Clarissa Pérez Faria

MOLECULAR CHARACTERIZATION OF *Giardia lamblia* FROM PATIENTS OF A REFERRAL HOSPITAL OF RIO DE JANEIRO/BRAZIL: APPLICATION OF MULTILOCUS GENOTYPING TO STUDY THE INTER AND INTRA-ASSEMBLAGE VARIATIONS

Tese de doutoramento em Ciências Farmacêuticas, na área de especialização em Microbiologia e Parasitologia, orientada pela Professora Doutora Maria do Céu Sousa e pela Doutora Graziela Zanini e apresentada à Faculdade de Farmácia da Universidade de Coimbra

Setembro de 2016

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UNIVERSIDADE DE COIMBRA

FFUC FACULDADE DE FARMÁCIA
UNIVERSIDADE DE COIMBRA

Clarissa Pérez Faria

Molecular characterization of *Giardia lamblia* from patients of a referral hospital of Rio de Janeiro/Brazil: application of multilocus genotyping to study the inter and intra-assemblage variations

Dissertation presented to obtain a Ph.D. degree in Pharmaceutical Sciences, speciality Microbiology/Parasitology in University of Coimbra, Faculty of Pharmacy.

Supervisors: Professor Doctor Maria do Céu Sousa and Doctor Graziela Zanini

Coimbra, 2016

Aos meus pais

Ao Marcelo

Agradecimentos

Em primeiro lugar, gostaria de agradecer a minha orientadora Professora Doutora Maria do Céu Sousa por ter me aceitado para o doutoramento, pela sua amizade, constante incentivo, dedicação, apoio e por toda a ajuda que me deu para que eu pudesse alcançar este objetivo. Pela imensa admiração pessoal e profissional que lhe tenho (é um verdadeiro orgulho trabalhar consigo), certamente todas as palavras de agradecimento serão sempre poucas.

À Doutora Graziela Maria Zanini, minha orientadora, gostaria de igualmente agradecer pelo conhecimento científico compartilhado, por ter acreditado no meu trabalho e na minha motivação. Destes longos anos recordarei de nossa amizade, de nossas conversas e dos muitos bons momentos que passamos juntas. Por tudo, muito obrigada.

À Professora Doutora Paula Santana e seu doutorando Ricardo Almendra agradeço a disponibilidade e a colaboração ao longo de todo o meu trabalho. Uma ajuda inestimável prestada na análise espacial e geoprocessamento dos dados.

Ao Doutor André Almeida pela ajuda preciosa nos ensaios de qPCR, tanto na otimização das condições como na análise dos resultados obtidos.

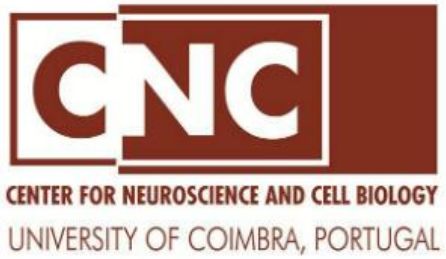
À Professora Doutora Paula Morais e ao Doutor Diogo Proença agradeço a ajuda prestada na análise filogenética das sequências.

Aos meus colegas dos laboratórios de Microbiologia (FFUC), Bioquímica (FFUC) e Parasitologia (INI/FIOCRUZ), pelo companheirismo, pelo apoio constante e amizade que desenvolvemos ao longo dos anos.

Eu gostaria de deixar um agradecimento especial aos meus pais, Herminia e Jorge (*in memoriam*), ao meu irmão e ao Marcelo, pelo apoio incondicional, carinho e amor que eles sempre me deram.

This work was performed at the Faculty of Pharmacy, within the activities of the Centre for Neuroscience and Cell Biology of Coimbra, University of Coimbra, Coimbra, Portugal, under the supervision of Professor Doctor Maria do Céu Rodrigues de Sousa e Professor Doctor Graziela Maria Zanini.

This work was supported financially by FCT POCTI (FEDER) and COMPETE (PEst-C/SAU/LA000172013-2014). Clarissa Pérez Faria was supported by grant 245531/2012-2 from National Council for Scientific and Technological Development (CNPq).



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Abbreviations

ADI	Arginine deiminase
AIDS	Acquired immune deficiency syndrome
ART	Antiretroviral therapy
ASH	Allelic sequence heterozygosity
ATP	Adenosine triphosphate
bg	β-giardin gene
catB	Cathepsin B
CCL	Chemokine (C-C motif) ligand
CEDAE	State Company of Water and Sewage
CI	Confidence interval
COX-2	Cyclooxygenase-2
CT	Census tracts
CXCL8	Chemoattractant interleukin-8
CWP	Cyst wall proteins
DNA	Deoxyribonucleic acid
ef-1α	Elongation factor 1 alpha gene
ELISA	Enzyme-Linked Immunosorbent Assay
FIOCRUZ	Fundação Oswaldo Cruz
flavoHb	Flavohemoglobin protein
gdh	NADPH-dependent glutamate dehydrogenase gene
HCMPs	High-cysteine membrane proteins
HDI	Human development index
HIV	Human immunodeficiency virus
IBGE	Brazilian Institute of Statistics and Geography
IBS	Irritable bowel syndrome
IEC	Intestinal epithelium cells
Ig	Immunoglobulin
IGS	Intergenic ribosomal spacer
IL	Interleukin
INI	Evandro Chagas National Institute of Infectious Diseases
iNOS	Inducible nitric oxide synthase
ITS	Internal transcriber regions
lsu rDNA	Large subunit ribosomal DNA
MAPKs	Mitogen-activated protein kinases
MDI	Material deprivation index

MHDI	Municipal human development index
MIF	Mertiolate-iodine-formaldehyde
MLC	Myosin light chain
MLCK	Myosin-light-chain kinase
MLG	Multilocus genotype
MLST	Multilocus sequence typing
NAD^+	Nicotinamide adenine dinucleotide
$NADP^+$	Nicotinamide adenine dinucleotide phosphate
NF-κB	Transcription nuclear factor-κB
NO	Nitric oxide
NOS	Nitric oxide synthase
OCT	Ornithine carbamoyltransferase
*orfC*4	Open reading frame C4 gene
PCR	Polymerase chain reaction
PMN	Polymorphonuclear
qPCR	Quantitative polymerase chain reaction
rDNA	Ribosomal DNA
RFLP	Restriction fragment length polymorphisms
RJ	Rio de Janeiro
ROS	Reactive oxygen species
SD	Standard deviation
SNP	Single-nucleotide polymorphism
SPSS	Statistical Package for the Social Sciences
ssu rRNA	Small-subunit ribosomal RNA gene
STD	Sexually transmitted disease
STH	Soil-transmitted helminths
TcdA	*Clostridium difficile* toxin A
TcdB	*Clostridium difficile* toxin B
TEM	Transmission electron microscopy
tim or *tpi*	Triose phosphate isomerase gene
TNF-α	Tumor necrosis factor alpha
USA	United States of America
VLF	Ventrolateral flange
VSP	Variant surface protein
ZO	Zonula–Occludens
WHO	World Health Organization

Abstract

Giardia lamblia is one of the most frequent human intestinal protozoa reported worldwide. Despite the high prevalence, the epidemiology of giardiasis and other intestinal parasites has been poorly documented in Brazil, principally in Rio de Janeiro State. *G. lamblia* is considered a species complex, whose members show little variation in their morphology, but have a remarkable genetic variability. The genetic characterization of *G. lamblia* has been scarcely reported in Rio de Janeiro (Brazil) and molecular epidemiology researches have been conducted only in the last few years. Therefore, the work aimed to (*i*) estimate the prevalence of intestinal parasites in Rio de Janeiro and provide a detailed analysis of their geographical distribution, considering demographic, socio-economic, and epidemiological factors; (*ii*) determine the prevalence of *G. lamblia* assemblages and detect mixed infections; (*iii*) identify inter- and intra-assemblage genetic variation in *G. lamblia*; and (*iv*) correlate assemblages with symptoms in patients with and without HIV/AIDS. The cross-sectional survey was conducted among patients attending the Evandro Chagas National Institute of Infectious Diseases (FIOCRUZ). For the diagnosis of intestinal parasites, stool samples were collected and processed by sedimentation, flotation in zinc sulphate solution, Kato-Katz, Baermann-Moraes, Graham methods, and iron haematoxylin and safranin stainings. The place of residence of infected and uninfected individuals was georeferenced, which allowed the assessment of the influence of socioeconomic conditions, through the material deprivation index (MDI), on the incidence of parasites. The positive stool samples for *G. lamblia* were genetic characterized by PCR-RFLP, qPCR and sequencing targeting β-giardin (*bg*), glutamate dehydrogenase (*gdh*), triose phosphate isomerase (*tpi*), open reading frame C4 (*orf*C4) and small-subunit ribosomal RNA (*ssu rRNA*) genes. Of the 3245 patients analysed, 569 (17.5%) were infected with at least one parasite. The most frequent species were *Endolimax nana* (28.8%), *Entamoeba coli* (14.8%), Complex *Entamoeba histolytica/Entamoeba dispar* (13.5%), *Blastocystis hominis* (12.7%), and *Giardia lamblia* (8.1%). Geospatial distribution of intestinal parasitic infections was not random or homogeneous, but was influenced by the MDI. Out of the 65 *G. lamblia* positive samples, 41 (63.1%) were successfully amplified by nested-PCR of *bg* and *gdh* genes, and subsequently characterized by PCR-RFLP. Among them, 16 were typed as sub-assemblage AII, 7 as BIII, 4 as BIV and 8 as a mixture of BIII and BIV. After the analysis by qPCR assay, a total of 55 (84.6%) samples were amplified using at least one locus. Multilocus genotyping (MLG)

results showed that 29 (52.7%) samples belonged to assemblage A and 26 (47.3%) to assemblage B. The intra-assemblage genetic variation was determined via multilocus sequence typing (MLST). Fourteen assemblage A samples were genotyped at the three MLST loci (*bg*/*tpi*/*gdh*). Two previously identified MLG were found (AII-1 and AII-4), and two novel MLG are proposed (AII-8, profile A2/A2/A4; and AII-9, profile A3/A2/A2). Sequence analysis showed that assemblage B isolates have a higher nucleotide variation, than assemblage A isolates. Novel assemblage B sequences were described (39 of 59; 72.2%) at the three loci. Of the sequences that did not present heterogeneous nucleotides, only 15 corresponded to previously reported sequences. The presence of double peaks prevented the unambiguous identification of MLGs. Thirty-eight of 65 positive samples for *G. lamblia* were from HIV positive patients and 27 were from HIV negative patients. Of the HIV infected patients, 19 (55.9%) isolates were genotyped as assemblage B of which 9 (47.4%) patients had a CD4 T cell count below 200 cells/mm^3. In addition, we found a greater number of samples belonging to assemblage B in symptomatic cases (11 of 19; 57.9%). In HIV negative patients, 17 (65.4%) *G. lamblia* isolates were classified as assemblage A and 9 were classified as assemblage B. This work provides the first epidemiological information on the prevalence and distribution of intestinal parasitic infections in Rio de Janeiro, pointing out that the prevalence of these parasites remain high. MDI showed that intestinal parasites were strongly associated with the socioeconomic status of the population, thus making it possible to identify social vulnerable areas. This is the first time that assemblage B was detected and that MLG tools has been used to genetically characterise *G. lamblia* isolates in human clinical samples from Rio de Janeiro. The use of several molecular markers and the qPCR allowed us to reliably determine the distribution of assemblages in the population and confirmed that mixed infections of assemblages A and B were not present. MLST enabled the identification of novel subtypes in both assemblages and the description of two novel assemblage A MLGs. Our work has provided a new insight into the genetic diversity of parasite and improved our understanding of the epidemiology of the disease by elucidating the dynamics of giardiasis in the population of Rio de Janeiro. Our data suggest that assemblage B is very likely to be found in HIV positive patients and probably the lower CD4 T cell count gives advantages for assemblage B replication. To the best of our knowledge, this is the first study that provides information on *G. lamblia* assemblages and symptoms in patients with and without HIV/AIDS virus and their association with CD4 T cell counts. As HIV infection increases the risk of having intestinal parasitic infections, including *G. lamblia*, the detection and treatment of infections are important measures to improve the quality of life of HIV positive patients.

Resumo

Giardia lamblia é um dos protozoários intestinais mais frequentemente detectados no homem. Apesar da alta prevalência, a epidemiologia da giardíase e de outras parasitoses intestinais tem sido pouco documentada no Brasil, principalmente no Estado do Rio de Janeiro. *G. lamblia* é considerado um complexo de espécies, cujos membros apresentam pouca variação na sua morfologia, mas com uma extensa variabilidade genética. A caracterização genética de *G. lamblia* tem sido pouco relatada no Rio de Janeiro (Brasil) e estudos de epidemiologia molecular foram realizados somente nos últimos anos. Portanto, o trabalho teve como objectivos (*i*) determinar a prevalência de parasitas intestinais no Rio de Janeiro e fornecer uma análise detalhada da sua distribuição geográfica, considerando-se os factores demográficos, socioecónomicos e epidemiológicos; (*ii*) determinar a prevalência dos diferentes genótipos de *G. lamblia* e detectar infecções mistas; (*iii*) identificar as variações genéticas inter e intra-genotípicas em *G. lamblia*; (*iv*) correlacionar os genótipos com os sintomas em doentes com e sem VIH/SIDA. O levantamento transversal foi realizado em indivíduos atendidos no Instituto Nacional de Infectologia Evandro Chagas (FIOCRUZ). O diagnóstico de parasitoses intestinais foi realizado em amostras de fezes através dos métodos de sedimentação, flutuação em solução de sulfato de zinco, Kato-Katz, Baermann-Moraes e Graham e pela coloração da hematoxilina férrica e safranina. O local de residência dos indivíduos, infectados e não infectados, foi georreferenciado permitindo avaliar a influência das condições socioeconómicas, através do índice de privação material (MDI), na incidência de parasitas. As amostras de fezes positivas para *G. lamblia* foram caracterizadas geneticamente por PCR-RFLP, qPCR e sequenciação tendo como alvo os genes β-giardina (*bg*), glutamato desidrogenase (*gdh*), triose fosfato isomerase (*tpi*), quadro de leitura aberto C4 (*orf*C4) e subunidade menor do ARN ribossómico (*ssu rRNA*). Dos 3245 indivíduos analisados, 569 (17,5%) estavam infectados com pelo menos um parasita. As espécies mais frequentes foram *Endolimax nana* (28,8%), *Entamoeba coli* (14,8%), Complexo *Entamoeba histolytica/Entamoeba dispar* (13,5%), *Blastocystis hominis* (12,7%) e *Giardia lamblia* (8,1%). A distribuição geoespacial das parasitoses intestinais não foi aleatória ou homogénea, sendo influenciada pelo MDI. Das 65 amostras positivas para *G. lamblia*, 41 (63,1%) foram amplificadas com êxito pelo *nested*-PCR dos genes *bg* e *gdh* e subsequentemente caracterizadas por PCR-RFLP: 16 foram tipificadas no sub-genótipo AII, 7 no BIII, 4 no BIV e 8 uma mistura de BIII e BIV. Após a análise pela técnica de qPCR, um total de 55 (84,6%) amostras foram amplificadas usando pelo menos um locus. Resultados da genotipagem por múltiplos genes (MLG) mostraram que 29 (52,7%) amostras pertenciam ao genótipo A e 26 (47,3%) ao genótipo B. A variação intra-genotípica foi determinada através do sequenciação

de múltiplos genes (MLST). Quatorze amostras do genótipo A foram genotipadas nos três loci (*bg/tpi/gdh*). Dois MLG previamente descritos foram identificados (AII-1 e AII-4) e dois novos MLG foram propostos (AII-8, perfil A2/A2/A4 e AII-9, perfil A3/A2/A2). A análise das sequências mostrou que os isolados do genótipo B apresentaram um polimorfismo genético maior do que os isolados do genótipo A. Novas sequências do genótipo B (39 de 54; 72.2%) foram encontradas nos três *loci*. Das sequências que não apresentaram nucleótidos heterogéneos, somente 15 foram idênticas a sequências previamente descritas. A presença de picos duplos impediu a definição dos MLGs. Das 65 amostras positivas para *G. lamblia*, 38 eram de doentes VIH positivos e 27 de doentes VIH negativos. Nos doentes infectados pelo VIH, 19 (55,9%) isolados de *G. lamblia* foram genotipados como genótipo B e 9 (47,4%) doentes apresentaram uma contagem T CD4 menor do que 200 células/mm^3. Além disso, observou-se um maior número de amostras pertencentes ao genótipo B nos casos sintomáticos (11 de 19; 57,9%). Nos doentes VIH negativos, 17 (65.4%) isolados de *G. lamblia* foram classificados como genótipo A e 9 foram classificados como genótipo B. Este trabalho é o primeiro a fornecer dados epidemiológicos sobre a prevalência e distribuição das parasitoses intestinais no Rio de Janeiro. O MDI mostrou que os parasitas intestinais estão fortemente associados com o nível socioeconómico da população, identificando-se áreas de vulnerabilidade social. Pela primeira vez foi detectado o genótipo B no Rio de Janeiro e o MLG foi utilizado na caracterização genética de *G. lamblia*. O uso de vários marcadores moleculares e do qPCR permitiu-nos determinar com confiança a distribuição dos genótipos na população e confirmou que as infecções mistas pelos genótipos A e B não estavam presentes. O MLST possibilitou a identificação de dois novos subtipos em ambos genótipos e a descrição de novos MLGs no genótipo A. Globalmente, o nosso trabalho contribuiu para um melhor conhecimento sobre a diversidade genética do parasita e melhorou a nossa compreensão sobre a epidemiologia da doença através da elucidação da dinâmica da giardíase na população do Rio de Janeiro. Os nossos resultados sugerem que o genótipo B é mais detectado em doentes VIH positivos e que, provavelmente, o baixo número de células T CD4 favorece a replicação do genótipo B. De acordo com o nosso conhecimento, este é o primeiro estudo sobre os genótipos de *G. lamblia* e os sintomas em doentes com e sem vírus VIH/SIDA e a sua associação com a contagem de células CD4 T. Como a infecção pelo VIH é um factor de risco para parasitoses intestinais, incluindo *G. lamblia*, a detecção e tratamento destas infecções são medidas importantes para melhorar a qualidade de vida dos doentes VIH positivos.

