suggest that human transmission of giardiasis was primarily anthroponotic. Efforts to control *G. duodenalis* (and other fecal-orally transmitted pathogens) should focus on providing safe drinking water and improving sanitation and personal hygiene practices.

Keywords: intestinal parasites; giardiasis; transmission; diarrhea; epidemiology; Africa

1. Introduction

Giardia duodenalis (synonyms *G. intestinalis*, *G. lamblia*) is the etiological agent of giardiasis, a major contributor to the burden of diarrheal disease causing near 190 million symptomatic infections per year globally, adding up to ~171,100 daily-adjusted life years lost [1]. Clinical infection ranges from asymptomatic carrier state to severe abdominal pain, diarrhea, vomiting, flatulence, malabsorption, anorexia, and weight loss [2]. Long-term sequelae including postinfectious irritable bowel disease and chronic fatigue have also been reported [3]. Despite its medical importance, no human vaccine is available against

giardiasis [4], and clinical resistance against 5-nitroimidazoles (the most used drugs for first-line treatment) occurs in up to 50% of cases [5]. Transmission is via the fecal–oral route, either by ingestion of water or food contaminated with cysts of the parasite, or through direct contact with infected humans, animals, or fomites [6].

Giardiasis disproportionally affects young children, many of whom are undernourished, living in limited-resource settings without or with inadequate access to clean water or good sanitation [7]. In endemic settings, giardiasis has been consistently associated with childhood growth faltering, failure to thrive, and impaired cognitive development [8,9]. Findings from prospective longitudinal cohort studies conducted in sub-Saharan African countries indicate that *Giardia* was not an independent risk factor for diarrhea in children [10–12]. Integrating asymptomatic carriage of *Giardia* with its role as a diarrhea-causing agent in these settings is, therefore, challenging and controversial [13]. Several factors have been proposed to contribute to the outcome of the infection, including nutritional [14], microbial [15], metabolic [16], and pathogen-strain [17] variables.

Giardia duodenalis is now known as a multispecies complex comprising eight (A–H) genotypically distinct assemblages [18]. Most human *Giardia* infections are due to assemblages A or B, although human cases by animal-adapted assemblages C-F are less frequently reported [18,19]. Several molecular-based surveys have attempted to correlate the presence of diarrhea with the genotype of *G. duodenalis*. Large prospective case-control studies conducted in Bangladesh and Mozambique generated contradictory results [20,21], highlighting the need for additional studies aiming at investigating the molecular diversity of the parasite in symptomatic and asymptomatic individuals of all age groups.

In Nigeria, the annual number of deaths from diarrheal diseases per 100,000 people decreased from 275.3 in 1990 to 92.5 in 2019 [22]. Although *Giardia* infection is not usually associated with mortality, giardiasis seems widely present in the country, affecting both asymptomatic and clinical individuals and livestock (Table 1) [23–50]. In addition, very little is currently known on the genetic variability of *G. duodenalis* circulating in Nigerian human populations. This study aims at investigating the occurrence and molecular diversity of *G. duodenalis* in school children in Ibadan, a city in the Southwestern part of Nigeria.

Table 1. Occurrence and genetic diversity of *Giardia duodenalis* in human and animal hosts in Nigeria, 1994–2022.

Host Population	City/Region	Detection Method	Samples Analyzed (*n*)	Infection Rate (%)	Assemblage (*n*)	Reference
Human						
Asymptomatic children	Anambra State	CM	1536	0.1	ND	[23]
Asymptomatic children	Benin City	CM	184	59.8	ND	[24]
Asymptomatic children	Imo State	CM	300	2.7	ND	[25]
Asymptomatic children	Lagos City	CM	384	12.2	ND	[26]
Asymptomatic children	Niger Delta	CM	1062	9.2	ND	[27]
Asymptomatic children	Oyo State	PCR, DFA	199	37.2	ND	[28]
Diarrheic children	Ebonyi State	PCR, DFA	199	0.0	–	[29]
Diarrheic children	Ilesa City	CM	300	7.1	ND	[30]
Diarrheic children	Lagos City	CM	215	0.5	ND	[31]
Diarrheic children	Zaria City	CM	142	26.1	ND	[32]
HIV+ patients	Benin City	CM	2000	0.6	ND	[33]
HIV+ patients	Lagos City	CM	65	9.2	ND	[34]
HIV+ patients	Zaria City	CM	10	25.0	ND	[35]
Hospital outpatients	Enugu State	CM	500	47.8	ND	[36]

Table 1. *Cont.*

Host Population	City/Region	Detection Method	Samples Analyzed (*n*)	Infection Rate (%)	Assemblage (*n*)	Reference
Hospital outpatients	Ibadan City	CM	360,000	1.6	ND	[37]
Hospital outpatients	Kaduna State	PCR-RFLP	157	3.2	A (5)	[38]
Hospital outpatients	Lagos City	CM	1015	0.6	ND	[34]
Hospital outpatients	Ogun State	CM	479	4.2	ND	[39]
Food handlers	Ogun State	CM	100	13.0	ND	[40]
Game scouts	Kainji Lake National Park	CM	20	NS	ND	[41]
Rural dwellers	Plateau State	CM	300	2.3	ND	[42]
Urban dwellers	Enugu State	CM	403	35.2	ND	[43]
Urban dwellers	Jos City	CM	111	4.3–7.2	ND	[44]
Urban dwellers	Lagos City	CM	2099	7.9	ND	[45]
Livestock						
Goat	Ogun State	ELISA, PCR	302	45.7	A (5), B (13), E (40)	[46]
Pig	Ogun State	ELISA, PCR	209	25.4	B (4), E (37), B + E (2)	[47]
Rabbit	Ogun State	PCR	83	72.3	B (19)	[48]
Insects						
Cockroach	Lagos State	CM	749	18.7	ND	[49]
Fly	Ogun State	CM	7190	3.3	ND	[50]

CM: conventional microscopy; DFA: direct fluorescence assay; ELISA: enzyme-linked immunosorbent assay; ND: not determined; NS: not specified; PCR: polymerase chain reaction; PCR-RFLP: polymerase chain reaction-restriction fragment length polymorphism.

2. Materials and Methods

2.1. Study Design and Participants

In this two-point cross-sectional study, individual stool samples were collected from asymptomatic school children aged 5–17 from four districts in Ibadan metropolis: Boluwaji, Moniya, Orogun, and Yemetu. Ibadan is the capital and most populous city of Oyo State in Southwestern Nigeria. It is the third largest city by population in the country after Lagos and Kano, with a total population of 3,649,000 as of 2021. Ibadan has a tropical savanna climate, with an average annual temperature of 25.9 °C and an annual precipitation of about 1467 mm, mostly concentrated in the period August–October. The warmest month is February (average temperature: 28.9 °C) and the coolest month is August (average temperature: 24.4 °C). This study was carried out between August–December 2017 and May–July 2018.

2.2. Stool Sample Collection

Informative meetings were held at scheduled times with school pupils on the causes, symptoms, and mode of transmission of giardiasis. Those willing to participate in this study were provided with transparent, sterile stool recipients containing no preservatives and instructed on how to collect a stool sample in a simple and safe way. The stool recipients were labelled with individual numeric codes allocated to each participating child for easy identification of samples. Collected fresh stool samples were placed on ice and transported within one hour to the Cellular Parasitology Laboratory, Department of Zoology, University of Ibadan, for further analysis.

2.3. Epidemiological Questionnaires

Individual standardized questionnaires in Yoruba and English languages were completed by a member of our research team in face-to-face interviews with each participating child at sample collection. Questions included demographics, hand and vegetable washing, contact with domestic animals and livestock, source of drinking water, use of treated water, latrine use, and garbage disposal. The information obtained was entered into an Excel spreadsheet (Microsoft Corporation, Redmond, WA, USA) and used for risk association analyses.

2.4. Microscopy

Freshly collected stool samples were microscopically examined within 24 h of collection. The formol-ether concentration technique was used [51]. Briefly, 1–2 g of the fecal sample was homogenized in 7 mL of 10% formalin and sieved through surgical gauze as a mechanical filter to remove fecal debris. The sieved suspension was transferred into a clean 15 mL centrifuge tube and 3 mL of ethyl acetate were added. After vigorous shaking, the mixture was centrifuged for 3 min at 3000 rpm. The supernatant was carefully discarded, and fecal smears made from the sediment. Smears were then stained with 1% Lugol's iodine and examined at 200× for screening and 400× for confirmation of *Giardia* cysts. Each slide was examined by two independent microscopists. Smears yielding discrepant results were resolved by a third microscopist.

2.5. DNA Extraction and Purification

Genomic DNA was isolated from about 200 mg of fecal samples yielding positive/discrepant results for *G. duodenalis* at microscopy examination by using the QIAamp DNA Stool Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Extracted and purified DNA samples were eluted in 200 μL of PCR-grade water. Fecal DNA samples were shipped to the Parasitology Reference and Research Laboratory of the National Centre for Microbiology (Majadahonda, Spain) in August 2022 for downstream molecular testing.

2.6. Molecular Confirmation and Characterization of Giardia duodenalis

Detection of *G. duodenalis* DNA was achieved using a real-time PCR (qPCR) method targeting a 62-bp region of the gene, codifying the small subunit ribosomal RNA (*ssu* rRNA) of the parasite [52].

For assessing the molecular diversity of the parasite, we adopted a sequence-based multilocus genotyping (MLST) scheme targeting the genes encoding for the *ssu* rRNA, the glutamate dehydrogenase (*gdh*), β-giardin (*bg*), and triose phosphate isomerase (*tpi*) proteins of the parasite. For assessing the molecular diversity of *G. duodenalis* at the assemblage level, a nested PCR was used to amplify a 300-bp fragment of the *ssu* rRNA gene [53,54]. The molecular diversity of the parasite at the sub-assemblage level was investigated only in *Giardia* isolates that tested positive by qPCR and yielded cycle threshold (C_T) values ≤ 32. A semi-nested PCR was used to amplify a 432-bp fragment of the *gdh* gene [55], and nested PCRs were used to amplify 511 and 530 bp fragments of the *bg* and *tpi* genes, respectively [56,57].

2.7. General PCR and Electrophoretic Procedures

Detailed information on the PCR cycling conditions and oligonucleotides used for the molecular identification and/or characterization of the protozoan parasites investigated in the present study is presented in Tables S1 and S2, respectively. All the direct, semi-nested, and nested PCR protocols described above were conducted on a 2720 Thermal Cycler (Applied Biosystems Foster City, CA, USA). Reaction mixes always included 2.5 units of MyTAQ^{TM} DNA polymerase (Bioline GmbH, Luckenwalde, Germany), and 5–10 μL MyTAQ^{TM} Reaction Buffer containing 5 mM dNTPs and 15 mM MgCl$_2$. Laboratory-confirmed positive and negative DNA samples of human origin were routinely used as

controls and included in each round of PCR. PCR amplicons were visualized on 1.5% D5 agarose gels (Conda, Madrid, Spain) stained with Pronasafe (Conda) nucleic acid staining solutions.

2.8. Sanger Sequencing Analyses

Positive PCR products of the expected size were directly sequenced in both directions using appropriate internal primer sets (Table S1). DNA sequencing was conducted by capillary electrophoresis using the BigDye® Terminator chemistry (Applied Biosystems) on an ABI PRISM 3130 automated DNA sequencer. Generated DNA consensus sequences were aligned to appropriate reference sequences using MEGA X [58] for species confirmation and genotype identification. The *G. duodenalis* sequences obtained in this study have been deposited in GenBank under accession numbers OP946920–OP946928 (*ssu* rRNA locus), OP947099–OP947117 (*gdh* locus), OP947118–OP947130 (*bg* locus), and OP947131–OP947138 (*tpi* locus).

2.9. Haplotype Variability

Significant variables for haplotype variability analysis were selected applying Categorical Principal Components Analysis (CATPCA) and conducting the TwoStep Cluster Analysis procedure through IBM SPSS Statistics for Windows, Version 28.0 (IBM Corp., Armonk, NY, USA) [59], with the purpose of revealing natural groupings within the dataset of continuous and categorical variables and their most relevant input predictors. Cronbach's Alpha overall value was considered to measure the internal dataset's consistency.

For the TwoStep Cluster Analysis, the number of clusters was not provided in advance, and Bayesian Information Criterion (BIC) was used to determine the best cluster solution. The input predictor with the highest values of importance was then considered for the haplotype variability analysis and network.

3. Results

In this study, 311 stool samples and corresponding questionnaires were collected from asymptomatic school children. Of these, 145 (46.6%) were male and 165 (53.1%) were female. Gender was unknown for a single (0.3%) child. The age of the participating school children ranged from five to 17 years with a median age of 10 years. This rate did not differ between *Giardia*-infected and uninfected school children.

3.1. Microscopy

Overall, 31.5% (98/311, 95% Confidence Interval (CI): 26.4–36.7) of fecal samples examined by microscopy tested positive for at least one intestinal parasite (Table 2). *Giardia duodenalis* was the most prevalent parasite found (29.3%, 91/311; 95% CI: 24.3–34.7), followed by *Entamoeba* spp. (18.7%, 58/311; 14.5–23.4), *Ascaris lumbricoides* (1.3%, 4/311; 0.4–3.3), and *Taenia* sp. (0.3%, 1/311; 0.01–1.8). *Giardia duodenalis* was more frequently found in coinfection with *Entamoeba* spp. (15.8%, 49/311) than in monoinfection (11.9%, 37/311). Out of the 91 *Giardia*-positive samples, 18.7% (17/91) corresponded to smears yielding discrepant results at microscopy examination (Table S3).

Table 2. Detection of intestinal parasites by microscopy examination.

Parasite Species	Positive (*n*)	Frequency (%) [1]
None	213	68.5
In monoinfection		
G. duodenalis	37	11.9
Entamoeba complex	7	2.3
In coinfection		
G. duodenalis + *A. lumbricoides*	2	0.6
G. duodenalis + *Entamoeba* complex	49	15.8
G. duodenalis + *Taenia* sp.	1	0.3
G. duodenalis + *A. lumbricoides* + *Entamoeba* complex	2	0.6
Total	311	100

[1] Over the total of fecal samples (*n* = 311) examined. *Entamoeba* complex: *Entamoeba histolytica/dispar/moshkovskii*.

3.2. Confirmation of G. duodenalis by qPCR

All 91 fecal DNA samples yielding positive/discrepant results for *G. duodenalis* at microscopy examination were subjected to qPCR testing to confirm the presence of the parasite. qPCR positive results were obtained in 76.9% (70/91) of the samples, including 58.8% (10/17) of those generating discrepant results at microscopy. Yielded C_T values ranged from 20.0 to 39.6 (median: 30.0; standard deviation: 5.1). Most (65.7%, 46/70) of the qPCR-positive samples yielded C_T values $\leq$ 32.