Chapter 1

General Introduction

1.1. Introduction

Intestinal parasitic infections remain among the most common infectious diseases worldwide. This situation is observed particularly in developing countries where inadequate water treatment, poor sanitation and lack of adequate health services are common. Additionally, it is more difficult to implement enteric parasite-control actions in these regions due to the high cost of improvements in infrastructure, and the lack of educational projects offered to the population (Ostan *et al*., 2007; Mehraj *et al*., 2008; Speich *et al*., 2016). The scenario in Brazil is no different, even with the medical and pharmaceutical advances and developments in sanitary engineering.

Giardia lamblia (also called *G. intestinalis* and *G. duodenalis*) is the causative agent of giardiasis and considered one of the leading causative agents of diarrhoea. It is a flagellated unicellular eukaryotic intestinal parasite found in the gastrointestinal tract of humans and a range of other vertebrates (Levine *et al*., 1980; Feng and Xiao, 2011).

In developing countries giardiasis infects in early childhood, with prevalence of 15 to 20% in children under ten years. Children between six months and five years old and/or malnourished are more susceptible. Chronic infection can result in serious consequences on the nutritional status, physical and mental development presumably due to malabsorption of nutrients (de Carvalho *et al*., 2006; Robertson *et al*., 2010; Nkrumah *et al*., 2011).

Clinical manifestations range from asymptomatic infections to acute or chronic diarrhoea. However, the majority of cases of giardiasis have no symptoms making difficult to eradicate and control this parasite. It is difficult to explain this heterogeneity and one hypothesis is that the parasite assemblages (or genotypes) could contribute to the development of symptoms. Molecular analyses have suggested that the level of genetic diversity among the assemblages A and B is sufficient to recognize them as two distinct species of *Giardia*. Some authors believe that the genomic differences between strains WB (assemblage A) and GS (assemblage B) could explain some of the phenotypic differences (Jerlström-Hultqvist *et al*., 2010; Xu *et al*., 2012). Certainly, the understanding of the epidemiology of giardiasis is committed by the uncertainty of taxonomy. Several studies correlating *G. lamblia* assemblages with symptoms development did not show conclusive results (Haque *et al*., 2005; Puebla *et al*., 2014; Minetti *et al*., 2015).

Particular attention has focused on the possible relationship between *G. lamblia* infections in human and companion animals, especially dogs and cats (Cacciò *et al*., 2005; Cacciò and Ryan, 2008). Genetic variability and zoonotic potential of *G. lamblia* could differ

between geographical regions, thereby the classification of circulating parasites in different areas is essential.

Despite the high prevalence of the infection in Brazil, the genetic characterization of the parasite has been poorly documented and the molecular epidemiology research has only been conducted in the last few years (Souza *et al*., 2007; Kohli *et al*., 2008; Santos *et al*., 2012; Durigan *et al*., 2014; Colli *et al*., 2015; David *et al*., 2015; Oliveira-Arbex *et al*., 2015; Nunes *et al*., 2016). So far only one study was performed with samples from Rio de Janeiro (Volotão *et al*., 2007), consequently information on the current distribution and prevalence of *G. lamblia* assemblages in the city is still scarce.

1.2. Intestinal parasitic infections

Neglected tropical diseases including the intestinal parasitic infections (e.g., *Cryptosporidium* spp., *Entamoeba histolytica*, and *G. lamblia*) are significant cause of morbidity and mortality worldwide. Globally about one third of the total population is estimated to be infected with intestinal parasites, the majority being people living in tropical and sub-tropical parts of the world (Chan *et al*., 1997). Approximately 500 million people worldwide are infected with *E. histolytica,* nearly 50 million people suffer from invasive amoebic infection, resulting in 40–70 thousand deaths annually (WHO, 1997; Duc *et al*., 2011). *Cryptosporidium* is another important protozoan parasite that causes gastrointestinal illness in humans, especially among young children and immunocompromised patients with specific T-cell deficiencies (Hunter and Nichols, 2002).

About 819 million people are infected with *Ascaris lumbricoides*, 464.6 million people with *Trichuris trichiura*, and 438.9 million people with hookworm infection (Pullan and Brooker, 2012). Soil-transmitted helminths (*A. lumbricoides*, *T. trichiura*, hookworms and *Strongyloides stercoralis*) infections rarely result in death, but they affect nutrition, resulting in anaemia, loss of appetite, intestinal damage and reduced absorption of vitamin A (Hotez *et al*., 2006).

Intestinal parasitic infections are transmitted by the faecal-oral route and through drinking contaminated water, thus infections are more present where access to clean water and sanitation is inadequate (Bethony *et al*., 2006; Pullan and Brooker, 2012; Speich *et al*., 2016). Both conditions have long been associated with diarrhoea (Cairncross *et al*., 2010), which is among the main contributors to global child mortality (Liu *et al*., 2012). Recently, a systematic review and meta-analysis showed that sanitation facilities and water treatment are

associated with lower risks of infection with intestinal protozoa, and could also prevent diarrhoeal diseases (Speich et *al*., 2016). The same relationships were observed for soil-transmitted helminths (Strunz *et al*., 2014).

Water is essential to life but is also a major vehicle for pathogen dissemination. The potential for waterborne parasite transmission is high since infective helminth eggs and protozoa (oo)cysts are distributed through water in the environment. Pathogens like *G. lambia* and *Cryptosporidium* spp. are seen as important waterborne disease pathogens and are associated with severe gastrointestinal illness. Amoebiasis, balantidiosis, cyclosporidiosis and microsporidiosis outbreaks have been reported throughout the world (Baldursson and Karani, 2011; Kumar *et al*., 2014). It is well documented that conventional water and sewage treatment process are not completely effective in destroying protozoa (oo)cysts and helminth eggs (Betancourt and Rose, 2004; Savioli *et al*., 2006; Hatam-Nahavandi *et al*., 2015). Improper disposal of human and animal waste has also been identified as a source of infection, contaminating water sources (Smith et al., 2007) and recreational waters such as swimming pools, water parks and lakes (Savioli *et al*., 2006). Occasionally, sewer overflows also contribute to contamination of surface water and agricultural lands, which leads to potential human infection. Food contamination is also important and can occur directly in the handling process such as contaminated equipment, infected food handlers or wash water, or indirectly through contaminated irrigation water (Dawson, 2005).

The urban context with its particular living conditions, that includes uncontrolled expansion of urban slums and informal settlements, residential overcrowding, or environmental degradation also explains the high prevalence of intestinal parasites. In addition, with the insertion of women in the labour market, a higher number of toddlers and pre-schoolers are enrolled in kindergartens and day-care centres. These spaces for child development have come to play an important role in host-parasite relationships, since they represent the external environment more frequented by young children (Mehraj *et al*., 2008; Gonçalves *et al*., 2011).

Multiple infections are very common and usually can be used as indicators of transmission through the faecal-oral route, thereby pointing to the transmission of intestinal parasites via the supply of water for human consumption, or to the ingestion of contaminated food. In many countries polyparasitism had been described, for example, in Kenya, 7% of the studied population was infected with multiple parasites (LaBeaud *et al*., 2015). Mejia Torres and colleagues (2014) observed in Honduras that 14.6% of children were infected with more than one parasite.

In Brazil, intestinal parasite infections persist, although their frequency has decreased due improvement of sanitary conditions (Barreto *et al*., 2007; Basso *et al*., 2008; Barreto *et al*., 2010). Until now, studies of enteric parasites in Brazil have been limited, isolated and fairly rare, generally reflecting the situation in small towns. Due the enormous expanse of territory of Brazil, prevalence of enteric parasites varies between regions and contrasting data are observed: 42% was reported in São Paulo (southeast) (Castro *et al*., 2015); 73.5% in Mato Grosso do Sul (midwest) (Aguiar *et al*., 2007); 75.3% in Paraná (south) (Buschini *et al*., 2007); and 77.2% in Bahia (northeast) (Mariano *et al*., 2015). Previously studies conducted in Rio de Janeiro showed intestinal parasites prevalence ranging from 18.3% to 66% (Santos *et al*., 1984; Macedo and Rey, 1996; Costa-Macedo *et al*., 1998; Uchôa *et al*., 2001; Carvalho-Costa *et al*., 2007; Volotão *et al*., 2007).

1.3. *Giardia* spp.

1.3.1. History of nomenclature

Giardia was first observed and described by Antonie van Leeuwenhoeck in 1681 in his own diarrhoeal stool under the microscopy (Dobell, 1920), but only in 1859 the genus was described in greater detail by Vilem Lambl, who named it *Cercomonas intestinalis* (Lambl, 1859). In 1888 the name *Lamblia intestinalis* was given to the species by Blanchard in commemoration of the first accurate description of the parasite by Lambl (Meyer and Jarrol, 1980). However, six years earlier the generic name *Giardia* was established by Kunstler (1882) for a flagellate found in the intestine of tadpoles, being the first time that *Giardia* was used as a genus. Only in 1914, Alexeieff pointed out the error and synonymized *Lamblia* Blanchard, 1888 and *Giardia* Kunstler, 1882, which was accepted by the majority of early workers.

Giardia is one of the first protozoans to be described. In spite of its early discovery, only in the late 1970s that *Giardia* became universally accepted as a human pathogen once the majority of infections were non-invasive and frequently asymptomatic. In 1978, Kulda and Nohynková concluded that this parasite causes disease in humans based on symptoms such as malabsorption and pathology observed in the upper part of the small intestine in patients to which the organism was isolate. The finding of severe giardiasis in patients with hypogammaglobulinemia, the occurrence of waterborne outbreaks of diarrhoea, and the

fulfilment of Koch's postulates in experimental human infections have definitively confirmed its association with human disease (Nash *et al*., 1987).

1.3.2. Taxonomy

Giardia is a unicellular eukaryotic parasite belonging to the kingdom Protista, subkingdom Protozoa, phylum Sarcomastigophora, subphylum Mastigophora, class Zoomastigophora, order Diplomonadida, family Hexamitidae, which can infect a variety of vertebrate hosts (Levine *et al*., 1980).

A recent classification establishes the Protozoa as the basal eukaryotic kingdom and recognizes 11 phyla. According to the new systematic, based on genetic, structural and biochemical data, *Giardia* belongs to infrakingdom Excavata, Phylum Metamonada, Subphylum Trichozoa, Superclass Eopharyngia, Class Trepomonadea, Subclass Diplozoa, Order Giardiida and Family Giardiidae (Cavalier-Smith, 2003).

In 2005, Adl and colleagues updated the classification of unicellular eukaryotes for the protozoa and they expanded it to include other protists, incorporating the results of ultra-structural researches and molecular phylogenetic studies. The authors recognize six clusters of eukaryotes: (*i*) Opisthokonta (animals, fungi, choanoflagellates, and Mesomycetozoa), (*ii*) Amoebozoa (most traditional amoebae, slime moulds, many testate amoebae, some amoebo-flagellates, and several species without mitochondria), (*iii*) Excavata (oxymonads, parabasalids, diplomonads, jakobids, and several other genera of heterotrophic flagellates, and possibly including the Euglenozoa and Heterolobosea), (*iv*) Rhizaria (Foraminifera, most of the traditional Radiolaria, and the Cercozoa with filose pseudopodia, such as many amoebo-flagellates and some testate amoebae), (*v*) Archaeplastida (Glaucophyta, red algae, green algae, and Plantae), (*vi*) Chromalveolata (Alveolata [ciliates, dinoflagellates, Apicomplexa], the Stramenopiles [brown algae, diatoms, many zoosporic fungi, and the opalinids amongst others], with the Haptophyta and Cryptophyceae). Therefore, based on these new proposal, the flagellated protozoan belonging to genus *Giardia* Kunstler 1882, were classified as:

Excavata Cavalier-Smith, 2002, emend. Simpson, 2003

- Fornicata Simpson, 2003
  - Eopharyngia Cavalier-Smith, 1993
    - Diplomonadida Wenyon, 1926, emend. Brugerolle *et al.*, 1975
      - Giardiinae Kulda and Nohynková, 1978

It is important to note that Adl *et al*. (2005) adopted a hierarchical system without formal rank designations, such as ‘‘class’’, ‘‘sub-class’’, ‘‘super-order’’, or ‘‘order’’.

Cavalier-Smith (2003) and Adl *et al*. (2005) updated the previous classification proposed by Levine *et al*. (1980) although the latter still official being used.

1.3.3. *Giardia* species

Initially, *Giardia* was divided into species on the basis of the host of origin. More than 50 species of *Giardia* were described, the majority between 1920 and 1930 (Kulda and Nohynková, 1996). However, it was recognised that the *Giardia* sp. obtained from different hosts were morphologically similar. The taxonomy of *Giardia* genus was based on morphology of trophozoites. Evaluated by light microscopy, trophozoites were differentiated primarily on the shape of median bodies, body shape and the size of the ventral adhesive disc relative to the length of the cell (Kulda and Nohynková, 1996; Adam, 2001). Using these criteria, Filice (1952) defined three species: *Giardia agilis* (long and slender), *Giardia muris* (short and nearly round), and *Giardia lamblia* (pear-shaped). Subsequently two species were described on the basis of ultra-structural features identified by scanning electron microscopy of ventrolateral flange, marginal groove, ventral disc and flagella characteristics: the *Giardia psittaci* (Erlandsen and Bemrick, 1987) and the *Giardia ardeae* (Erlandsen *et al*., 1990). An additional species, *Giardia microti*, has been proposed on the basis of cyst morphology (Feely, 1988) and on small-subunit ribosomal RNA (*ssu rRNA*) sequence analysis (van Keulen *et al*., 1998). Thereafter all species were defined using *ssu rRNA* gene sequencing. The use of DNA-based analysis solidified largely the taxonomy and organization of *Giardia* species. Nevertheless, the morphological uniformity of these isolates masks a considerable genetic diversity within the *G. lamblia* group (Monis *et al*., 1999; Thompson, 2004).

G. agilis is found in amphibians, *G. ardeae* and *G. psittaci* infect birds (herons and parakeets, respectively), *G. microti* and *G. muris* are observed in voles and muskrats (Adam, 2001; Plutzer *et al*., 2010). *G. lamblia* is the only species found in humans, although it is also detected in other domestic and wild mammals (Thompson, 2000). Another species, *Giardia varani*, has been described from a water monitor (*Varanus salvator*) (Upton and Zien, 1997). This parasite is similar in size and shape to *G. lamblia*, however, lacks median bodies and had binucleated cysts. Its identity has not been confirmed genetically.