3.3. Genotyping and Subgenotyping of G. duodenalis Isolates

All 91 fecal DNA samples yielding positive/discrepant results for *G. duodenalis* at microscopy examination were subjected to nested *ssu*-PCR to ascertain the assemblage of the parasite involved. Of them, 63.7% (58/91) were successfully genotyped at this locus. These included two isolates that tested negative by qPCR (Table S3). Sequence analyses revealed the presence of assemblage A (29.3%, 17/58) and assemblage B (70.7, 41/58). Table S4 summarizes the molecular data generated at the *ssu* rRNA locus. Out of the 17 assemblage A sequences, 16 showed 100% identity with the reference sequence used (GenBank accession number AF199446), with the remaining one differing from it by a single nucleotide polymorphism (SNP). Out of the 41 assemblage B sequences, 85.4% (35/41) were identical to reference sequence AF199447, with the remaining six sequences differing by 1–2 SNPs from it. All SNPs detected corresponded to ambiguous (double peak) positions (Table S4).

The molecular diversity of *G. duodenalis* at the sub-assemblage level was investigated at three (*gdh*, *bg*, and *tpi*) genetic markers. All 46 *Giardia*-positive DNA samples yielding qPCR C_T values $\leq$ 32 were re-assessed under this scheme. Successful PCR amplifications and sequencing data were generated for 56.5% (26/46), 50.0% (23/46), and 26.1% (12/46) of the samples investigated at the *gdh*, *bg*, and *tpi* loci, respectively.

Overall, 65.9% (60/91) of the *Giardia*-positive samples were successfully genotyped at one locus at least (Table 3). MLST data at the four assessed loci were available for 11.7% (7/60) of samples. Subtyping data at a single locus, two loci, or three loci were available for 48.3% (29/60), 18.3% (11/60), and 21.7% (13/60) of samples, respectively. Assemblage B (68.3%, 41/60) was more prevalent than assemblage A (28.3%, 17/60). Mixed A + B infections were detected in two samples (3.3%, 2/60). No host-adapted assemblages of canine (C, D), feline (F), or livestock (E) origin were detected.

Table 3. Multilocus sequence typing results of the 60 *G. duodenalis*-positive samples of pediatric origin successfully genotyped at any of the four loci investigated in the present survey. The age and gender of the infected children are also shown.

Sample ID	Age (Years)	Gender	C_T Value in qPCR	*ssu* rRNA	*gdh*	*bg*	*tpi*	Assigned Genotype
2	8	M	25.5	–	–	B	–	B
4	9	F	29.4	B	–	–	–	B
5	12	F	27.8	B	BIII	–	–	BIII
6	11	F	–	B	–	–	–	B
9	10	M	23.6	B	BIII	–	–	BIII
11	9	F	32.6	B	–	–	–	B
13	12	M	27.9	B	BIII	–	–	BIII
15	7	M	30.3	–	BIII	–	–	BIII
17	11	F	25.8	B	–	–	–	B
25	6	M	34.8	B	–	–	–	B
27	5	F	31.5	B	–	–	–	B
28	7	M	31.5	B	–	–	–	B
29	8	F	32.7	B	–	–	–	B
30	8	M	38.6	B	–	–	–	B
31	7	M	21.8	B	BIII/BIV	B	–	BIII/BIV
32	10	M	–	A	–	–	–	A
33	9	M	21.2	A	AII	AII	–	AII
34	12	F	30.6	B	BIII	–	–	BIII
36	10	M	23.6	B	–	B	–	B
37	11	F	25.7	A	–	–	–	A
39	10	F	22.2	A	AII	AII	–	AII
41	10	M	20.0	B	–	–	–	B
44	7	M	24.9	B	–	AII	–	AII + B
46	5	F	31.2	B	BIII	–	–	BIII
47	7	F	24.7	B	BIII	B	–	BIII
49	15	M	29.8	B	–	–	–	B
50	8	M	24.7	A	AII	AII	–	AII
51	5	M	27.8	B	BIII	–	–	BIII
52	9	F	21.2	A	AII	AII	AII	AII
53	10	F	23.7	A	AII	AIII	–	AII/AIII
54	10	F	23.1	A	AII	AIII	–	AII/AIII
55	8	F	23.1	A	AII	AIII	–	AII/AIII
56	14	F	35.8	A	–	–	–	A
58	11	F	36.3	A	–	–	–	A
59	12	F	24.4	B	BIII/BIV	B	BIII	BIII/BIV
61	10	F	24.1	B	BIII/BIV	B	–	BIII/BIV
63	12	F	31.4	B	BIV	–	–	BIV
68	13	M	26.8	B	BIII	B	BIII	BIII
70	11	M	27.3	A	–	AII	AII	AII
77	11	F	34.3	A	–	–	–	A
78	11	M	24.9	A	AII	AII	AII	AII
79	12	M	33.6	B	–	–	–	B
80	14	F	34.6	B	–	–	–	B
81	5	M	33.3	A	–	–	–	A
82	10	M	22.3	B	–	–	BIII	BIII
83	13	F	31.0	B	–	–	–	B
84	9	M	26.7	B	BIII/BIV	B	BIII	BIII/BIV
85	14	F	23.2	B	–	B	BIII	BIII
88	6	F	22.0	B	BIV	–	BIII/BIV	BIII/BIV
89	10	M	23.0	B	BIII/BIV	B	BIII	BIII/BIV
91	8	F	22.5	A	AII	AII	AII	AII
92	7	F	32.6	B	–	B	–	B
94	7	F	36.3	B	–	–	–	B
95	9	F	28.3	B	–	–	–	B
96	11	F	29.9	B	–	–	–	B
97	10	F	32.5	B	BIII	–	AII	AII + BIII
98	10	M	30.6	B	–	–	–	B
99	3	M	30.2	B	–	–	–	B
100	10	M	29.1	B	–	–	–	B
102	9	F	24.7	A	–	AIII	–	AIII

Table S5 shows the frequency and molecular diversity of *G. duodenalis* at the *gdh* locus. Out of the 26 *gdh* sequences, nine (34.6%) were assigned to the sub-assemblage AII. Of them,

eight showed 100% identity with reference sequence L40510. Assemblages BIII and BIV were identified in 10 (38.5%) and two (7.7%) isolates, respectively. All 10 BIII sequences were different among them, differing by 2–8 SNPs with reference sequence AF069059. The same degree of high genetic variability was observed in the two BIV sequences, which differed by 1–4 SNPs with reference sequence L40508. Discordant BIII/BIV results were identified in an additional five sequences showing 8–13 SNPs when compared with reference sequence L40508.

Table S6 shows the frequency and molecular diversity of *G. duodenalis* at the *bg* locus. Out of the 23 *bg* sequences, eight were identified as sub-assemblage AII. Seven of them were identical to reference sequence AY072723. Four additional sequences were assigned to sub-assemblage AIII, all of them showing 100% identity with reference sequence AY072724. A total of 11 sequences were identified as assemblage B (the *bg* locus is not suitable for sub-assemblage discrimination), of which two were identical to reference sequence AY072727. The remaining nine sequences differed from it by 1–13 SNPs.

Table S7 shows the frequency and molecular diversity of *G. duodenalis* at the *tpi* locus. Out of the 12 *tpi* sequences, five were identified as sub-assemblage AII, all of them identical to reference sequence U57897. Six sequences were unambiguously characterized as sub-assemblage BIII, differing by 1–4 SNPs with reference sequence AF069561. An additional sequence yielded a discordant BIII/BIV result, differing by five SNPs with reference sequence AF069560. As in the case of *gdh*, most of these SNPs corresponded to double peaks.

3.4. Haplotype Variability

CATPCA Cronbach's Alpha overall value (alpha = 0.99) showed evidence of good dataset reliability. The TwoStep Cluster Analysis revealed the existence of two clusters with a silhouette measure of cohesion and separation of 0.3 (fair). The first one represented 43.7% of individuals and the second one included 56.3% of children, with a relationship between the two of 1.29. The variable *Sampled area* resulted to be the input predictor with the highest value (Table S8); for this reason, it was selected as the main variable for the haplotype variability analysis.

Twenty-six different haplotypes were identified at the *gdh* locus, with the highest haplotype diversity (Hd) value of 0.9917 detected in the *tpi* locus. Haplotypes variability results are displayed in detail in Table 4 and visualized as network representations in Figure 1.

Table 4. Haplotypes distribution for *gdh*, *bg*, and *tpi* sequences.

Marker	Sequences (n)	Sites (n)	Monomorphic Sites (n)	Segregating Sites (n)	Segregating Sites with >2 Variants (n)	Positions with Gaps (n)	Positions with Missing Data (n)	Variable Sites (n)	Haplotypes (n)	Haplotype Diversity
gdh	38	433	238	63	15	132	0	180	26	0.9815
bg	26	637	388	53	10	196	0	240	19	0.9785
tpi	16	531	299	108	27	124	0	208	15	0.9917

The haplotypes analysis shows assemblage B as having the highest variability, with few mutation steps between the several haplotypes detected, in contrast with assemblage A's low diversity levels.

For all three loci considered, the haplotypes presenting the highest frequency of detection were not specific for a given area; indeed, they were near equally distributed between at least two different sampling areas, excepting assemblage A at the *tpi* locus (Figure 1C). There is no evidence of a specific haplotype segregation pattern related to a single geographical area, to contiguous sampling areas or to a specific molecular marker.
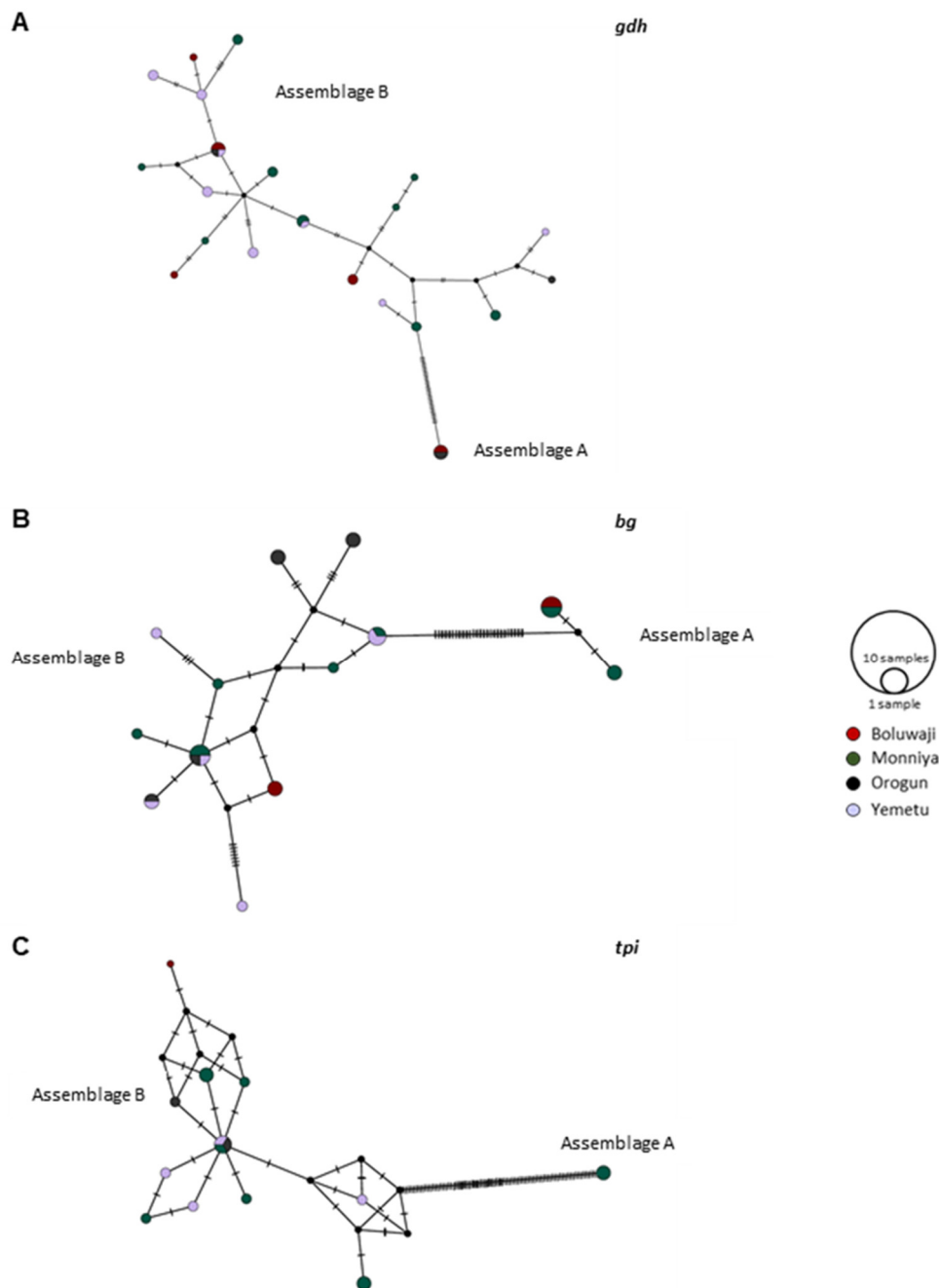
Figure 1. Median-joining haplotype networks built in PoPArt software using *gdh* (**A**), *bg* (**B**), and *tpi* (**C**) sequences generated in the present study. Haplotypes are represented by circles proportional to relative haplotypes abundance; different colors indicate different sampling areas. Hatch marks refer to the mutational steps between haplotypes. Black circles represent hypothetical missing haplotypes predicted.

4. Discussion

In this microscopy-based study, one out of three apparently healthy school children were infected with intestinal parasites in Ibadan, Nigeria. *Giardia duodenalis* was the most frequent pathogen detected (29.3%), being present (alone or in combination) in 91 out of the 97 cases of the subclinical parasitic infections observed. *Giardia duodenalis* was more frequently found in association with members of the *Entamoeba* complex (*n* = 49) than in monoinfection (*n* = 37). Infections with nematode (*A. lumbricoides*) and cestode (*Taenia* sp.) parasites were sporadic (less than five cases).

The microscopy-based *G. duodenalis* infection rate detected (29.3%) was in line with those (3–60%) previously reported in Nigerian asymptomatic children [24–27]. Other studies conducted in the country have documented infection rates of 0.5–26% in children with diarrhea [29–32], of 0.6–25% in HIV+ patients [33–35], and of 0.6–48% in hospital outpatients [36–39]. These data were obtained in surveys conducted using microscopy (mostly on single stool samples from participating individuals), a diagnostic method known to lack diagnostic sensitivity [60]. These facts strongly suggest that current microscopy-based figures likely underestimate the true burden of *G. duodenalis* infection in Nigeria, particularly in apparently healthy individuals. Indeed, asymptomatic carriers are known to inadvertently spread the infection either in endemic [61] or non-endemic [62] areas. Under this scenario, highly sensitive PCR-based methods coupled with Sanger sequencing can be useful to (i) improve the detection of asymptomatic cases, and by (ii) determining genetic variants and assessing sources of infection, transmission pathways, and zoonotic potential.

The molecular characterization of microscopy-positive *G. duodenalis* isolates at the assemblage and sub-assemblage levels is the most important contribution of this study. This is particularly relevant in Nigeria, a country where molecular-based surveys are scarce. Indeed, human genotyping data is only available for five hospital outpatients in Kaduna state, all of them infected by the assemblage A of the parasite [37]. Zoonotic assemblages A and B have also been reported in goats and pigs [46,47] and in farmed rabbits [48]. Our study is also the first investigation adopting an MLST scheme to assess the genetic diversity of *G. duodenalis* in isolates of human origin, as the only previous survey available used the *tpi* locus only [37]. Of note, MLST analyses are relevant for investigating the epidemiology of *G. duodenalis* because they assist in detecting mixed infections involving different assemblages/sub-assemblages of the parasite and in characterizing sources of infection and zoonotic potential [63].