The morphological characteristics of the six species of *Giardia* currently accepted by most researches, and their hosts are shown in Table 1.1.

Table 1.1. Species in the genus *Giardia* (Adapted from Thompson *et al*., 2000; Adam, 2001).

Species	Hosts	Morphological characteristics	Trophozoite dimensions (µm)	
			Lenght	Width
G. lamblia	Human Domestic mammals Wild mammals	Pear-shaped trophozoites with claw-shaped median bodies	12-15	6-8
G. agilis	Amphibians	Long, narrow trophozoites with club-shaped median bodies	20-30	4-5
G. psittaci	Birds	Pear-shaped trophozoites, with no ventrolateral flange. Claw-shaped median bodies	~14	~6
G. ardeae	Birds	Rounded trophozoites, with prominent notch in ventral disc and rudimentary caudal flagellum. Median bodies round-oval to claw shaped	~10	~6,5
G. muris	Rodents	Short and rounded trophozoites with small round median bodies	9-12	5-7
G. microti	Rodents	Trophozoites similar to *G. lamblia*. Mature cysts contain fully differentiated trophozoites.	12-15	6-8

1.4. *Giardia lamblia*

1.4.1. Morphology

G. lamblia has two major stages during their life cycle, trophozoite (Figure 1.1A) and cyst (Figure 1.1B). The vegetative trophozoite is the motile form and inhabits the small intestine of the host. Cyst is the environmental resistant stage and it is responsible for transmission of the parasite among susceptible hosts (Adam, 2001).

Trophozoites are pear-shaped, measure around 12 to 15 µm long and 5 to 9 µm wide (Figure 1.2A). Two nuclei nearly identical in appearance are seen and a delicate ventrolateral flange (VLF) surrounds the cell externally (Figure 1.2B). The cytoskeleton includes a median body, a ventral adhesive disk and four pairs of flagella (anterior, posterior, caudal and ventral) (Figures 1.2A - 1.2C) (Adam, 2001).

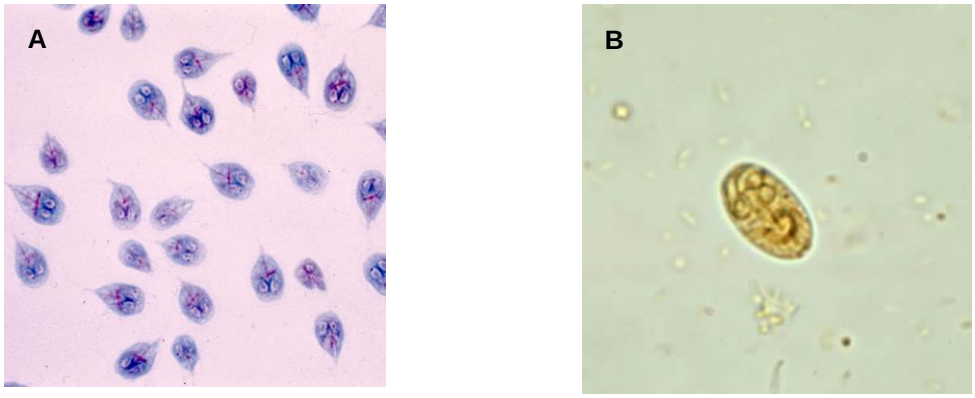

Figure 1.1. The two morphological forms of *Giardia lamblia*. (A): trophozoites and (B): cyst (Adapted from CDC, available from http://www.cdc.gov).

Giardia has a very simple intracellular organization. Common eukaryotic subcellular compartments such as mitochondria, peroxisomes and nucleoli are not found. Other organelles like an elaborated secretory system, composed of endoplasmic reticulum and Golgi complex are incipient. The Golgi apparatus was observed in encysting trophozoites but its presence was not confirmed in vegetative trophozoites, although it has been observed membranes suggestive of such complex. Lysosomal vacuoles, ribosomal and glycogen granules are found in the cytoplasm (Gillin *et al*., 1996; Soltys *et al*., 1996; Lanfredi-Rangel *et al*., 1999; Adam, 2001).

The *G. lamblia* trophozoite has two diploid nuclei that contain five chromosomes each. The nuclei have been shown to be equivalent in size and in the amount of DNA that they contain, and both nuclei are transcriptionally active. DNA replication is initiated almost simultaneously in each nucleus (Adam, 2001; Yu *et al*., 2002).

Median bodies (or parabasal bodies) are microtubular structures situated in the middle of the body, just below the ventral adhesive disk (Figure 1.2A). They are unique structures of the *Giardia* genus. No specific function has been ascribed for median bodies and its morphology varies according to the species of *Giardia* (Table 1.1) (Adam, 2001; Dawson, 2010).

The funis, another unique structure of *Giardia*, made up of sheets of microtubules following the axonemes of the caudal flagella has no known function, yet has been suggested to have either a structural role in maintaining the giardial cell shape or a potential role in the flexion of the posterior ‘tail’ region during detachment (Benchimol *et al*., 2004).

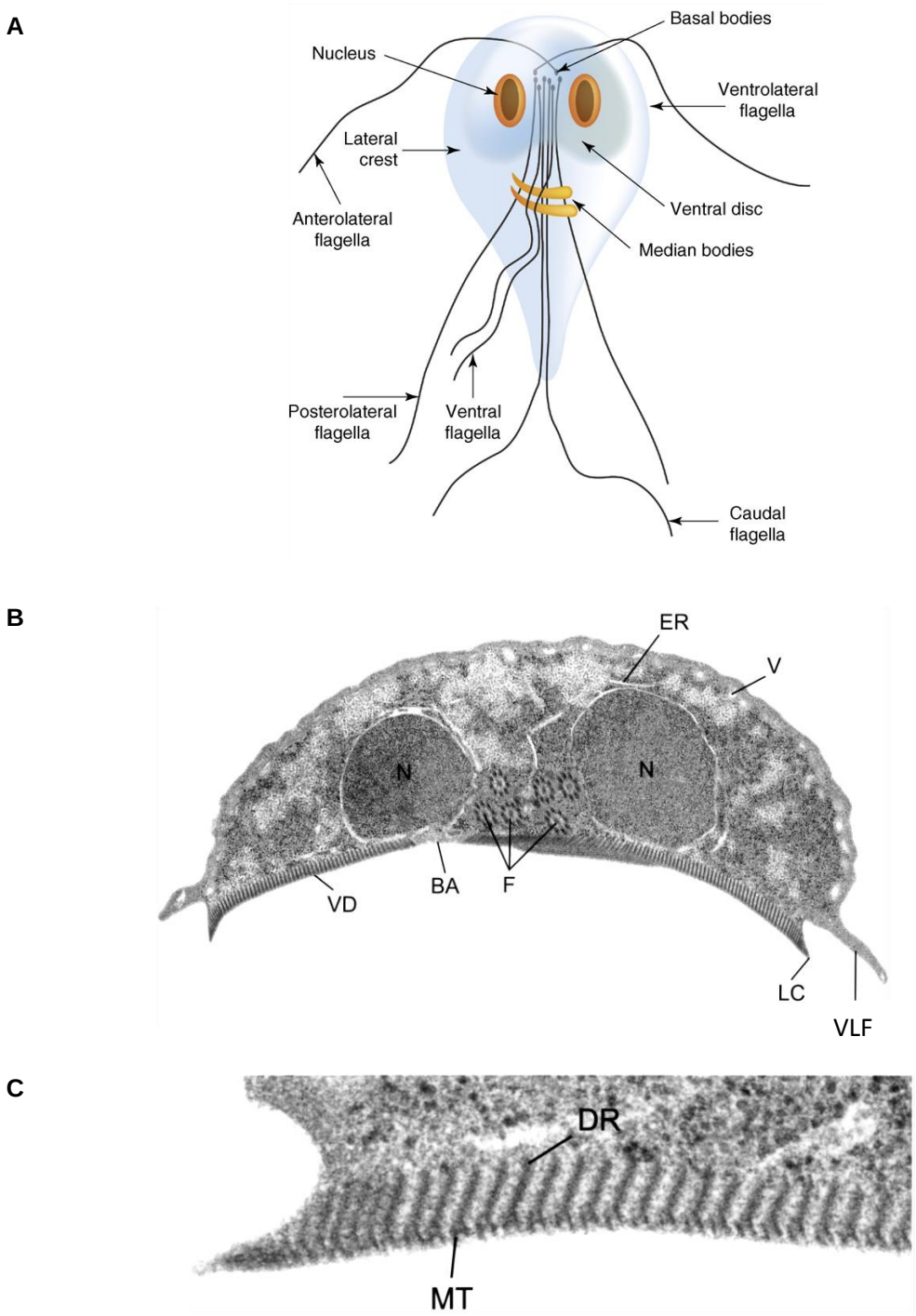

Figure 1.2. (A) Diagram of *Giardia lamblia* trophozoite; (B) Trophozoite coronal section by transmission electron microscopy (TEM). A coronal view of a trophozoite demonstrates the nuclei (N), endoplasmatic reticulum (ER), flagella (F), and vacuoles (V). A mechanical suction is formed when the ventral disk (VD) attaches to an intestinal or glass surface. Components of the ventral disk include the bare area (BA), lateral crest (LC), and ventrolateral flange (VLF); (C) Close-up of the ventral disk by TEM. A magnified view of the ventral disk shows the microtubules (MT) and microribbons or dorsal ribbons (DR) (Adapted from Adam, 2001; Monis *et al*., 2009).

Flagella originate from basal bodies that are close to the nucleus and emerge from the anterior, posterior, ventral and caudal regions of the trophozoite (Figure 1.2A). Flagella appear to be important for motility (for positioning and orienting trophozoites) but not for attachment (Adam, 2001; House *et al*., 2011). This role seems to be played by ventral adhesive disk, which has a crucial role in parasite survival, allowing colonization and resistance to peristalsis. The ventral adhesive disk is also an exclusive structure of the *Giardia* genus, appears as a concave structure and covering most of the anterior ventral surface of the parasite (Figures 1.2A and 1.2B). Contractile proteins are present in ventral disc such as actinin, α-actinin, myosin and tropomyosin for the contraction of the disk involved in adherence (Adam, 2001; House *et al*., 2011).

The cyst has an ellipsoid or oval shape, measure about 8 to 12 μm long and 7 to 10 μm wide, is covered by a cyst wall (0.3 to 0.5 μm thick) and the internal structures are duplicated in relation to the trophozoite. The wall is mainly composed of *N*-acetylgalactosamine and three different cyst wall proteins (CWP1, CWP2 and CWP3). Furthermore, the ventral adhesive disk is disassembled and flagella become intracytoplasmic (Ankarklev *et al*., 2010). Metabolism also decreases as cells round up and enters dormancy (Adam, 2001). It can be found in the faeces and may survive in the environment for three months at 4°C in water (Faubert, 2000).

1.4.2. Life cycle

Trophozoites colonize the small intestine of their hosts, especially the duodenum and the first portion of the jejunum, being responsible for illness called giardiasis. The trophozoites can be free or attached to the intestinal mucosa by ventral sucking disk, where they obtain the necessary nutrients for their survival and avoid transport beyond the jejunum (Adam, 2001).

The cyst formation, also called encystment, occurs in the passage of the parasite, still in its trophozoite form, to the colon. They are eliminated in the stool and they are the infective stage of *Giardia*, being responsible for transmitting the parasite. Trophozoites and cysts can be found in stool samples usually five to seven days after the initial infection. Trophozoites can be found in liquid stools, however in non-diarrhoeal faeces there is a predominance of cysts (Adam, 2001). To adapt to both environments, internal (digestive system of their hosts) and external (environment), the parasite undergoes a significant change during its life cycle.

Giardia infection is initiated when the vertebrate host ingests the cysts present in contaminated water or, less commonly, food or through direct faecal-oral contact (Figure 1.3)

(Thompson, 2004). After exposure to the acidic environment of the stomach, the cysts excyst into excyzoite in the upper small intestine, where the conditions for survival and proliferation are optimal. Flagella first appear through an opening in one of the poles of the cyst, followed by the excyzoite body. The short-lived excyzoite is oval, has eight flagella and a metabolism intermediate between a trophozoite and a cyst. The excyzoite has four nuclei with a total ploidy of 16N and undergoes cell division twice without DNA replication, generating four disease-causing trophozoites with a ploidy of 4N (Ankarklev *et al*., 2010). During this division process, the excyzoite increases its metabolism and gene expression, segregates organelles, upregulates proteins associated with motility and assembles the adhesive disc. Trophozoites replicate asexually by longitudinal binary division and initiate infection. Each new parasite repeats the process, and at the height of *Giardia* infections, millions of trophozoites colonize the intestine, exceeding 10^6 trophozoites per centimetre of gut (Adam, 2001; Ankarklev *et al*., 2010).

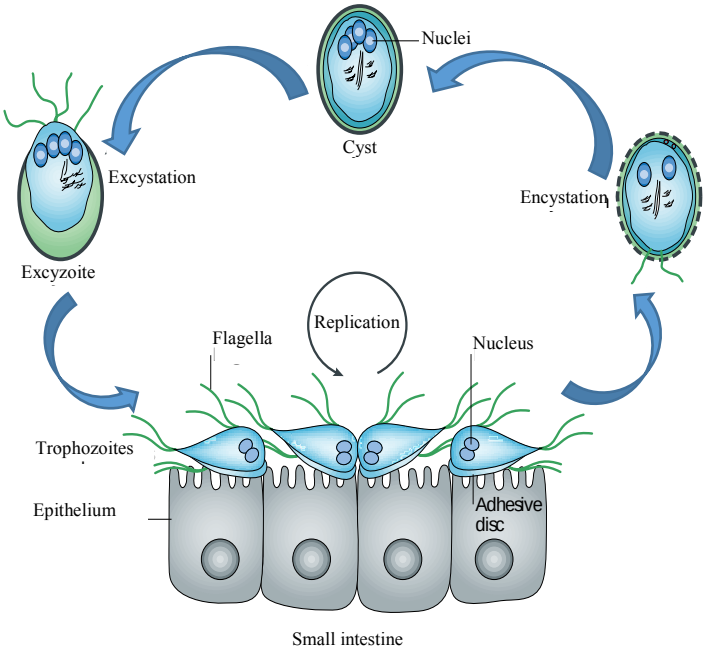

Figure 1.3. Life cycle of *Giardia* sp. Cysts are ingested through contaminated water and food or faecal-oral transmission. Cysts are exposed to gastric acid during their passage through the host`s stomach, and in the upper small intestine occurs excystation. Cysts differentiate into trophozoites via the short-lived excyzoite stage. The excyzoite is different from the trophozoite in that it has not yet assembled the adhesive disc and it contains four tetraploid nuclei. The excyzoite divides twice, without DNA replication between the divisions, giving rise to four trophozoites containing two diploid nuclei each. The parasite replicate asexually by longitudinal binary fission, with a generation time of 6-12 hours *in vitro*. Trophozoites start to encyst when they migrate to the lower part of the small intestine (Adapted from Ankarklev *et al*., 2010).

After the exposure to biliary fluid some of the trophozoites form cysts in the jejunum. This process is induced in response to host-specific factors such as high levels of bile, low levels of cholesterol and a basic pH. Initially flagella start to be internalized, the adhesive disc disassembles into four crescent-shaped structures that are kept in the cytoplasm, and after the cytoplasm condenses there is production of a cyst wall (Adam, 2001). The newly formed cysts have two nuclei, but then there are nuclear division inside the cyst and four nuclei are formed. Subsequently, the ventral adhesive disc, basal bodies, median bodies and the locomotor apparatus (flagella) are duplicated. The cysts are released through the faeces intermittently, allowing completion of the transmission cycle by infecting a new host. Giardiasis patient can shed 1 x 10^8 viable cysts per gram of faecal material (Adam, 2001).

1.4.3. Pathogenesis and symptomatology

Symptomatology of *G. lamblia* infection is highly variable between individuals and can range from asymptomatic (60-80% of the cases), mild and self-limited, to severe infections with acute or chronic diarrhoea. Diarrhoea is the major symptom of giardiasis, and can occur with or without the intestinal malabsorption syndrome. Clinical signs of infection may include vomiting, dehydration, abdominal pain, flatulence usually accompanied by nausea and weight loss. There is no appearance of blood in the stool since *Giardia* is a non-invasive parasite and few virulence factors have been identified (Eckmann, 2003; Cacciò and Ryan, 2008). However, infected individuals may also develop extra-intestinal and post-infectious gastrointestinal complications. Ocular complications, arthritis, allergies, myopathy, chronic fatigue and irritable bowel syndrome (IBS) can follow an episode of giardiasis, and the mechanisms remain unknown (Wensaas *et al*., 2012; Halliez and Buret, 2013).