In our hands, the *ssu*-PCR yielded a higher PCR amplification success rate (63%) than the PCR protocols targeting the *gdh*, *bg*, and *tpi* loci (26–57%), even considering that the latter were only attempted in fecal DNA samples with qPCR C_T values $\leq$ 32. This discrepancy can be explained by the superior diagnostic sensitivity of the multi-copy *ssu* rRNA gene compared to the single-copy *gdh*, *bg*, and *tpi* genes. Our typing analyses revealed that assemblage B was more prevalent than assemblage A (72% vs. 28%) in the pediatric population surveyed. This finding is in line with those previously reported in molecular surveys conducted in human populations in other sub-Saharan African countries including Angola (64% vs. 36%) [64], Ethiopia (82% vs. 18%) [65], Mozambique (90% vs. 8%) [21], Rwanda (86% vs. 13%) [9], Tanzania (79% vs. 21%) [66], and Zambia (73% vs. 27%) [67], among others. Remarkably, no canine-adapted (C, D), feline-adapted (F), or livestock-adapted (F) assemblages were found, suggesting that transmission of giardiasis in the surveyed pediatric population is primarily of anthroponotic nature. However, it should be noted that both assemblages A and B have zoonotic potential [68], so we cannot rule out the possibility that an unknown fraction of the *G. duodenalis* infections detected here were indeed of non-human origin. Additionally, previous molecular-based epidemiological studies conducted in Brazil and Syria have evidenced that sub-assemblage AII was more prevalently found in young children than in individuals of older ages [69,70]. In the present study, AII results were found in 20% (12/60) of the participating children. It would be interesting to see whether this proportion remains unchanged in further studies targeting individuals of all age groups.

In line with the results generated in previous molecular studies in endemic areas [21,71–74], a much higher molecular variability was observed in assemblage B sequences than in assemblage A sequences regardless the genetic marker used and the geographical origin of the samples. A significant proportion of B sequences (5/17 within the *gdh* marker and 1/12 within the *tpi* marker) corresponded to inconsistent BIII/BIV results. Most of the ambiguous nucleotidic positions in discordant BIII/BIV sequences involved double peaks, indicative of either true intra-assemblage BIII + BIV mixed infections, or the direct result of a genetic recombination mechanism [75,76]. Of note, prior analyses of sequenced *G.*

duodenalis assemblage A [63,77] and assemblage B [78] genomes have demonstrated low phylogenetic resolution at the *gdh*, *bg*, and *tpi* loci. These results evidenced the limitations of current MLST schemes and highlighted the necessity of identifying new markers for accurate and robust molecular typing.

The main strengths of this study include a relatively elevated sample size from an endemic area where little epidemiological information was previously available, and the adoption of a classical MLST scheme to assess the molecular diversity within *G. duodenalis*. However, some limitations might have biased the results obtained and the conclusions reached. First, *G. duodenalis* typing and sub-typing analyses were conducted on microscopy-positive samples only. It is likely that performing PCR and Sanger sequencing in all samples would have yielded different frequencies and diversities of *G. duodenalis* assemblages/sub-assemblages. Second, our MLST approach was based on single-copy markers. Adopting more sensitive MLST schemes can results on the assignation of different genetic variants. Third, interviewed children might have provided inaccurate answers to some of the questions included in the epidemiological questionnaire, potentially biasing the results of our statistical analyses. Fourth, epidemiological and molecular data generated in the present study is restricted to the age group and geographical area investigated, and might not be representative of the entire population in Nigeria. And fifth, initial detection of intestinal parasites was based on microscopy examination of single stool samples. Because of the limited diagnostic sensitivity of this method and the irregular shedding of the parasite's transmission stages (eggs, cysts, oocysts, spores) in stools from infected patients, reported prevalence rates might represent an underestimation of the true ones.

5. Conclusions

Our results indicate that *G. duodenalis* is a common finding in apparently healthy school children in Ibadan, Nigeria. This study provides the most comprehensive and thorough account on the molecular diversity of *G. duodenalis* in this country. As in other sub-Saharan African regions, assemblage B is the predominant genetic variant of the parasite circulating in humans. This fact, together with the absence of animal-adapted assemblages in the surveyed pediatric population, strongly suggests that human transmission of *G. duodenalis* is primarily anthroponotic. Therefore, efforts to control giardiasis (and other fecal-orally transmitted pathogens) should focus on providing safe drinking water and improving sanitation and personal hygiene practices including hand washing.

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References

1. Kirk, M.D.; Pires, S.M.; Black, R.E.; Caipo, M.; Crump, J.A.; Devleesschauwer, B.; Döpfer, D.; Fazil, A.; Fischer-Walker, C.L.; Hald, T.; et al. World Health Organization estimates of the global and regional disease burden of 22 foodborne bacterial, protozoal, and viral diseases, 2010: A data synthesis. *PLoS Med.* **2015**, *12*, e1001921.
2. Allain, T.; Buret, A.G. Pathogenesis and post-infectious complications in giardiasis. *Adv. Parasitol.* **2020**, *107*, 173–199. [CrossRef] [PubMed]
3. Hanevik, K.; Wensaas, K.-A.; Rortveit, G.; Eide, G.E.; Mørch, K.; Langeland, N. Irritable Bowel Syndrome and Chronic Fatigue 6 Years After Giardia Infection: A Controlled Prospective Cohort Study. *Clin. Infect. Dis.* **2014**, *59*, 1394–1400. [CrossRef] [PubMed]
4. Davids, B.J.; Liu, C.M.; Hanson, E.M.; Le, C.H.Y.; Ang, J.; Hanevik, K.; Fischer, M.; Radunovic, M.; Langeland, N.; Ferella, M.; et al. Identification of Conserved Candidate Vaccine Antigens in the Surface Proteome of *Giardia lamblia*. *Infect. Immun.* **2019**, *87*, e00219-19. [CrossRef] [PubMed]
5. Mørch, K.; Hanevik, K. Giardiasis treatment: An update with a focus on refractory disease. *Curr. Opin. Infect. Dis.* **2020**, *33*, 355–364. [CrossRef] [PubMed]
6. Dixon, B.R. *Giardia duodenalis* in humans and animals—Transmission and disease. *Res. Veter Sci.* **2021**, *135*, 283–289. [CrossRef] [PubMed]
7. Speich, B.; Croll, D.; Fürst, T.; Utzinger, J.; Keiser, J. Effect of sanitation and water treatment on intestinal protozoa infection: A systematic review and meta-analysis. *Lancet Infect. Dis.* **2016**, *16*, 87–99. [CrossRef] [PubMed]
8. Berkman, D.S.; Lescano, A.G.; Gilman, R.H.; Lopez, S.L.; Black, M.M. Effects of stunting, diarrhoeal disease, and parasitic infection during infancy on cognition in late childhood: A follow-up study. *Lancet* **2002**, *359*, 564–571. [CrossRef]
9. Ignatius, R.; Gahutu, J.B.; Klotz, C.; Steininger, C.; Shyirambere, C.; Lyng, M.; Musemakweri, A.; Aebischer, T.; Martus, P.; Harms, G.; et al. High Prevalence of *Giardia duodenalis* Assemblage B Infection and Association with Underweight in Rwandan Children. *PLoS Negl. Trop. Dis.* **2012**, *6*, e1677. [CrossRef]
10. Kotloff, K.L.; Nataro, J.P.; Blackwelder, W.C.; Nasrin, D.; Farag, T.H.; Panchalingam, S.; Wu, Y.; Sow, S.O.; Sur, D.; Breiman, R.F.; et al. Burden and aetiology of diarrhoeal disease in infants and young children in developing countries (the Global Enteric Multicenter Study, GEMS): A prospective, case-control study. *Lancet* **2013**, *382*, 209–222. [CrossRef]
11. Platts-Mills, J.A.; Babji, S.; Bodhidatta, L.; Gratz, J.; Haque, R.; Havt, A.; McCormick, B.J.; McGrath, M.; Olortegui, M.P.; Samie, A.; et al. Pathogen-specific burdens of community diarrhoea in developing countries: A multisite birth cohort study (MAL-ED). *Lancet Glob. Health* **2015**, *3*, e564–e575. [CrossRef] [PubMed]