The clinical manifestations are self-limiting in most of the cases, with transient intestinal complications that are usually solved completely, but because of the potential for chronic or intermittent symptoms, treatment is recommended (Eckmann, 2003; Robertson *et al*., 2010). Asymptomatic individuals are important reservoirs for spread of the parasite.

The giardiasis incubation period is one to three weeks after the patient ingests the cyst, and symptoms usually occur six to fifteen days after infection (Flanagan, 1992; Eckmann, 2003). Children are more vulnerable to giardiasis than adults, and may have more serious consequences. *Giardia* infection has an adverse impact on child growth and psychomotor development, and associated with diarrhoea and malabsorption syndrome can cause iron-deficiency anaemia, micronutrient deficiencies and growth retardation (Koruk *et al*., 2010). In

Brazil, the child population has been the group most affected by the high incidence of parasitic infections (de Carvalho *et al*., 2006; Barreto *et al*., 2010; Santos *et al*., 2012).

It still unclear why some individuals develop clinical manifestations while others remain asymptomatic, but there is no single and simple explanation for this broad spectrum of symptoms observed in infections caused by *G. lamblia*. Host and parasite factors may be responsible for the severity of the infection: host factors such as age, immune and nutritional status, as well as previous infection with other *Giardia* strain and/or concurrent enteric infections with other intestinal pathogens; parasite factors, probably associated to the assemblage, virulence, pathogenicity, number of ingested cysts, replication rate, presence of a particular variant surface proteins (VSPs), and the ability to evade the host immune system (Thompson, 2004; Cacciò and Ryan, 2008).

Giardia infections cause intestinal barrier dysfunction via a variety of mechanisms, including increased rates of intestinal epithelial apoptosis (Figure 1.4A) and disruption of apical junctional complexes (Figure 1.4B). The pathogenesis may be linked to factors such as: (*i*) the large amount of trophozoites attached to the intestinal epithelium being a physical barrier to absorption of nutrients; (*ii*) intestinal barrier disfunction; (*iii*) activation of the host lymphocytes; (*iv*) shortening of brush border microvilli with or without coinciding villous atrophy; (*v*) disaccharide deficiencies; (*vi*) anion hypersecretion and (*vii*) increased intestinal transit rates. The association of all these factors trigger a series of events that culminate with diarrhoea (Figure 1.4C) (Chin *et al*., 2002; Cotton *et al*., 2011; Bhargava *et al*., 2015).

The tight attachment between *G. lamblia* trophozoites and intestinal epithelial cells through its adhesive disk reduce the small intestinal absorptive surface area causing disaccharidase deficiencies and malabsorption of nutrients (fat-soluble vitamins, fatty acids, B12 vitamin and folic acid), water, and electrolytes. The increased quantity of these nutrients in the lumen of the intestine can determine steatorrhea (Ortega-Pierres *et al*., 2009; Cotton *et al*., 2011).

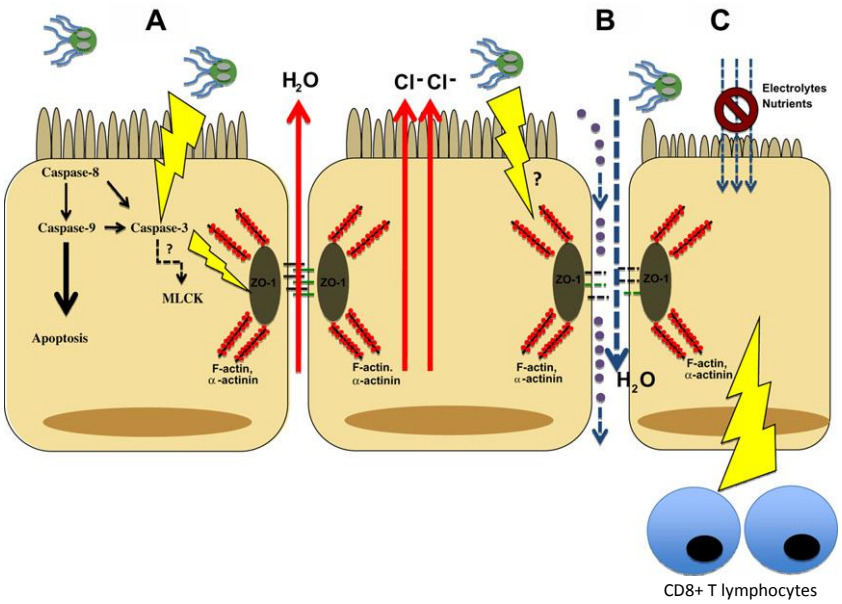

Figure 1.4. Mechanisms involved in the pathophysiology of giardiasis. (A): Heightened rates of epithelial cell apoptosis thought to occur via activation of Caspases-9 and 3. Parasitic factors activate hyper-secretion of chloride, which may contribute a secretory component to diarrhoea. (B): *Giardia* increases intestinal permeability by disrupting apical junctional complex components (including F-actin, ZO-1, claudin-1, and α-actinin) in a Caspase-3 dependent manner and via activation of myosin light chain kinase (MLCK). The increased permeability allows the passive diffusion of luminal antigens into the sub-epithelial compartment. (C): All these gathered events lead to a shortening of the brush border microvilli, an effect mediated by host CD8+ T lymphocytes. Consequently, total absorptive surface area of the small intestine is reduced during giardiasis, resulting in impairment of the production of multiple digestive enzymes and contributing to maldigestion commonly associated with these conditions. Additionally, microvillus injury impairs glucose and electrolytes absorption, resulting in decreased water uptake and eventual malabsorptive diarrhoea (Adapted from Cotton *et al*., 2011).

The parasite establishes a complex and dynamic interaction with host enterocytes. Cellular F actin, tight junctional Zonula–Occludens (ZO)-1, alpha-actinin and claudin protein (all of them are critical components of the sealing properties of tight junctions) disruption is modulated by a pro-apoptotic caspase 3 and a myosin-light-chain kinase (MLCK) (Figure 1.4B). *Giardia* products activate MLCK, which phosphorylates myosin light chain (MLC) and disrupts cytoskeletal and apical tight junctional elements in enterocytes, and subsequently increase permeability across the epithelial monolayers. Therefore, the intestinal barrier disruption may indeed facilitate the translocation of luminal antigens into underlying host tissue. In addition, increased enterocyte apoptosis in a caspase-3 and -9 dependent manner also modulate the permeability of the intestinal barrier (Figure 1.4A) (Chin *et al*., 2002; Cotton *et al*., 2011; Bhargava *et al*., 2015).

The diffuse shortening of epithelial brush border microvilli also reduce the total intestinal absorptive surface (Figure 1.4C). This process, as well as the microvillar disaccharidase deficiencies, is mediated by activated of CD8+ T lymphocytes of the host via parasite secretory/excretory products. These data suggest that the induction of the diffuse shortening of host brush border microvilli by the parasite may cause diarrhoeal disease via malabsorption and maldigestion (Cotton *et al*., 2011).

The malabsorption of nutrients and electrolytes, due parasite attachment or shortening of the brush border microvilli, creates an osmotic gradient that leads the water into the intestinal lumen resulting small intestine distension and increased peristalsis. It is also possible that the diarrhoea disease in infected individuals may be a result from increased intestinal transit rate due to massive mast cell degranulation and adaptive immune responses of the host. Finally, the impairment in the transport of electrolytes caused by *Giardia* leads to increased chloride hypersecretion that, with malabsorption of glucose, sodium and water may be responsible for the luminal fluid accumulation during infection (Ortega-Pierres *et al*., 2009; Cotton *et al*., 2011).

As aforementioned, the pathophysiological mechanisms of *Giardia* infection are clearly multi-factorial, and involve host and parasite factors, as well as immunological and non-immunological mucosal processes.

Every three to five days host intestinal cells are constantly being renewed, in that way *G. lamblia* must move and re-attach frequently to avoid the transport beyond the jejunum by the peristalsis. Likewise, the mucus layer prevents the parasite from obtaining immediate access to the epithelium (Figure 1.5). Trapping of *Giardia* by mucus and their subsequent removal from the intestine by peristalsis is a good strategy by host to reduce the quantity of parasites. The enteric bacterial flora, the normal small-intestinal microbiota, may be involved in the inhibition of *Giardia* growth. Intestinal Paneth cell-derived, defensins, lactoferrin, proteases, lipases and bile salts kill most other microbes in the upper small intestine and possess anti-giardial activity *in vitro*. In addition, reactive oxygen species (ROS) and nitric oxide (NO) have both cytotoxic and immunomodulatory activities during intestinal infections (Muller and Allmen, 2005; Roxstrom-Lindquist *et al*., 2006).

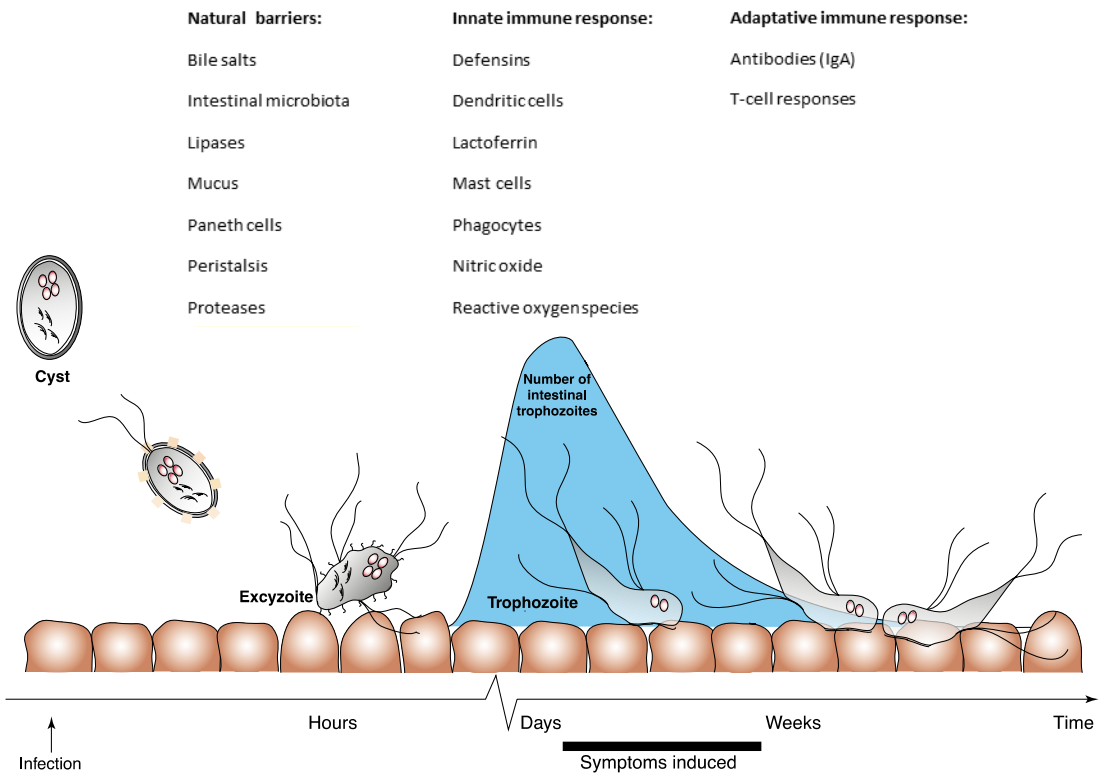

Figure 1.5. *Giardia lamblia* interaction with the human intestine. *G. lamblia* inhabits the upper part of the small intestine after the host ingests cysts. The excyzoites emerge from the cysts and quickly differentiates into trophozoites that replicate in the intestinal lumen in the duodenum. At a certain intestinal concentration of trophozoites, symptoms are induced, usually six to 15 days after infection. In addition to being a physical barrier, the intestinal epithelium has a central role in innate and adaptive mucosal immunity in response to external stimuli. The different parts of the intestinal immune system are shown in the order in which they interact with *Giardia* during infection. The number of trophozoites in the intestine during the infection is represented by the area in blue (Adapted from Roxstrom-Lindquist *et al*., 2006).

The host immune response plays a key role on the clinical form or asymptomatic infection, once the most severe cases affect children and immunosuppressed. The main elements of the immune response include: IgA immunoglobulins that interfere with parasite adhesion to the mucosa, immunoglobulin M (IgM) and immunoglobulin G (IgG) performing cellular immune response; macrophage activity, involved in phagocytosis and antigen presentation; the activity of monocytes and neutrophils (Faubert, 2000; Eckmann, 2003).

A higher incidence of giardiasis has been observed in patients with hypogammaglobulinemia and impaired secretion of IgA. The parasite causes a greater damage on the microvilli of these patients than in immunocompetent patients (Char *et al*., 1993; Faubert, 2000).

1.4.4. Evasion mechanisms

Despite the wide spectrum of clinical symptoms, *Giardia* infection in human is typically characterized by little or no mucosal inflammation (Cotton *et al*., 2011). Previous works have shown that *Giardia* can actively down-regulate inflammatory signals in epithelial and immune cells in order to be able to survive and colonize small intestine (Roxstrom Lindquist *et al*., 2006; Kamda and Singer, 2009). Surprisingly, microarrays analysis demonstrated that epithelial cells, when exposed to parasites *in vitro*, produce cytokines that are chemotactic for immune cells being therefore expected an increase in inflammatory status. The authors suggested that *Giardia* parasites may actively subvert/limit the inflammatory response in small intestine allowing its effective colonization (Roxstrom-Lindquist *et al*., 2005; Roxstrom Lindquist *et al*., 2006).

For instance, *Giardia* trophozoites were shown to evade host immune responses by inhibiting NO production in human intestinal epithelium cells (IEC) (Eckmann *et al*., 2000). The L-arginine is the primary substrate for the production of NO by the enzyme nitric oxide synthase (NOS), however *G. lamblia* trophozoites are able to degrade the amino acid as an energy source. The parasite rapidly reduces the amount of arginine in the growth medium decreasing the proliferation of IECs (Stadelmann *et al*., 2012). NO is an immunoregulatory substance of the immune system, inhibiting both growth and encystation of *G. lamblia* trophozoites as well as excystation of cysts *in vitro*, and is responsible for the cytotoxicity of the macrophages (Eckmann *et al*., 2000).

It is well documented that *G. lamblia* secretes enzymes of the arginine metabolic pathway (arginine deiminase [ADI] and ornithine carbamoyltransferase [OCT]), upon interaction with intestinal epithelial cells (Ringqvist *et al*., 2008; Cotton *et al*., 2015) (Figure 1.6). These two enzymes are able to metabolize arginine to produce adenosine triphosphate (ATP) thereafter decreasing the amount of arginine. Moreover, it was proposed that *G. lamblia* trophozoites produce a flavohemoglobin protein (flavoHb) capable of degrading NO (Mastronicola *et al*., 2010; Cotton *et al*., 2015) and attenuating T-cell proliferation (Stadelmann *et al*., 2013) (Figure 1.6). This protein has a well-known NO reductase activity and degrades NO to nitrate, protecting the parasite from nitrosative stress (Mastronicola *et al*., 2010). So, dendritic cell cytokine production (Banik *et al*., 2013) and T cell proliferation is affected due to reduced arginine-availability (Stadelmann *et al*., 2013).

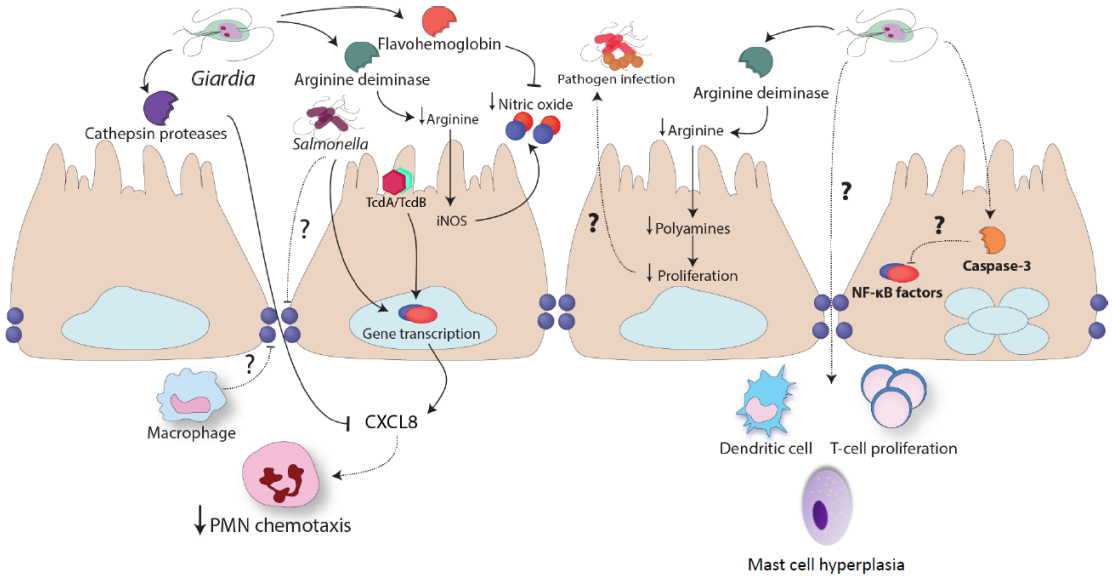

Figure 1.6. Immunomodulation by *Giardia* sp.. *Giardia* infections have been shown to attenuate granulocyte infiltration *in vivo* following intra-rectal instillation of *Clostridium difficile* toxin A and B (TcdA/TcdB). *Giardia* trophozoites release cathepsin cysteine proteases that attenuate PMN chemotaxis. Arginine deiminase (ADI) released by the parasite consumes L-arginine and this results in attenuated nitric oxide (NO) production. Furthermore, flavohemoglobins released by the parasite decrease the levels of NO. *Giardia* arginine deiminase also decreases intestinal epithelial proliferation, and this may affect the ability of other pathogens to colonize the intestinal tract. Similarly, during trophozoite-induced intestinal epithelial apoptosis, the activation of caspase proteins may cleave pro-inflammatory transcription factors. Multiple reports have shown that *Giardia* trophozoites modulate dendritic cell and helper T cell function, and cause mast cell hyperplasia (Adapted from Cotton *et al*., 2015).

Regarding direct interaction with immune cells, *Giardia* parasites inhibit the production of pro-inflammatory IL-12 in dendritic cells, resulting in an immune response able to control the infection but devoid of strong inflammatory signals (Kamda and Singer, 2009). Recently, a study demonstrated that *G. lamblia* trophozoites cathepsin B (catB) cysteine proteases degraded chemoattractant interleukin-8 (CXCL8) induced by pro-inflammatory interleukin-1β, or by *Salmonella enterica* serovar *Typhimurium*, and attenuated CXCL8-induced neutrophil/polymorphonuclear leukocyte (PMN) chemotaxis (Cotton *et al*., 2014a; Cotton *et al*., 2015) (Figure 1.6). In the same year, it was reported that *G. lamblia* infection decreased granulocyte tissue infiltration and cytokines and chemokines involved in PMN recruitment after intra-rectal instillation of *Clostridium difficile* toxin A and B in an isolate-dependent manner (Cotton *et al*., 2014b; Cotton *et al*., 2015) (Figure 1.6). Both studies demonstrate that *Giardia* infections may attenuate PMN accumulation by decreasing the expression of the mediators responsible for their recruitment.

Our group observed that *G. lamblia* does not trigger macrophages activation via canonical pro-inflammatory signaling cascades such as mitogen-activated protein kinases (MAPKs) and transcription nuclear factor-κB (NF-κB), being this reflected by minimal induction of inducible nitric oxide synthase (iNOS), cyclooxygenase-2 (COX-2) and of cytokines/chemokines such as IL-1β, IL-6, tumor necrosis factor alpha (TNF-α) and chemokine (C-C motif) ligand 4 (CCL4) (Faria *et al*., 2014).

Another important mechanism for evasion of the host immune response is the antigenic variation, a clonal phenotypic variation which the parasite expressed successively alternative forms of their surface antigens without genotype changes. Antigenic variation in *G. lamblia* involves variant surface proteins (VSPs) that are cysteine-rich integral membrane proteins. These proteins cover the entire surface of the parasite and conferred protection against IgA and proteases produced by the host (Ankarklev *et al*., 2010).

Modulation of the host immune response will benefit the parasite by extending the length of infection and allowing greater time for transmission to a new host. Unlike most intestinal pathogens, *Giardia* induces diarrhoea without necessarily causing significant infiltration of neutrophils or macrophages. As aforementioned, the recent findings indicate that *Giardia* actively modulates host inflammatory responses.

1.4.5. Genetic diversity

Studies based on molecular analysis have shown that *G. lamblia* is considered a species complex, whose members show little variation in their morphology but have a remarkable genetic variability (Thompson, 2004; Cacciò and Ryan, 2008). Due to its invariant morphology, investigations of aspects such as host specificity and transmission patterns require the direct genetic characterisation of parasites from faecal samples.

In the last years, genetic characterisation has been extensively used to evaluate the genetic variability of *G. lamblia*. The vast majority of studies have relied on the analysis of conserved genes loci like small subunit ribosomal RNA (*ssu rRNA*) and a variety of housekeeping genes, including β-giardin (*bg*), elongation factor 1 alpha (*ef*-1α), open reading frame C4 (*orfC*4), NADPH-dependent glutamate dehydrogenase (*gdh*) and triose phosphate isomerase (*tim* or *tpi*) (Thompson, 2004; Cacciò and Ryan, 2008).

In previous studies based on the hybridization of chromosomes separated by pulse field gel electrophoresis, the *tpi* gene was mapped to chromosome 5, the *gdh* and *bg* have been mapped to chromosome 4, and most of the *ssu rRNA* gene copies were mapped to chromosome 1 (Adam, 2000). These genes are used in accordance with the purpose of each

study, once they differ widely in terms of genetic variability and in sensitivity. The *ssu rRNA* and *ef*-1α genes are strongly conserved and usually used to distinguish *Giardia* species and assemblages, but are of little use for studies where genetic variation within assemblages needs to be determined. *Tpi* and *gdh* genes were the more variable, followed by *bg* and *orf*C4 genes, thus highly discriminatory genes. Commonly the latter loci were used to distinguish *G. lamblia* assemblages and sub-assemblages and to infer their phylogenetic relationship (Monis *et al*., 1999; Wielinga and Thompson, 2007; Thompson and Ash, 2016).

The *ssu rRNA* has been the most useful gene for molecular comparisons, because rRNA sequences are highly conserved across life and because the function of the rRNA is central to the biology of the organism. Therefore, most widely accepted in classification scheme and due to the multicopy nature, the Polymerase Chain Reaction (PCR) that targets this locus has a high sensitivity (Adam, 2001).

Giardins were first described and isolated in 1985 (Crossley and Holberton, 1985; Peattie *et al*., 1989) and were defined as a family of 30 kDa structural proteins found at the edges of microribbons (or dorsal ribbons), which are an integral part of the ventral disk of the trophozoite (Figure 1.2C) (Faubert, 2000; Adam, 2001). Three classes of giardins have been characterized: α, β, and γ. They are unique proteins of *G. lamblia* cells and important determinant in pathogenicity once the ventral disk allows the parasite to tightly adhere to the surface of epithelial cells in the vertebrate duodenum (Holberton *et al*., 1988; Aggarwal *et al*., 1989; Alonso and Peattie, 1992; Nohria *et al*., 1992).

The proteins tpi and gdh are important metabolically enzymes of *G. lamblia*. Tpi is an efficient catalyst that is absolutely essential for efficient energy production through glycolysis, catalysing a reversible conversion between dihydroxyacetone phosphate and D-glyceraldehyde-3-phosphate (Mowatt *et al*., 1994; Adam, 2001), while gdh play an important role in carbohydrate metabolism and ammonia assimilation, amino acid synthesis, and/or catabolism. The gdh enzyme catalyses the interconversion between α-ketoglutarate and L-glutamate and utilize either nicotinamide adenine dinucleotide phosphate ($NADP^+$) or nicotinamide adenine dinucleotide (NAD^+) as coenzyme (Yee and Denis, 1992).

Elongation factor are a set of proteins involved in the regulation of the rate of transcription elongations. Ef-1α plays a central role for the protein synthesis within eukaryotic cells, being responsible for aminoacyl-tRNA loading onto the A site of the ribosome, thereby regulating the fidelity and rate of polypeptide elongation during translation (Hashimoto *et al*., 1994; Condeelis, 1995).

The orfC4 protein was initially described as specific to *G. lamblia* assemblage B (Nash and Mowatt, 1992), but further two studies pointed out the error and demonstrated that it is expressed in the other assemblages (Nores *et al*., 2000; Yong *et al*., 2002). The gene is considered to be specific of *G. lamblia. Orf*C4 encodes a cytoplasmic 22 kDa protein that seems to interfere in the differentiation of trophozoites and cysts. Besides, contains a region structurally similar to the α-crystallin domain of small heat-shock proteins that indicates the potential role of *orf*C4 as a small chaperone involved in the response to stress, including encystation (Yong *et al*., 2002; Nores *et al*., 2009).

The use of targets that are considered to be unique to *G. lamblia* is an advantage for the molecular detection of the parasite. In the past few years, several studies using multiple genes in various combinations allowed the identification of intra-specific genetic variation (Wielinga and Thompson, 2007; Adam *et al*., 2013; Thompson and Ash, 2016).

Studies adopting genetic and phenotypic criteria demonstrated that *G. lamblia* isolates from humans and other mammalian species belong to one of the two major genetic groups or assemblages. Distributed worldwide, these two groups were also described in North America as Groups 1/2 and 3 groups (Nash *et al*., 1995), in Europe as "Polish" and "Belgian" (Homan *et al*., 1992) and Australia as assemblages A and B (Mayrhofer *et al*., 1995; Monis *et al*., 1996). The comparative analysis of these different denominations groups showed that they were genetically equivalent (Monis *et al*., 2009) (Table 1.2). Within the two groups, isolates have been reported to be heterogeneous as well (Homan *et al*., 1992; Mayrhofer *et al*., 1995; Monis *et al*., 1996).

Table 1.2. Pseudonyms of the two major *Giardia lamblia* genetic groups recovered from humans and various animals hosts[§] (Adapted from Thompson *et al*., 2000).

Geographical location	***G. lamblia* divisions**		**References**
North America	Groups 1/2	Group 3	Nash *et al*., 1995
Europe	Polish	Belgian	Homan *et al*., 1992
Australia	Assemblage A	Assemblage B	Mayrhofer *et al*., 1995
			Monis *et al*., 1996

[§] Pseudonyms in the same row are genetically equivalent.

Regarding the need to reach a consensus on the nomenclature for these groups, the term assemblage (or genotype) is the one that best reflects the fact that each major group contains a genetically diverse collection of isolates of *G. lamblia* that is not limited to any particular geographic location (Thompson *et al*., 2000). The term assemblage seems to have

greater acceptance among researchers, and for that reason was adopted in this work.

Actually *G. lamblia* is divided into at least eight distinct genetic assemblages (A-H), but only assemblages A and B are known to infect humans (Monis *et al*., 1999; Thompson, 2004; Cacciò and Sprong, 2010). The others are likely to be host specific, as assemblages C and D occur mostly in dogs and other canids, assemblage E in hoofed livestock, assemblage F in cats, assemblage G in rats and assemblage H in marine mammals (Table 1.3) (Monis *et al*., 1999; Thompson, 2004; Cacciò and Ryan, 2008; Cacciò and Sprong, 2010; Lasek-Nesselquist *et al*., 2010).

Table 1.3. Genetic classification of *Giardia lamblia* (Adapted from Monis *et al*., 1999; Thompson, 2004; Lasek-Nesselquist *et al.*, 2010).

Assemblage	Host range
A	Humans and others primates, livestock, cats, dogs and some species of wild animals
B	Humans and others primates, cats, dogs and some species of wild animals
C	Dogs and other canids
D	Dogs and other canids
E	Hoofed livestock
F	Cats
G	Rats
H	Marine mammals

There is an ongoing discussion as to whether at least some *G. lamblia* assemblages may represent distinct *Giardia* species. A taxonomy revision was proposed, recognizing some assemblages as separate species due to significant genetic diversity observed in *G. lamblia* and specificity to their hosts (Table 1.4). This suggestion seems to be more logical and may create less confusion with regard to the taxonomy of *Giardia* and a better understanding of the epidemiology of giardiasis. However, the existence of some controversial points, most of the them concerning the names to be given to assemblage H and the fact that the two distinct assemblages (C and D) that infect dogs will be grouped in a single species, *G. canis*, despite the rather large genetic variability observed at the genes investigated (Ryan and Cacciò, 2013). Actually, it is still under debate, whether presently available data are sufficient for sound species differentiation.

Table 1.4. The currently recognized assemblages of *Giardia lamblia* and the new taxonomy proposed by Monis and colleagues (2009).

Assemblage	Proposed species name
A	*G. duodenalis*
B	*G. enterica*
C	*G. canis*
D	*G. canis*
E	*G. bovis*
F	*G. cati*
G	*G. simondi*
H	*?*

The distribution of the assemblages A and B varies greatly from one country to another, although assemblage B seems to be more common overall (Haque *et al*., 2005; Singh *et al*, 2009; Ryan and Cacciò, 2013). A study conducted in Brazil reported that children with assemblage B shed significantly more cysts than children infected with assemblage A (Kohli *et al*., 2008). This coupled with faecal-oral transmission, may contribute to the higher prevalence and dispersion of assemblage B.

The development and use of molecular tools to characterize different *Giardia* isolates, and the knowledge of the spectrum of symptoms associated with giardiasis, has led to the hunt for associations between assemblage type and specific symptoms. While various reports from around the world correlated assemblage B infections with more severe symptomatology, others have correlated severe symptoms with assemblage A infections, but a definitive conclusion at the moment concerning the data were not obtained (Cacciò and Ryan, 2008; Robertson *et al*., 2010).

Several studies have shown a strong correlation between assemblage A and symptomatic infection, and between assemblage B and asymptomatic infection (Read *et al.*, 2002; Aydin *et al*., 2004; Haque *et al*., 2005; Sahagún *et al.*, 2008; Breathnach *et al*., 2010). In contrast, other studies have reported an association between assemblage B and symptomatic infections (Homan and Mank, 2001; Gelanew *et al*., 2007; Lebbad *et al*., 2011; Puebla *et al*., 2014). No correlation was observed between assemblages and symptoms in the studies conducted in Brazil (Kohli *et al*., 2008), Cuba (Pelayo *et al*., 2008) and Nicaragua (Lebbad *et al*., 2008).

Recently, it was demonstrated that *G. lamblia* assemblage A trophozoites attenuate secretion of the CXCL8 through a secreted catB cysteine protease, directly attenuating

CXCL8-induced PMNs chemotaxis (Cotton *et al*., 2014a). In contrast, these effects were not observed with *G. lamblia* assemblage B GS/M infections. Moreover, *Giardia* assemblage A decreased granulocyte infiltration and cytokines and chemokines involved in PMN recruitment (Cotton *et al*., 2014b). Certainly further studies are needed to show whether the different assemblages induce different immune responses and whether these differences are related to differences in symptoms.

Enzyme electrophoretic studies have shown that *G. lamblia* assemblage A is divided into two sub-assemblages: AI has been found in both humans and animals, and received a particular attention due its zoonotic potential; AII was commonly anthroponotic, although it has been reported, in a few studies, on animals. The host distribution of assemblage B, which is divided into two sub-assemblages, BIII and BIV, is predominantly found in humans and much less commonly in animals (Adam, 2001). Further, a new sub-assemblage referred to as "AIII" was described using DNA-based analysis (van der Giessen *et al*., 2006; Robertson *et al*., 2007; Cacciò *et al*., 2008), and found only in wild hoofed animals.

Molecular techniques for the direct typing of *G. lamblia* cysts were developed, and were useful for studying the genetic variability of the parasite. *G. lamblia* molecular characterization in human faecal samples using *bg*, *tpi* and *gdh* genes revealed that some sequences had between one and four single-nucleotide polymorphisms (SNPs) in relation to sequences previously described, raising the question of what are the appropriate criteria to describe new assemblages or sub-assemblages (Robertson *et al*., 2006). Incongruent genotyping results was also observed, one isolate was classified as B3 subtype at *bg* gene and B2 subtype at the *gdh* gene (Robertson *et al*., 2006). The lack of concordance in the assignment of isolates to a specific assemblage in different loci was attributed to the presence of genetically different cysts in the same sample, called mixed infection, or the allelic sequence heterozygosity (ASH). Some years earlier, similar results had already been detected, with 25% of the faecal isolates derived from cats and dogs genotyped differently at the *gdh* compared with *ssu rRNA*, that were explained by the possible presence of mixed assemblages, with one assemblage being preferentially amplified over another at one locus (Read *et al*., 2004).