12. Donowitz, J.R.; Alam, M.; Kabir, M.; Ma, J.Z.; Nazib, F.; Platts-Mills, J.A.; Bartelt, L.A.; Haque, R.; Petri, W.A. A Prospective Longitudinal Cohort to Investigate the Effects of Early Life Giardiasis on Growth and All Cause Diarrhea. *Clin. Infect. Dis.* **2016**, *63*, 792–797. [CrossRef] [PubMed]

13. Bartelt, L.A.; Platts-Mills, J.A. Giardia: A pathogen or commensal for children in high-prevalence settings? *Curr. Opin. Infect. Dis.* **2016**, *29*, 502–507. [CrossRef] [PubMed]

14. Veenemans, J.; Mank, T.; Ottenhof, M.; Baidjoe, A.; Mbugi, E.V.; Demir, A.Y.; Wielders, J.P.M.; Savelkoul, H.F.J.; Verhoef, H. Protection against Diarrhea Associated with *Giardia intestinalis* Is Lost with Multi-Nutrient Supplementation: A Study in Tanzanian Children. *PLoS Negl. Trop. Dis.* **2011**, *5*, e1158. [CrossRef] [PubMed]

15. Ianiro, G.; Iorio, A.; Porcari, S.; Masucci, L.; Sanguinetti, M.; Perno, C.F.; Gasbarrini, A.; Putignani, L.; Cammarota, G. How the gut parasitome affects human health. *Ther. Adv. Gastroenterol.* **2022**, *15*, 17562848221091524. [CrossRef] [PubMed]

16. Soto-Méndez, M.J.; Aguilera, C.M.; Mesa, M.D.; Campaña-Martín, L.; Martín-Laguna, V.; Solomons, N.W.; Schümann, K.; Gil, Á. Interaction of *Giardia intestinalis* and Systemic Oxidation in Preschool Children in the Western Highlands of Guatemala. *J. Pediatr. Gastroenterol. Nutr.* **2016**, *63*, 118–122. [CrossRef]

17. Reynoso-Robles, R.; Ponce-Macotela, M.; Rosas-López, L.E.; Ramos-Morales, A.; Martínez-Gordillo, M.N.; González-Maciel, A. The invasive potential of *Giardia intestinalis* in an in vivo model. *Sci. Rep.* **2015**, *5*, 15168. [CrossRef]

18. Ryan, U.M.; Feng, Y.; Fayer, R.; Xiao, L. Taxonomy and molecular epidemiology of *Cryptosporidium* and *Giardia*—A 50 year perspective (1971–2021). *Int. J. Parasitol.* **2021**, *51*, 1099–1119. [CrossRef]

19. Squire, S.A.; Ryan, U. Cryptosporidium and Giardia in Africa: Current and future challenges. *Parasites Vectors* **2017**, *10*, 195. [CrossRef]

20. Haque, R.; Mondal, D.; Karim, A.; Molla, I.H.; Rahim, A.; Faruque, A.S.G.; Ahmad, N.; Kirkpatrick, B.D.; Houpt, E.; Snider, C.; et al. Prospective Case-Control Study of the Association between Common Enteric Protozoal Parasites and Diarrhea in Bangladesh. *Clin. Infect. Dis.* **2009**, *48*, 1191–1197. [CrossRef]

21. Messa, A., Jr.; Köster, P.C.; Garrine, M.; Gilchrist, C.; Bartelt, L.A.; Nhampossa, T.; Massora, S.; Kotloff, K.; Levine, M.M.; Alonso, P.L.; et al. Molecular diversity of *Giardia duodenalis* in children under 5 years from the Manhiça district, Southern Mozambique enrolled in a matched case-control study on the aetiology of diarrhoea. *PLoS Negl. Trop. Dis.* **2021**, *15*, e0008987. [CrossRef] [PubMed]

22. Vos, T.; Lim, S.S.; Abbafati, C.; Abbas, K.M.; Abbasi, M.; Abbasifard, M. GBD 2019 Diseases and Injuries Collaborators. Global burden of 369 diseases and injuries in 204 countries and territories, 1990–2019: A systematic analysis for the Global Burden of Disease Study 2019. *Lancet* **2020**, *396*, 1204–1222. [CrossRef] [PubMed]

23. Enekwechi, L.C.; Azubike, C.N. Survey of the prevalence of intestinal parasites in children of primary school age. *W. Afr. J. Med.* **1994**, *13*, 227–230.

24. Edosomwan, E.U.; Evbuomwan, I.O.; Agbalalah, C.; Dahunsi, S.O.; Abhulimhen-Iyoha, B.I. Malaria coinfection with Neglected Tropical Diseases (NTDs) in children at Internally Displaced Persons (IDP) camp in Benin City, Nigeria. *Heliyon* **2020**, *6*, e04604. [CrossRef]

25. Ihejirika, O.C.; Nwaorgu, O.C.; Ebirim, C.I.; Nwokeji, C.M. Effects of intestinal parasitic infections on nutritional status of primary children in Imo State Nigeria. *Pan Afr. Med. J.* **2019**, *33*, 34. [CrossRef]

26. Gyang, V.P.; Chuang, T.-W.; Liao, C.-W.; Lee, Y.-L.; Akinwale, O.P.; Orok, A.; Ajibaye, O.; Babasola, A.J.; Cheng, P.-C.; Chou, C.-M.; et al. Intestinal parasitic infections: Current status and associated risk factors among school aged children in an archetypal African urban slum in Nigeria. *J. Microbiol. Immunol. Infect.* **2019**, *52*, 106–113. [CrossRef]

27. Arene, F.O.; Akabogu, O.A. Intestinal parasitic infections in pre-school children in the Niger Delta. *J. Hyg. Epidemiol. Microbiol. Immunol.* **1986**, *30*, 99–102.

28. Efunshile, M.A.; Ngwu, B.A.F.; Kurtzhals, J.A.L.; Stensvold, C.R.; Sahar, S.; König, B. Molecular Detection of the Carriage Rate of Four Intestinal Protozoa with Real-Time Polymerase Chain Reaction: Possible Overdiagnosis of *Entamoeba histolytica* in Nigeria. *Am. J. Trop. Med. Hyg.* **2015**, *93*, 257–262. [CrossRef]

29. Efunshile, A.M.; Ezeanosike, O.; Onyekachi, O.N.I.; Ugwu, M.I.; König, B.; Robertson, L.J. Apparent absence of Giardia infections among children under 5-years of age with acute watery diarrhoea in Abakaliki, Nigeria. *Epidemiol. Infect* **2018**, *147*, 58. [CrossRef]

30. Tinuade, O.; John, O.; Saheed, O.; Oyeku, O.; Fidelis, N.; Olabisi, D. Parasitic etiology of childhood diarrhea. *Indian J. Pediatr.* **2006**, *73*, 1081–1084. [CrossRef]

31. Ogunsanya, T.I.; Rotimi, V.O.; Adenuga, A.; Birkholz, S.; Knipp, U.; Lemma, E.; Kroger, A.; Opferkuch, W. A study of the aetiological agents of childhood diarrhoea in Lagos, Nigeria. *J. Med. Microbiol.* **1994**, *40*, 10–14. [CrossRef] [PubMed]

32. Yakubu, A.M.; Sathiakumar, N. Chronic diarrhoea in Nigerian children. *J. Diarrhoeal Dis. Res.* **1985**, *3*, 145–148. [PubMed]

33. Akinbo, F.O.; Okaka, C.E.; Omoregie, R. Prevalence of intestinal parasitic infections among HIV patients in Benin City, Nigeria. *Libyan J. Med.* **2010**, *5*, 5506. [CrossRef] [PubMed]

34. Sanyaolu, A.O.; Oyibo, W.A.; Fagbenro-Beyioku, A.F.; Gbadegeshin, A.H.; Iriemenam, N.C. Comparative study of entero-parasitic infections among HIV sero-positive and sero-negative patients in Lagos, Nigeria. *Acta Trop.* **2011**, *120*, 268–272. [CrossRef] [PubMed]

35. Keshinro, I.B.; Musa, B.O. Cellular immunity and diarrhoeal disease amongst patients infected with the human immunodeficiency viruses 1 and 2 in Zaria, Nigeria. *Niger. J. Med.* **2003**, *12*, 22–26.