As the amount of sequence information increased, incongruent genotyping results began to be reported and to reliably determine the distribution of assemblages across the population the analysis of a single locus was not sufficient (Cacciò *et al*., 2008; Sprong *et al*., 2009; Almeida *et al*., 2010; Huey *et al*., 2013). Consequently, the use of multilocus genotypes (MLGs) tools became imperative.

Based on MLG analysis of the four molecular markers most widely used for genotyping *G. lamblia* isolates (*ssu rRNA*, *bg*, *gdh*, and *tpi*), it was proposed a subtype nomenclature system for assemblage A isolates (Cacciò *et al*., 2008); 10 different MLGs could be identified (AI-1 and 2, AII- 1 to 7 and AIII-1) (Table 1.5). So, it was possible to define three sub-assemblages (AI, AII and AIII) for assemblage A, and six subtypes (A1 to A6) when the analysis was performed by each gene, with the exception of the *ssu rRNA* locus. The *ssu rRNA* gene demonstrated low intra-variability in both assemblages A and B (Cacciò *et al*., 2008). The presence of heterogeneous templates, that are overlapping nucleotide peaks at specific positions in the chromatograms, and SNPs make impossible to propose a precise nomenclature for the MLGs of assemblage B isolates (Cacciò *et al*., 2008; Sprong *et al*., 2009).

Table 1.5. Sub-assemblages, multilocus genotypes (MLGs) and subtypes in *Giardia lamblia* assemblage A isolates (Adapted from Cacciò *et al*., 2008).

Sub-assemblages	**MLG**	**Subtypes**		
		bg	***tpi***	***gdh***
AI	AI-1	A1	A1	A1
	AI-2	A5	A5	A5
AII	AII-1	A2	A2	A2
	AII-2	A3	A2	A3
	AII-3	A2	A2	A3
	AII-4	A3	A2	A4
	AII-5	A3	A1	A3
	AII-6	A3	A3	A3
	AII-7	A3	A4	A3
AIII	AIII-1	A6	A6	A6

Subtyping is based on similarity and a single point mutation has been considered sufficient to describe a new subtype (Cacciò and Ryan, 2008; Monis *et al*., 2009; Sprong *et al*., 2009).

The intra-isolate sequence heterogeneity that was observed in *G. lamblia*, especially in assemblage B, can be explained by two principal mechanisms: (*i*) allelic sequence heterozygosity (ASH) or (*ii*) mixed infections. *Giardia* has two diploid nuclei which may accumulate specific independently mutations and this could generate ASH, while mixed infections are the presence of genetically different cysts in the same sample (Cacciò and Ryan, 2008). Although the presence of more than one cyst on a single sample is the most

accepted explanation (Read *et al*., 2004; Robertson *et al*., 2006), it was reported the possibility to occur genetic recombination (Poxleitner *et al*., 2008; Birky, 2010).

The alleles of a gene in different nuclei are expected to accumulate different mutations, but surprisingly, the degree of heterozygosity is very low for a polyploidy asexually replicating organism like *G. lamblia* and the mechanism(s) responsible remained undetermined (Adam, 2001; Jerlström-Hultqvist *et al*., 2010). Indeed, in asexual eukaryotes, the two allelic gene copies at a locus are expected to become highly divergent as a result of the independent accumulation of mutations in the absence of segregation (Meselson's effect). Therefore, substantial genetic differences are expected to accumulate among the chromosome homologues in asexual organisms with a ploidy of two or higher, as it has been shown for bdelloid rotifers (Welch and Meselson, 2000). This suggests that there may be a biological mechanism for maintaining genome fidelity and reducing heterozygosity between the four genome copies.

According to previous studies, the assemblage A genome displays a very low level of ASH compared with assemblage B (less than 0.01% versus 0.5% in B) (Cacciò and Sprong, 2010; Ankarklev *et al*., 2012). The occurrence of ASH between the two nuclei of a single cyst and also between different cysts was demonstrated recently (Ankarklev *et al*., 2012), but the reasons for different ASH levels among assemblages A and B are not yet understood. The low level of allelic sequence divergence in assemblage A suggests that the parasite has some kind of control mechanism to reduce sequence divergence of the four alleles, whereas this mechanism is not as efficient in the assemblage B. The author suggested that assemblages A and B have different mechanisms in exchanging genetic material, or that assemblage B isolates can fuse and form a tetraploid cell that undergoes parasexual reduction to diploid or often aneuploid cells in a process similar to sexual process in *Candida albicans* and other pathogenic fungi (Forche *et al*., 2008). Aneuploidy has already been detected, presumably resulting from mitotic nondisjunction (Tumová *et al*., 2007).

The occurrence of ASH complicates the assignment of isolates to specific subtypes, especially for assemblage B, and is very difficult to distinguish between ASH and mixed infections. Another possible explanation for the presence of double peaks and the low level of ASH is the occurrence of meiotic recombination, which suggests the potential for sexual reproduction in *Giardia*, but the evidence of sex still elusive. Sexual reproduction has never been observed in *Giardia* spp. but might not be detectable if it is rare (Poxleitner *et al*., 2008; Birky, 2010; Cacciò and Sprong, 2010). Moreover, meiosis-associated proteins are present in

Giardia, although they may have alternative functions such as DNA damage repair (Ramesh *et al*., 2005).

In the last years it has been suggested that the level of genetic diversity between assemblage A and B parasites are sufficient to recognize them as different species of *Giardia*. The divergence in the level of allelic heterozygosity, coding capacity, phylogenetic distance and the degree of genomic diversity, as well as several biological differences were detected between WB strain (isolated from human patient infected in Afghanistan and classified as assemblage A) and GS strain (isolated from human patient infected in Alaska and classified as assemblage B) and/or assemblage A and B isolates (Jerlström-Hultqvist *et al*., 2010; Adam *et al*., 2013; Ankarklev *et al*., 2015). The two genomes have 77% nucleotide and 78% amino acid identity in protein-coding regions. The main differences were seen in the large gene families encoding VSPs, NEK kinases and high-cysteine membrane proteins (HCMPs). The VSP repertoires seem to be completely different in the two isolates and the rate of antigenic variation is much faster for GS, with an average of 6 generations, than for WB, with an average 13 of generations (Nash *et al*., 1990; Franzén *et al*., 2009). WB strain has a higher growth and it is more easily stably transfected by episomal plasmids than strain GS (Singer *et al*., 1998). Cytogenetic studies have shown that certain assemblages A and B differ in the number of chromosomes in each nucleus and pulsed field electrophoresis detected differences in chromosome sizes, suggesting that these differences were due to the recombination of regions of rDNA in the telomeres (Adam, 1992; Le Blancq *et al*., 1992). The WB strain can efficiently be encysted *in vitro* but efficient encystation of GS strain has not been possible in standard encystation medium (Luján *et al*., 1996).

The phenotypic definition of species has been widely used for eukaryotic microorganisms based on morphological characteristics, such as *G. lamblia*. The genetic distances that separate assemblages are very large and recent comparisons at the whole genome level have reinforced the evidence that assemblages A and B, the two assemblages for which genome sequences are currently available, represent distinct species (Franzén *et al*., 2009; Jerlström-Hultqvist *et al*., 2010; Adam *et al*., 2013). All results obtained to date support the revision of the taxonomy, suggesting that *G. lamblia* assemblages should be classified as different species (Table 1.4) (Monis *et al*., 2009). However, the information available so far is not sufficient to define them as separate species according to the concept used for species classification (Jerlström-Hultqvist *et al*., 2010).

1.4.6. Zoonotic potential

G. lamblia is a parasite commonly found in domestic animals such as dogs, cats and livestock, and in several species of wildlife. The role that animal infections may play in zoonotic transmission remains controversial. So far, it is not known how these cycles could interact with each other or what the role of zoonotic transmission in the epidemiology of infections by *Giardia* in humans (Thompson and Ash, 2016).

In order to better understand the zoonotic potential of *Giardia* infection in wild and domestic animals, it has been important to determine whether humans and other animals are susceptible to infection with genetically identical parasites. The application of molecular techniques to *Giardia* isolated from human and other mammalian hosts in different parts of the world has clearly demonstrated the occurrence of zoonotic species. It was found genetically similar isolates in human and non-human hosts such as dogs, cats, livestock and wildlife, in the same geographical areas (Traub *et al*., 2004; Lebbad *et al*, 2010; Johnston *et al*., 2010; Soares *et al*., 2011). Thus, supporting the potential for zoonotic transmission.

The first studies in human isolates identified sub-assemblage AII and assemblages B. Later, infections with sub-assemblage AI were observed. Subsequently, sub-assemblage AII and assemblage B were observed in animals (Table 1.6) (Sulaiman *et al*., 2003; Traub *et al*., 2004; Read *et al*., 2004; Lalle *et al*., 2005; Fayer *et al*., 2006; Eligio-García *et al*., 2008). The sequencing of phylogenetically informative genes has revealed transmission between humans, dogs (*Canis familiaris*) and cattle (*Bos taurus*) in Italy (Lalle *et al*., 2005) and among humans, cattle and mountain gorillas (*Gorilla beringei beringei*) in Uganda (Graczyk *et al*., 2002).

Table 1.6. Several *Giardia lamblia* assemblages A and B and sub-assemblages identified in fish, marine mammals, wildlife, farm and companion animals (Adapted from Ryan and Cacciò, 2013).

Host	Geographic location	Assemblage	References
Fish			
Fingerlings	Australia	A, AII, B (various sub-assemblages), E	Yang *et al.*, 2010
Freshwater fish	Australia, Africa	A, B	Yang *et al.*, 2010; Ghoneim *et al.*, 2012
Marine fish	USA, Australia, Africa	A, AII, B (various sub-assemblages)	Lasek-Nesselquist *et al.*, 2008; Lasek-Nesselquist *et al.*, 2010; Yang *et al.*, 2010; Ghoneim *et al.*, 2012
Marine mammals			
Seals	USA	AI, AII, B, C, D and H	Dixon *et al.*, 2008; Gaydos *et al.*, 2008; Lasek-Nesselquist *et al.*, 2010
Shark	USA	B	Lasek-Nesselquist *et al.*, 2008
Porpoises, dolphins	USA	A, AII, B	Lasek-Nesselquist *et al.*, 2008; Lasek-Nesselquist *et al.*, 2010
Wildlife			
Beaver	Canada, USA	A, B	Appelbee *et al.*, 2002; Sulaiman *et al.*, 2003; Fayer *et al.*, 2006
Birds	Australia, USA	A, AII, B	Lasek-Nesselquist *et al.*, 2008; Nolan *et al.*, 2011
Hoofed animals	Europe, USA	A, AI, AIII	Trout *et al.*, 2003; Lalle *et al.*, 2007; Cacciò *et al.*, 2007; Lebbad *et al.*, 2010; Solarczyk *et al.*, 2012
Non-human primates	Africa, Brazil, Canada, China, Europe, Japan	A, AI, AII, B	Graczyk *et al.*, 2002; Itagaki *et al.*, 2005; Levecke *et al.*, 2007; Levecke *et al.*, 2009; Cacciò *et al.*, 2008; Kutz *et al.*, 2008; Volotão *et al.*, 2008; Lebbad *et al.*, 2010; Ye *et al.*, 2012
Marsupials	Australia	A, AI, B	McCarthy *et al.*, 2008; Thompson *et al.*, 2008; Thompson *et al.*, 2010

Table 1.6 (*Continued*)

Rodents	Croatia, Europe, USA	A, B	Sulaiman *et al*., 2003; Beck *et al*., 2011; Levecke *et al*, 2011
Wild felids	Croatia	AI	Beck *et al*., 2011
Wild canids	Africa, Australia, Croatia, Europe	AI, AII, B (various sub-assemblages)	Hamnes *et al*.,2007; Thompson *et al*., 2009; Ash *et al*., 2010; Beck *et al*., 2011
Farm animals			
Cattle, pigs	Canada, Ethiopia, Europe	AI, AII, AIII, B	Lalle *et al*., 2005; Uehlinger *et al*., 2006; Geurden *et al*., 2008; Sprong *et al*., 2009; Minetti *et al*., 2014; Wegayehu *et al*., 2016
Chicken, ducks	Italy	A, B, A+B	Berrilli *et al*., 2012
Sheep, goats, pigs	China, Europe	A, AI, AII, B (various sub-assemblages)	Giangaspero *et al*., 2005; Geurden *et al*., 2008; Sprong *et al*., 2009; Lebbad *et al*., 2010; Minetti *et al*., 2014; Wang *et al*., 2016
Companion animals			
Cats	Brazil, China, Europe	AI, AII, AIII, B	van Keulen *et al*., 2002; Volotão *et al*., 2007; Cacciò *et al*., 2008; Sprong *et al*., 2009; Lebbad *et al*., 2010; Xu *et al*, 2016
Dogs	Brazil, Canada, China, Europe and Thailand	A, AI, AII, B	Volotão *et al*., 2007; Traub *et al*., 2009; Himsworth *et al*., 2010; McDowall *et al*., 2011; Lalle *et al*., 2005; Leonhard *et al*., 2007; Lebbad *et al*., 2010; Xu *et al*, 2016
Horses	Australia	AI, AII, B (various sub-assemblages)	Traub *et al*., 2005; Scorza *et al*., 2012
Rabbits	China	B (various sub-assemblages)	Sulaiman *et al*., 2003; Lebbad *et al*., 2010; Zhang *et al*. 2012
Ferrets	Japan	A1	Abe *et al*., 2005; Abe *et al*., 2010

A study based on MLGs in Sweden has detected the AI-1 profile in a sheep isolate (Lebbad *et al*., 2010) (Table 1.6), which has been previously found in human isolates (Cacciò *et al*., 2008). Besides, sub-assemblage AIII found only in wild hoofed animals was observed in a cat isolate (Lebbad *et al*., 2010) (Table 1.6). Two different assemblage A MLGs previously detected in cats and ruminants in Sweden (Lebbad *et al*., 2010) were also identified in some Swedish patients (Lebbad *et al*., 2011). In addition, a cross-sectional study conducted in Brazil suggested a probable transmission of sub-assemblage AI between cats, dogs and humans (Volotão *et al*., 2007). Although the World Health Organization (WHO) considered giardiasis to have a zoonotic potential for over 30 years (WHO, 1979), direct evidence has been lacking.

As mentioned in the previous section, the host distribution of assemblage B is predominantly human and less extensive to animals, while assemblage A is less restricted. This data suggests that humans are major source of assemblage B and that domestic animals play a major role in the host range of assemblage A (Sprong *et al*., 2009) (Table 1.6). More recently, assemblages C, D, E and F were reported in human although these results can be considered unusual since these assemblages are likely to be host specific (Table 1.3) (Gelanew *et al*., 2007; Sprong *et al*., 2009; Traub *et al*., 2009; Durigan *et al*., 2014).

Particular attention has focussed on the relationship between *Giardia* infections in humans and companion animals, principally dogs and cats. Earlier researches observed that cats and dogs were infected mostly with host specific assemblages (Monis *et al*., 2003; Sulaiman *et al*., 2003; Thompson *et al*., 2008; Xiao and Fayer, 2008). However, assemblage A and occasionally B have been more recently identified, thus representing a potential sources of zoonotic infection in people (Table 1.6). For instance, a study conducted in Temple communities in Bangkok observed that most of the canine population harboured at least one potentially zoonotic assemblage of *G. lamblia*; assemblages A (79%) and B (21%) (Traub *et al*., 2009). Among the assemblage A isolates subtyped, the majority belonged to sub-assemblage AI, with sub-assemblage AII being occasionally noticed (Ryan and Cacciò, 2013).

Giardia infection in livestock is common and it is believed to be associated with economic losses (Feng and Xiao, 2011; Geurden *et al*., 2012). For example, its prevalence can reach 100% in calves (Ralston *et al*., 2003; Geurden *et al*., 2012). Although infections in cattle are often subclinical, *Giardia* is able to induce diarrhoea and a reduction in weight gain in calves (Geurden *et al*., 2009). A longitudinal study reported that 43% of the isolates collected from adult dairy cattle were classified as assemblage A and 57% were assemblage

E, indicating the existence of a potential risk of infection to humans (Uehlinger *et al*., 2006). Another study observed that three quarter of the livestock isolates belonged to sub-assemblage AI, and the remaining quarter to sub-assemblage AII (Sprong *et al*., 2009).

The association between infected beavers and waterborne outbreaks of human giardiasis in campers in Canada is that led the WHO to classify *Giardia* as a zoonotic parasite (Issac-Renton *et al*., 1993). Studies have shown that assemblages A and B are commonly found in wild mammals (Table 1.6). The impact of these zoonotic species of *Giardia* on wildlife is not known, but *Giardia* has been reported in several species of non-human primates in Africa (Johnston *et al*., 2010).

Giardia in wild primates *Gorilla beringei beringei* (Nizeyi *et al*., 1999, 2002) and *Alouatta pigra* (Vitazkova and Wade, 2006) has been linked to increased contact with humans and livestock and may be found more often in primates in disturbed forest fragments than in forest blocks (Salzer *et al*., 2007). Anthropogenic disturbance leading to habitat loss, crowding, contact with new reservoirs of parasites (humans and livestock), nutritional and other stress can result in altered host–parasite dynamics and could have negative consequences for wildlife conservation (Eley *et al*., 1989; Gillespie and Chapman, 2006). These studies highlighted that "reverse zoonotic transmission" (zooanthroponotic) is an important factor that must be considered in understanding the epidemiology of infections with *Giardia*, particularly in wildlife (Thompson, 2013).

In contrast, little information is available on the prevalence and genetic diversity of *Giardia* assemblages infecting marine mammals and fish species. The few performed works detected *G. lamblia* assemblages C and D in harbour seals (Gaydos *et al*., 2008), assemblages A and B in the faeces of dolphins, porpoises, seals and a thresher shark (Lasek-Nesselquist *et al*., 2008; Dixon *et al*., 2008) (Table 1.6). In five different fish species from Australia were identified assemblages A, B and D (Yang *et al*., 2010), and similar results were observed in Egypt (Ghoneim *et al*., 2012) (Table 1.6). These data suggest that there may be a health threat to both marine and terrestrial life. The possibility of human infection from these animals is very low, however they can contaminate water that is used by humans for recreation activities (Lasek-Nesselquist *et al*., 2008; Yang *et al*., 2010). Transmission of *Giardia* through drinking and recreational water is well documented (Smith and Grimason, 2003; Karanis *et al*., 2007). Anthropogenic activity such as the contamination of aquatic ecosystems with *G. lamblia* cysts through untreated or improperly treated wastewater and agricultural runoff could increase the role of marine animals as reservoirs of disease in humans (Lasek-Nesselquist *et al*., 2008).

Taking into account the knowledge about the prevalence of *Giardia* in different animal species, including humans, and our current understanding for the major genetic groupings in *G. lamblia*, there are four major cycles of transmission that can be maintained independently and that do not require interaction between them (Figure 1.7). Thus, *G. lamblia* can be maintained in independent cycles involving wildlife or domestic animals (Thompson, 2004; Thompson and Ash, 2016). As previously referred, numerous studies have characterised isolates of *Giardia* collected from different hosts and they have demonstrated the occurrence of the same species/assemblages in humans and other animals (Monis and Thompson, 2003). These data are indicative of zoonotic potential but they do not give information about the frequency of zoonotic transmission.

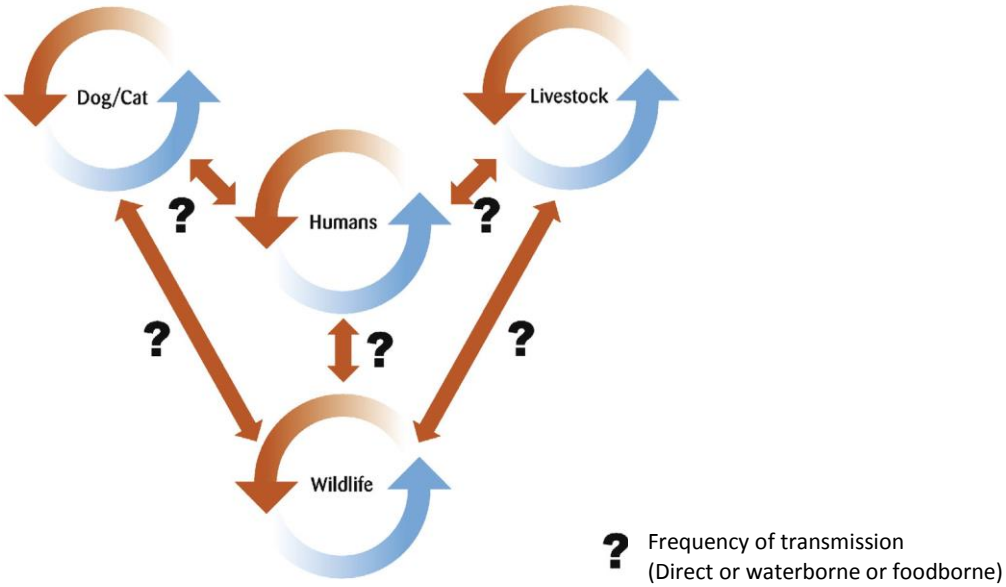

Figure 1.7. Major cycles of transmission of *Giardia* species. Some species are host specific and cycle between their respective hosts (blue) whereas others have low host specificity and capable of zoonotic transmission (red). Frequency of transmission between host species is largely unknown, and it is exacerbated by transmission via water and food (Adapted from Thompson and Ash, 2016).

1.4.7. Epidemiology

G. lamblia has a global distribution causing an estimated $2{,}8x10^{8}$ cases per annum (Lane and Lloyd, 2002), and is a leading cause of diarrhoeal disease worldwide (Feng and Xiao, 2011). In Asia, Africa and Latin America, about 200 million people have symptomatic giardiasis with some 500.000 new cases reported each year (WHO, 1996). In developing countries, the prevalence of human giardiasis is approximately 20-30% compared with 2-5% in developed countries, where it is associated mainly with traveling and waterborne outbreaks

(Flanagan, 1992; Karanis *et al*., 2007; Baldursson and Karanis, 2011). Giardiasis was included as part of the WHO's Neglected Disease Initiative since 2004 because of its significant public health and socioeconomic implications (Savioli *et al*., 2006).

G. lamblia, as well as other intestinal parasites, are considered indicators of health and socio-environmental vulnerability, and are associated with precarious sanitation and water quality of a country. They continue to pose a serious public health problem, especially in developing countries where sanitation is not expanded in line with population growth, such that access to basic services becomes more difficult.

Some epidemiological studies have shown that control strategies of local managements involving improvement in infra-structure, such as drinking water and sewage system, education of the society in order to enhance their personal hygiene, have been related to the reduction of incidence of intestinal parasites. In Thailand, a decrease in the prevalence of giardiasis was observed due the government investment in improvements in sanitary conditions, healthcare, and education in the country (Tungtrongchitr *et al*., 2010). Moreover, a study carried out in Brazil (Barreto *et al*., 2010) evaluated the impact of a sanitation intervention, known as Blue Bahia, on the prevalence of intestinal parasites infections, showing a substantial impact on the reduction of giardiasis.

Furthermore, the socio-economic level of a society affects the incidence of intestinal parasites favouring the acquisition of different pathogens and often patients are multiply infected (polyparasitized). A study conducted in São Paulo State (Brazil) (de Carvalho *et al*., 2006) observed a significant association between enteric parasites, family income, maternal education and age; the lowest intestinal parasites frequency occurred in children of families with higher income and higher education.

Additionally, *G. lamblia* is one of the most frequently parasite encountered in Brazil and is more prevalent in children from 0 to 4 years (de Carvalho *et al*., 2006; Mascarini and Donalísio, 2006; Marino *et al*., 2015). For instance, a polyparasitism of 51.2% was observed in children from Bahia (northeast) and among the positive samples there was higher frequency of contamination by protozoa (51.2%), especially for *G. lamblia* (31.2%). Similar results were observed in Minas Gerais (southeast) where 29.3% of the children were parasitized with intestinal parasites, 6.7% of the children presented polyparasitism and *G. lamblia* was the most prevalent (Gonçalves *et al*., 2011).

Characteristics of *G. lamblia* that may also influence the epidemiology of infection are the low infective dose, since 10 to 100 cysts are sufficient to establish infection. Besides, the cysts are immediately infectious when excreted in faeces, different from coccidian and

helminths, *Giardia* does not need a maturation period for cysts after excretion; and cysts are resistant and can survive for weeks to months in the environment. The environment dispersal can also lead to the contamination of drinking water and food (Cacciò *et al*., 2005; Ballweber *et al*., 2010). Its simple life cycle involving an environmentally resistant cyst provides ample opportunities for the parasite be transmitted directly from one infected individual to another (faecal-oral transmission), or indirectly through contamination of the environment or food (Thompson, 2004).

Potential mechanisms of *Giardia* cysts transmission include person to person, animal to animal, zoonotic (animal to human, human to animal), waterborne from humans or animals through drinking water or recreational contact such as in swimming, foodborne from contamination of water used in food preparation and manufacture or from food handlers (Karanis *et al*., 2007; Takizawa *et al*., 2009). Some activities have an increased risk for *Giardia* infection including travellers going to endemic areas, children in child care settings, close contacts with infected persons, persons taking part in outdoor activities who consume unfiltered, untreated water or who fail to practice good hygienic behaviours and persons who have contact with infected animals (Stuart *et al*., 2003).

G. lamblia has been recognized as one of the predominant causes of protozoan waterborne diseases, having a life cycle which is suited to waterborne and foodborne transmission (Karanis *et al*., 2007). The potential for transmission through drinking water is high since infective cysts are widely distributed in the environment; and due their size, they can overpass physical barriers in conventional water treatment processes and are disinfectant resistant. Deficiencies in the drinking water treatment process are among the most frequently reasons for giardiasis outbreaks. Improper disposal of human and animal waste can contaminate water sources with cysts, and this form of transmission remains a significant public health issue and animal health in many parts of the world, both in developed and in developing countries (Smith *et al*., 2007). For this reason, the role of wildlife and domestic animals play as reservoirs of *Giardia* and their potential source of waterborne outbreaks of giardiasis is of increasing concern.

In Brazil, the Ministry of Health, through Ordinance No. 518 of March 25, 2004, recommended the inclusion of *Giardia* cysts research on water for human consumption, in order to achieve a standard absence of cysts of this parasite, stimulating water treatment companies have more attention to this parasite.

1.5. Aim of the thesis

Although Brazil is a country with a high prevalence of intestinal parasitic infections, the prevalence in the metropolitan region of Rio de Janeiro, the second largest metropolitan area in the country, has not been estimated, and the genetic characterization of *Giardia lamblia*, one of the most common parasite in Brazil, has been poorly documented in Rio de Janeiro. Studies trying to associate *G. lamblia* assemblages and symptoms were done all over the world but results were inconclusive. Additionally, there have been very few studies trying to correlate *G. lamblia* assemblages with clinical manifestation among patients with the human acquired immunodeficiency virus (HIV) and, again, conclusive results were not achieved. This work aimed to: (*i*) estimate the prevalence of intestinal parasites in the metropolitan region of Rio de Janeiro and provide a detailed analysis of geographical distribution, considering demographic, socio-economic, and epidemiological contextual factors; (*ii*) determine the prevalence of different *G. lamblia* assemblages and sub-assemblages among patients with giardiasis attending the referral hospital from Rio de Janeiro (Evandro Chagas National Institute of Infectious Diseases, INI/FIOCRUZ); (*iii*) identify *G. lamblia* inter- and intra-assemblage genetic variation among patients with giardiasis; and (*iv*) correlate *G. lamblia* assemblages with symptoms in patients with and without HIV/AIDS. Certainly the knowledge of the geographic distribution and the prevalence of intestinal parasites, as well as the recognition of vulnerable areas are the first steps, and a prerequisite for development of appropriate control strategies by the Brazilian government. Besides, this study will greatly improve our understanding of the epidemiology of the disease and elucidate the dynamics of giardiasis in Rio de Janeiro population.

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Chapter 2

Geospatial distribution of intestinal parasitic infections in Rio de Janeiro (Brazil) and its association with social determinants

Clarissa P Faria, Graziela M Zanini, Gisele S Dias, Sidnei Silva, Marcelo B Freitas, Ricardo Almendra, Paula Santana, Maria C Sousa (**2016**), "Geospatial distribution of intestinal parasitic infections in Rio de Janeiro (Brazil) and its association with social determinants", submitted to PLOS Neglected Tropical Disease.

2.1. Abstract

Intestinal parasitic infections remain among the most common infectious diseases worldwide. The scenario in Brazil is no different, and this study aimed to estimate their prevalence and provide a detailed analysis of geographical distribution of intestinal parasites in the metropolitan region of Rio de Janeiro, considering demographic, socio-economic, and epidemiological contextual factors. The cross-section survey was conducted among individuals attending the Evandro Chagas National Institute of Infectious Diseases (FIOCRUZ, RJ) during the period from April 2012 to February 2015. Stool samples were collected and processed by sedimentation, flotation, Kato-Katz, Baermann-Moraes and Graham methods, iron haematoxylin staining and safranin staining. Of the 3245 individuals analysed, 569 (17.5%) were infected with at least one parasite. The most common protozoa were *Endolimax nana* (28.8%), *Entamoeba coli* (14.8%), Complex *Entamoeba histolytica/Entamoeba dispar* (13.5%), *Blastocystis hominis* (12.7%), and *Giardia lamblia* (8.1%). *Strongyloides stercoralis* (4.3%), *Schistosoma mansoni* (3.3%), *Ascaris lumbricoides* (1.6%), and hookworms (1.5%) were the most frequent helminths. There was a high frequency of contamination by protozoa (87%), and multiple infections were observed in 141 participants (24.8%). A positive association between age and gender with intestinal parasites was observed. Geospatial distribution of the detected intestinal parasitic infections was not random or homogeneous, but was influenced by socioeconomic conditions (through the material deprivation index (MDI)). Participants classified in the highest levels of deprivation had higher risk of having intestinal parasites. This study provides the first epidemiological information on the prevalence and distribution of intestinal parasitic infections in the Rio de Janeiro metropolitan area. Intestinal parasites, especially protozoa, are highly prevalent, indicating that parasitic infections are still a serious public health problem. MDI showed that intestinal parasites were strongly associated with the socioeconomic status of the population, thus making it possible to identify social vulnerable areas.

2.2 Author summary

Intestinal parasitic infections are considered indicators of health and socio-environmental vulnerability, and are associated with precarious sanitation and water quality of a country. They continue to pose a serious public health problem, especially in developing countries where sanitation is not expanded in line with population growth, such that access to basic services becomes more difficult. Although Brazil is a country with a high prevalence of intestinal parasitic infections, the prevalence in the metropolitan region of Rio de Janeiro (the second largest metropolitan area in the country) has not been estimated. Based on the identification of social determinants (income, education and sanitation), our group was able to identify vulnerable areas for intestinal parasitic infection in the metropolitan region of Rio de Janeiro. Infections caused by intestinal parasites are not included in the list of diseases compulsory notification in Brazil. However, special attention should be focused on this topic, and information on the geographic distribution and prevalence of intestinal parasites, as well as the recognition of vulnerable areas, are the first steps, and a prerequisite for development of appropriate control strategies by the government.

2.3. Introduction

Neglected tropical diseases, including intestinal parasitic infections, are a significant cause of morbidity and mortality in endemic countries (Speich *et al*., 2016). Intestinal parasitic infections have particular relevance as they affect the poorest and most deprived areas in tropical and subtropical regions (Speich *et al*., 2016). It is increasingly recognized that both protozoan and helminthic diseases are common among children under the age of five years. Children are more vulnerable to soil-transmitted helminths (STHs) than adults, and the nutritional impairment caused by the parasite can lead to iron-deficiency anaemia, malnutrition, and a negative impact on growth and cognitive development (Bethony *et al*., 2006; Pullan *et al*., 2011).

Despite all the medical and pharmaceutical advances and developments in sanitary engineering, intestinal parasitic infections remain among the most common infectious diseases worldwide, particularly in developing countries, where inadequate water treatment, poor sanitation and lack of adequate health services are common. Additionally, it is more difficult to implement enteric parasite-control actions in these regions due to the high cost of improvements in infrastructure, and the lack of educational projects offered to the population (Ostan *et al*., 2007; Mehraj *et al*., 2008; Speich *et al*., 2016).