36. Nzeako, B.C. Seasonal prevalence of protozoan parasites in Nsukka, Nigeria. *J. Commun. Dis.* **1992**, *24*, 224–230.

37. Ogunba, E.O. The prevalence of human intestinal protozoa in Ibadan, Nigeria. *J. Trop. Med. Hyg.* **1977**, *80*, 187–191.

38. Maikai, B.V.; Umoh, J.U.; Lawal, I.A.; Kudi, A.C.; Ejembi, C.L.; Xiao, L. Molecular characterizations of *Cryptosporidium*, *Giardia*, and *Enterocytozoon* in humans in Kaduna State, Nigeria. *Exp. Parasitol.* **2012**, *131*, 452–456. [CrossRef]

39. Reinthaler, F.F.; Mascher, F.; Klem, G.; Sixl, W. A survey of gastrointestinal parasites in Ogun State, southwest Nigeria. *Ann. Trop. Med. Parasitol.* **1988**, *82*, 181–184. [CrossRef]

40. Idowu, O.A.; Rowland, S.A. Oral fecal parasites and personal hygiene of food handlers in Abeokuta, Nigeria. *Afr. Health Sci.* **2006**, *6*, 160–164. [CrossRef]

41. Ogunji, F.O.; Akinboade, O.A.; Dipeolu, O.O.; Ayeni, J.; Okaeme, A. The prevalence of gastro-intestinal parasites and bacteria in the game scouts at the Kainji Lake National Park of Nigeria. *Int. J. Zoonoses* **1984**, *11*, 119–122.

42. Okafor, C.N.; Azubike, C.N. Studies in intestinal parasitic disease agents in stools of people in a rural area of Nigeria. *West Afr. J. Med.* **1992**, *11*, 106–111.

43. Okpasuo, O.J.; Aguzie, I.O.; Joy, A.T.; Okafor, F.C. Risk assessment of waterborne infections in Enugu State, Nigeria: Implications of household water choices, knowledge, and practices. *AIMS Public Health* **2020**, *7*, 634–649. [CrossRef] [PubMed]

44. Ikeh, E.I.; Obadofin, M.O.; Brindeiro, B.; Baugherb, C.; Frost, F.; Vanderjagt, D.; Glew, R.H. Intestinal parasitism in Magama Gumau rural village and Jos township in north central Nigeria. *Niger. Postgrad. Med. J.* **2007**, *14*, 290–295. [PubMed]

45. Oyerinde, J.P.; Ogunbi, O.; Alonge, A.A. Age and sex distribution of infections with *Entamoeba histolytica* and *Giardia intestinalis* in the Lagos population. *Int. J. Epidemiol.* **1977**, *6*, 231–234. [CrossRef] [PubMed]

46. Akinkuotu, O.A.; Takeet, M.I.; Otesile, E.B.; Olufemi, F.; Greenwood, S.J.; McClure, J.T. Multi-locus genotyping and phylogenetic analyses of *Giardia intestinalis* isolates from indigenous goats in Ogun State, Nigeria. *Acta Trop.* **2019**, *195*, 15–22. [CrossRef] [PubMed]

47. Akinkuotu, O.A.; Takeet, M.I.; Otesile, E.B.; Olufemi, F.; Greenwood, S.J.; McClure, J.T. Prevalence and multilocus genotypes of *Giardia duodenalis* infecting pigs in Ogun state, Nigeria. *Infect. Genet. Evol.* **2019**, *70*, 53–60. [CrossRef] [PubMed]

48. Akinkuotu, O.A.; Greenwood, S.J.; McClure, J.T.; Takeet, M.I.; Otesile, E.B.; Olufemi, F. Multilocus genotyping of *Giardia duodenalis* infecting rabbits in Ogun State, Nigeria. *Veter Parasitol. Reg. Stud. Rep.* **2018**, *13*, 171–176. [CrossRef] [PubMed]

49. Adenusi, A.A.; Akinyemi, M.I.; Akinsanya, D. Domiciliary Cockroaches as Carriers of Human Intestinal Parasites in Lagos Metropolis, Southwest Nigeria: Implications for Public Health. *J. Arthropod-Borne Dis.* **2018**, *12*, 141–151. [CrossRef] [PubMed]

50. Adenusi, A.A.; Adewoga, T.O.S. Human intestinal parasites in non-biting synanthropic flies in Ogun State, Nigeria. *Travel Med. Infect. Dis.* **2013**, *11*, 181–189. [CrossRef]

51. Cheesbrough, M. *District Laboratory Practice in Tropical Countries. Part 1*, 2nd ed.; Cambridge University Press: New York, NY, USA, 2009.

52. Verweij, J.J.; Schinkel, J.; Laeijendecker, D.; van Rooyen, M.A.; van Lieshout, L.; Polderman, A.M. Real-time PCR for the detection of *Giardia lamblia*. *Mol. Cell. Probes* **2003**, *17*, 223–225. [CrossRef] [PubMed]

53. Appelbee, A.; Frederick, L.; Heitman, T.; Olson, M. Prevalence and genotyping of *Giardia duodenalis* from beef calves in Alberta, Canada. *Veter Parasitol.* **2003**, *112*, 289–294. [CrossRef] [PubMed]

54. Hopkins, R.M.; Meloni, B.P.; Groth, D.M.; Wetherall, J.D.; Reynoldson, J.A.; Thompson, R.C.A. Ribosomal RNA Sequencing Reveals Differences between the Genotypes of *Giardia* Isolates Recovered from Humans and Dogs Living in the Same Locality. *J. Parasitol.* **1997**, *83*, 44–51. [CrossRef] [PubMed]

55. Read, C.M.; Monis, P.T.; Thompson, R.C. Discrimination of all genotypes of *Giardia duodenalis* at the glutamate dehydrogenase locus using PCR-RFLP. *Infect. Genet. Evol.* **2004**, *4*, 125–130. [CrossRef]

56. Lalle, M.; Pozio, E.; Capelli, G.; Bruschi, F.; Crotti, D.; Cacciò, S.M. Genetic heterogeneity at the beta-giardin locus among human and animal isolates of *Giardia duodenalis* and identification of potentially zoonotic subgenotypes. *Int. J. Parasitol.* **2005**, *35*, 207–213. [CrossRef]

57. Sulaiman, I.M.; Fayer, R.; Bern, C.; Gilman, R.H.; Trout, J.M.; Schantz, P.M.; Das, P.; Lal, A.A.; Xiao, L. Triosephosphate Isomerase Gene Characterization and Potential Zoonotic Transmission of *Giardia duodenalis*. *Emerg. Infect. Dis.* **2003**, *9*, 1444–1452. [CrossRef]

58. Kumar, S.; Stecher, G.; Li, M.; Knyaz, C.; Tamura, K. MEGA X: Molecular Evolutionary Genetics Analysis across Computing Platforms. *Mol. Biol. Evol.* **2018**, *35*, 1547–1549. [CrossRef]

59. IBM Corp. *IBM SPSS Statistics for Windows, Version 28.0*; IBM Corp.: Armonk, NY, USA, 2021.

60. Schuurman, T.; Lankamp, P.; van Belkum, A.; Kooistra-Smid, M.; van Zwet, A. Comparison of microscopy, real-time PCR and a rapid immunoassay for the detection of *Giardia lamblia* in human stool specimens. *Clin. Microbiol. Infect.* **2007**, *13*, 1186–1191. [CrossRef]

61. Popruk, S.; Thima, K.; Udonsom, R.; Rattaprasert, P.; Sukthana, Y. Does silent Giardia infection need any attention? *Open Trop. Med. J.* **2011**, *4*, 26–32. [CrossRef]

62. Reh, L.; Muadica, A.S.; Köster, P.C.; Balasegaram, S.; Verlander, N.Q.; Chércoles, E.R.; Carmena, D. Substantial prevalence of enteroparasites *Cryptosporidium* spp., *Giardia duodenalis* and *Blastocystis* sp. in asymptomatic schoolchildren in Madrid, Spain, November 2017 to June 2018. *Eurosurveillance* **2019**, *24*, 1900241. [CrossRef]

63. Ankarklev, J.; Lebbad, M.; Einarsson, E.; Franzén, O.; Ahola, H.; Troell, K.; Svärd, S.G. A novel high-resolution multilocus sequence typing of *Giardia* intestinalis Assemblage A isolates reveals zoonotic transmission, clonal outbreaks and recombination. *Infect. Genet. Evol.* **2018**, *60*, 7–16. [CrossRef]

64. Dacal, E.; Saugar, J.M.; de Lucio, A.; Hernández-De-Mingo, M.; Robinson, E.; Köster, P.C.; Aznar-Ruiz-De-Alegría, M.L.; Espasa, M.; Ninda, A.; Gandasegui, J.; et al. Prevalence and molecular characterization of *Strongyloides stercoralis*, *Giardia duodenalis*, *Cryptosporidium* spp., and *Blastocystis* spp. isolates in school children in Cubal, Western Angola. *Parasites Vectors* **2018**, *11*, 67. [CrossRef] [PubMed]

65. de Lucio, A.; Amor-Aramendía, A.; Bailo, B.; Saugar, J.M.; Anegagrie, M.; Arroyo, A.; López-Quintana, B.; Zewdie, D.; Ayehubizu, Z.; Yizengaw, E.; et al. Prevalence and Genetic Diversity of *Giardia duodenalis* and *Cryptosporidium* spp. among School Children in a Rural Area of the Amhara Region, North-West Ethiopia. *PLoS ONE* **2016**, *11*, e0159992. [CrossRef] [PubMed]

66. Di Cristanziano, V.; Santoro, M.; Parisi, F.; Albonico, M.; Shaali, M.; Di Cave, D.; Berrilli, F. Genetic characterization of *Giardia duodenalis* by sequence analysis in humans and animals in Pemba Island, Tanzania. *Parasitol. Int.* **2014**, *63*, 438–441. [CrossRef] [PubMed]

67. Tembo, S.J.; Mutengo, M.M.; Sitali, L.; Changula, K.; Takada, A.; Mweene, A.S.; Simulundu, E.; Chitanga, S. Prevalence and genotypic characterization of *Giardia duodenalis* isolates from asymptomatic school-going children in Lusaka, Zambia. *Food Waterborne Parasitol.* **2020**, *19*, e00072. [CrossRef]

68. Cai, W.; Ryan, U.; Xiao, L.; Feng, Y. Zoonotic giardiasis: An update. *Parasitol. Res.* **2021**, *120*, 4199–4218. [CrossRef]

69. Figueiredo Pacheco, F.T.; Novaes Rodrigues Silva, R.K.; Souza de Carvalho, S.; Carvalho Rocha, F.; Trindade das Chagas, G.M.; Chagas Gomes, D.; da Costa-Ribeiro, H., Jr.; Medrado Ribeiro, T.C.; Peixoto de Mattos, Â.; Kalabric Silva, L.; et al. The Predominance of *Giardia duodenalis* AII sub-assemblage in young children from Salvador, Bahia, Brazil. *Biomedica* **2020**, *40*, 557–568. [CrossRef]

70. Skhal, D.; Aboualchamat, G.; Al Mariri, A.; Al Nahhas, S. Prevalence of *Giardia duodenalis* assemblages and sub-assemblages in symptomatic patients from Damascus city and its suburbs. *Infect. Genet. Evol.* **2017**, *47*, 155–160. [CrossRef]

71. Cacciò, S.; Beck, R.; Lalle, M.; Marinculic, A.; Pozio, E. Multilocus genotyping of *Giardia duodenalis* reveals striking differences between assemblages A and B. *Int. J. Parasitol.* **2008**, *38*, 1523–1531. [CrossRef]

72. Sprong, H.; Cacciò, S.M.; van der Giessen, J.W.B.; on behalf of the ZOOPNET Network and Partners. Identification of Zoonotic Genotypes of *Giardia duodenalis*. *PLoS Negl. Trop. Dis.* **2009**, *3*, e558. [CrossRef]

73. Köster, P.C.; Malheiros, A.F.; Shaw, J.J.; Balasegaram, S.; Prendergast, A.; Lucaccioni, H.; Moreira, L.M.; Lemos, L.M.S.; Dashti, A.; Bailo, B.; et al. Multilocus genotyping of *Giardia duodenalis* in mostly asymptomatic indigenous people from the Tapirapé tribe, Brazilian Amazon. *Pathogens* **2021**, *10*, 206. [CrossRef] [PubMed]

74. Sarzhanov, F.; Köster, P.C.; Dogruman-Al, F.; Bailo, B.; Dashti, A.; Demirel-Kaya, F.; Carmena, D. Detection of enteric parasites and molecular characterization of *Giardia duodenalis* and *Blastocystis* sp. in patients admitted to hospital in Ankara, Turkey. *Parasitology* **2021**, *148*, 550–561. [CrossRef] [PubMed]

75. Morrison, H.G.; McArthur, A.G.; Gillin, F.D.; Aley, S.B.; Adam, R.D.; Olsen, G.J.; Best, A.A.; Cande, W.Z.; Chen, F.; Cipriano, M.J.; et al. Genomic Minimalism in the Early Diverging Intestinal Parasite *Giardia lamblia*. *Science* **2007**, *317*, 1921–1926. [CrossRef] [PubMed]

76. Franzén, O.; Jerlström-Hultqvist, J.; Castro, E.; Sherwood, E.; Ankarklev, J.; Reiner, D.S.; Palm, D.; Andersson, J.O.; Andersson, B.; Svärd, S.G. Draft genome sequencing of *Giardia intestinalis* assemblage B isolate GS: Is human giardiasis caused by two different species? *PLoS Pathog.* **2009**, *5*, e1000560. [CrossRef]

77. Seabolt, M.H.; Roellig, D.M.; Konstantinidis, K.T. Genomic comparisons confirm *Giardia duodenalis* sub-assemblage AII as a unique species. *Front. Cell. Infect. Microbiol.* **2022**, *12*, 1010244. [CrossRef]

78. Seabolt, M.H.; Konstantinidis, K.T.; Roellig, D.M. Hidden Diversity within Common Protozoan Parasites as Revealed by a Novel Genomotyping Scheme. *Appl. Environ. Microbiol.* **2021**, *87*, e02275-20. [CrossRef]