Water is essential to life, but is also a major vehicle for pathogen dissemination. The potential for waterborne parasite transmission is high since infective helminth eggs and protozoa (oo)cysts are distributed through water in the environment**.** Pathogens like *Giardia lambia* and *Cryptosporidium* spp. are recognized as important waterborne disease pathogens and are associated with severe gastrointestinal illness. Amoebiasis, balantidiosis, cyclosporidiosis and microsporidiosis outbreaks have been reported throughout the world (Baldursson and Karanis, 2011; Kumar *et al*., 2014). It is well documented that conventional water and sewage treatment process are not completely effective in destroying protozoa (oo)cysts and helminth eggs (Betancourt and Rose, 2004; Savioli *et al*., 2006; Hatam-Nahavandi *et al*., 2015). Improper disposal of human and animal waste has also been identified as a source of infection, contaminating water sources (Smith *et al*., 2007) and recreational waters such as swimming pools, water parks and lakes (Savioli *et al*., 2006). Occasionally, sewer overflows also contribute to contamination of surface water and agricultural lands, which leads to potential human infection. Food contamination is also important and can occur directly in the handling process (contaminated equipment, infected

food handlers or wash water), or indirectly through contaminated irrigation water (Dawson, 2005).

The lack of sanitary conditions to which the population is exposed favours the acquisition of various pathogens, and patients are often multiply infected (polyparasitized). Recently, a systematic review and meta-analysis showed that sanitation facilities and water treatment are associated with lower risks of infection with intestinal protozoa, and could also prevent diarrhoeal diseases (Speich *et al*., 2016). The same relationships were observed by Strunz *et al*. (2014) for soil-transmitted helminths.

In Brazil, intestinal parasite infections persist, although their frequency has decreased due improvement of sanitary conditions (Barreto *et al*., 2007; Basso *et al*., 2008; Barreto *et al*., 2010). Up until now, studies of enteric parasites in Brazil have been limited, isolated and fairly rare, generally reflecting the situation in small towns. Mariano and colleagues (2015) observed 77.2% of positive cases, and a polyparasitism of 51.2% in children from Itabuna (Bahia). Similar results were observed in two localities of São Paulo, where 65.9% of the individuals were positive for at least one parasite (David *et al*., 2015). In Rio de Janeiro, previous studies have shown intestinal parasite prevalence ranging from 18.3% to 66% (Santos *et al*., 1984; Macedo and Rey, 1996; Costa-Macedo *et al*., 1998; Uchôa *et al*., 2001; Carvalho-Costa *et al*., 2007; Volotão *et al*., 2007).

The aim of this study was to estimate the number of individuals infected with intestinal parasites who attended a referral hospital located in Rio de Janeiro (Brazil), and to provide a detailed analysis of the geographical distribution. The study also looked at the influence of demographic variables, socio-economic status and environmental factors on the intestinal parasitic infections. This knowledge will be essential for the development of effective prevention and control strategies to eliminate or reduce intestinal parasitic infection.

2.4. Methods

2.4.1. Study site

The cross-section survey was carried out from April 2012 to February 2015 in Evandro Chagas National Institute of Infectious Diseases (INI/FIOCRUZ), a reference hospital in infectious diseases in Brazil, located in Rio de Janeiro (RJ). Despite it being an infectious disease referral hospital, individuals also attend for routine consultations (cardiology, dermatologist, gynecology, neurology, ophthalmology, otolaryngologist, infectious disease speciality) or emergency situations. As the prevalence of intestinal parasites

in Brazil remains high, it is common the doctor´s submit requests for parasitological analysis in faeces, regardless of age or genera and of having or not symptoms suggestive of intestinal infections. The INI/FIOCRUZ hospital receives individuals from all municipalities, mainly the metropolitan area.

Rio de Janeiro State is composed of 92 municipalities. The metropolitan region of Rio de Janeiro is composed of 21 municipalities: Belford Roxo, Cachoeira de Macacu, Duque de Caxias, Guapimirim, Itaboraí, Itaguaí, Japeri, Magé, Maricá, Mesquita, Nilópolis, Niterói, Nova Iguaçu, Paracambi, Queimados, Rio Bonito, Rio de Janeiro, São Gonçalo, São João de Meriti, Seropédica and Tanguá (Figure 2.1). It is the second largest metropolitan area in Brazil with 11.812.482 inhabitants in an area of 8.147.356 km^2. This region has 2.746 slums, with a resident population of 1.702.073 inhabitants (14.4% from the total population) occupying 123.627km^2 (IBGE, 2010). The main characteristics of each municipality of the metropolitan region of Rio de Janeiro State are summarized in Table 2.1.

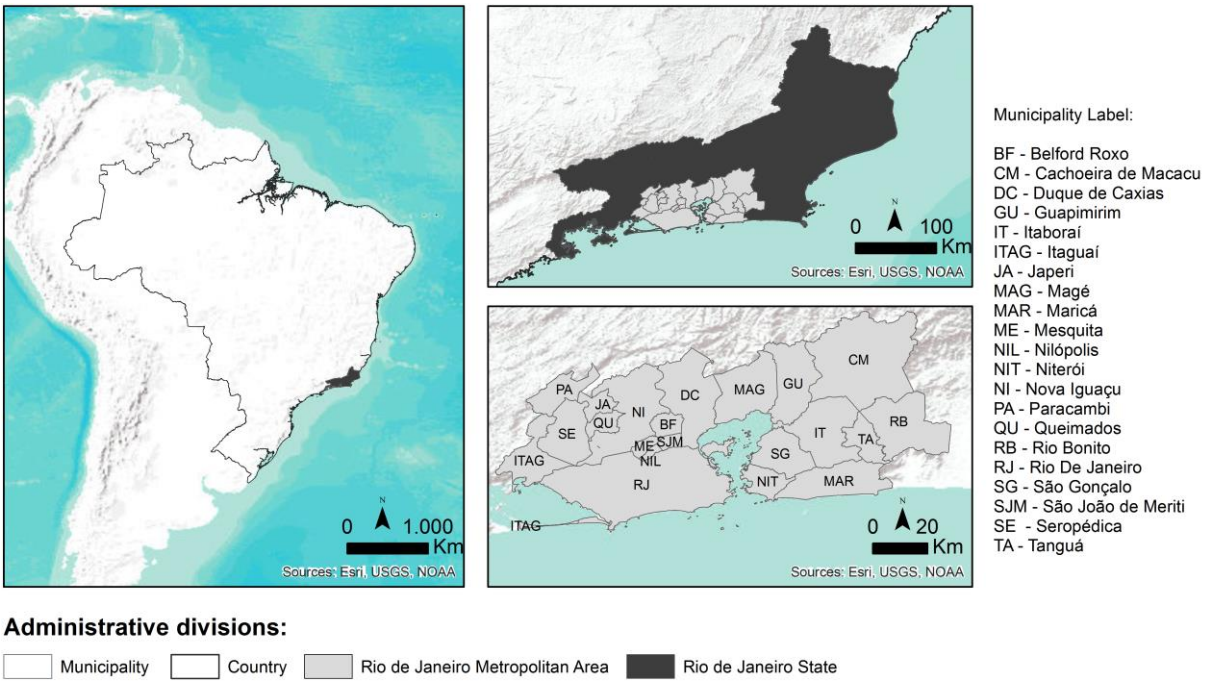

Figure 2.1. Localization of the metropolitan region of Rio de Janeiro State, Brazil.

According to the last census conducted in 2010, Rio de Janeiro municipality has a population of 6.320.446 inhabitants (Table 2.1) in an area of 1.197.463 km^2. The municipality has 2.227 slums, with a resident population of 1.393.314 inhabitants (11.8% from the total population) occupying 54.213 km^2 (IBGE, 2010).

Table 2.1. Main characteristics of municipalities of the metropolitan region of Rio de Janeiro State.

Municipalities	Population*	Population density (inhab./km^2)*	Drinking water coverage (%)*	Sanitation coverage (%)*	MHDI**	Gini coefficient*
Belford Roxo	469.332	6.031.38	76.8	39.3	0.684	0.4606
Cachoeira de Macacu	54.273	56.90	94.8	86.5	0.7	0.5077
Duque de Caxias	855.048	1.828.51	85.1	41.6	0.711	0.4875
Guapimirim	51.483	1.142.70	43.9	-	0.698	0.5232
Itaboraí	218.008	506.56	81.7	40.3	0.693	0.4967
Itaguaí	109.091	395.45	86.4	37.0	0.715	0.5004
Japeri	95.492	1.166.37	67.2	-	0.659	0.4578
Magé	227.322	585.13	79.7	40.6	0.709	0.5082
Maricá	127.461	351.55	58	12.3	0.765	0.5098
Mesquita	168.376	4.310.48	82.6	37.2	0.737	0.4919
Nilópolis	157.425	8.117.62	98.3	95.9	0.753	0.4805
Niterói	487.562	3.640.80	100	92.7	0.837	0.5983
Nova Iguaçu	796.257	1.527.60	92.1	42.0	0.713	0.5141
Paracambi	47.124	262.27	73.1	29.9	0.72	0.4718
Queimados	137.962	1.822.60	79.7	37.0	0.68	0.4584
Rio Bonito	55.551	121.70	87.2	-	0.71	0.5023
Rio de Janeiro	6.320.446	5.265.81	91.2	70.1	0.799	0.6391
São Gonçalo	999.728	4.035.90	85.1	36.8	0.739	0.4610
São João de Meriti	458.673	13.024.56	91.8	48.7	0.719	0.4620
Seropédica	78.186	275.53	69.6	31.1	0.713	0.4835
Tanguá	30.732	211.21	68.3	29.9	0.654	0.4615

* IBGE, 2010

** UNDP, 2013

Municipal human development index (MHDI) is a summary measure of average achievement in key dimensions of human development (a long and healthy life, being knowledgeable and have a decent standard of living), and gini index is a measure of statistical dispersion whose value ranges from zero (perfect equality) to one (perfect inequality). The MHDI of Rio de Janeiro is 0.799 according to the United Nations Development Programme (UNDP, 2013) and gini index is 0.6391 (IBGE, 2010). Most of the population (91.2%) has access to potable water and 70.1% has sanitation coverage (CEPERJ, 2012).

2.4.2. Study population, collection of faecal samples and laboratory methods

The study population included individuals (n=3245), of both genders and all age groups, attended in Evandro Chagas National Institute of Infectious Diseases, between April 2012 and February 2015. Stool samples were collected by the participant in plastic disposable flasks with or without preservatives and maintained at 4^{o}C until laboratory analysis on the same day. Flasks were labelled with the name, collection date and the hospital number. The parasitological tests were conducted at the Parasitology Laboratory of INI by experienced laboratory technologists and College of American Pathologist certifies the Laboratory. Moreover, participant' data (sex, age, educational level and residence) were obtained from the hospital's database.

For laboratory diagnosis of intestinal parasites, the fresh specimens were analysed by means of centrifugation sedimentation (de Carli, 2001), centrifugal flotation in zinc sulphate solution (Faust *et al*., 1938), Kato-Katz (Helm-TEST® kit, Fiocruz, Brazil) and Baermann-Moraes method (de Carli, 2001; Rey *et al*., 2002). All these techniques were routinely performed on all fresh stool samples. Specimens preserved in MIF solution (mertiolate-iodine-formaldehyde) were processed by the centrifugation sedimentation method (de Carli, 2001). The Graham method, faecal occult blood test, the iron haematoxylin staining and the safranin staining procedure was carried out depending on doctor request (de Carli, 2001). The slides were then observed under the optical microscope.

All individuals attended in INI/FIOCRUZ are dewormed when diagnosed (drugs are provided by the institution itself).

2.4.3. Data extraction and geospatial analysis

The zip code for each participant was obtained from the hospital's database and through Brazilian Institute of Statistics and Geography (IBGE) converted into geographic coordinates (latitude and longitude). IBGE was the source of data in respect of geography,

demography and socioeconomic conditions of the studied population (National Census of 2010).

The spatial distribution of the participants was assessed through a Kernel Density Function that allows to estimate the intensity of events across a surface by calculating the overall number of cases within a given search radius from a target point. To identify if the participants were spatially clustered or dispersed the Average Nearest Neighbor test was used.

To evaluate the social and economic conditions of the place of residence a material deprivation index (MDI) was constructed, at the census tract level, to the metropolitan region of Rio de Janeiro. The MDI is based upon the following indicators: (1) illiteracy rate/education (percentage of population older than 10 years that can read or write); (2) water supply/sanitation (percentage of permanent households without public water treatment plant); and (3) family income (percentage of households with per capita monthly income $\leq$1 minimum wage). Based on the Carstairs and Morris method, the indicators considered in each index were standardised (using the z-score method) so that each indicator has a weighted mean of zero and a variance of one, and exerted the same influence upon the final result (Carstairs and Morris, 1990). The MDI was analysed in quintiles: q1, lowest level of deprivation; q5, highest level of deprivation.

To address the potential effects of the socioeconomic conditions of the place of residence on the incidence of intestinal parasites, the proportion of participants living in each deprivation quintile was assessed. Simultaneously, the proximity to slums was analysed through geographical buffers of 50m and 100m. The spatial analysis was performed through the ArcMap 10.x software of ESRI.

2.4.4. Statistical analysis

The data entry was carried out using Excel software and analysed using Statistical Package for the Social Sciences (SPSS) version 16. Percentages were used to perform the exploratory analysis of the categorical variables and quantitative variables are presented as mean ± standard deviation (SD). Pearson´s chi-squared and Fisher's Exact Test were used for categorical data. The level of statistical significance was set as $p<0.05$, an odds ratio and 95% confidence interval (CI) was computed. Logistic regression was used to identify a potential contribution of each of the variables for the acquisition of intestinal parasite infections.

2.4.5. Ethical statement

The Research Ethics Committee Evandro Chagas National Institute of Infectious Diseases (INI/FIOCRUZ) approved the study (protocol number: 127.542). This project was in accordance with the Brazilian Ethical Resolutions, especially Resolution CNS 196/1996 and its complementary and the Code of Medical Ethics of 1988 (articles 122 - 1307). Study individuals provided a written signed informed consent prior to sample collection and for participants younger than 18 years, informed consent was provided by parents or guardians after a detailed explanation of the objectives of the work. A term of privacy and confidentiality was signed by the researches for individuals to whom it was not possible to obtain informed consent beforehand.

2.5. Results

2.5.1. Prevalence of intestinal parasitic infections

Between April 2012 and February 2015, a total of 3245 individuals (1564 female and 1681 male) had the parasitological tests done (Table 2.2). In 2012 a total of 995 samples were collected, with 193 positive samples; in 2013, 1189 individuals were collected being 187 positive samples; in 2014, 938 individuals with 168 positive samples; and in 2015, 123 individuals with 21 positive samples. Summarizing, we had 569 individuals (17.5%) with positive stool examination for one or more enteric parasite and 2676 individuals (82.5%) with negative results.

Table 2.2. Prevalence of intestinal parasites along the years of the survey.

Year of collection	Positive participants No. (%)	Negative participants No. (%)	Total
2012	193 (19.4)	802 (80.6)	995 (30.7)
2013	187 (15.7)	1002 (84.3)	1189 (36.6)
2014	168 (17.9)	770 (82.1)	938 (28.9)
2015	21 (17.1)	102 (82.9)	123 (3.8)
Total	**569 (17.5)**	**2676 (82.5)**	**3245 (100)**

The ages of the participants ranged from 1 to 93 years with an average of 41.34±15.54 (Mean±SD; median=41). The adults between 26-65 years were the majority of participants (n=2130) (Table 2.3). There were more male than female parasitized (64.5% versus 35.5%,

respectively) and seventy-five percent of participants (n=427) were educated above the primary grade (Table 2.3).

Table 2.3. Characteristics of positive and negative participants to intestinal parasites.

Characteristics	No. (%) positive participants (n= 569)	No. (%) negative participants (n= 2676)	Total (n= 3245)
Age group (years)			
0-14	16 (23.9)	51 (76.1)	67 (2.1)
15-25	81 (18.3)	361 (81.7)	442 (13.6)
26-65	417 (19.6)	1714 (80.4)	2131 (65.7)
>66	25 (13.6)	159 (80.5)	184 (5.7)
Missing	30 (7.1)	391 (92.9)	421 (12.9)
Gender			
Female	202 (12.9)	1362 (87.1)	1564 (48.2)
Male	367 (21.8)	1314 (78.2)	1681 (51.8)
Education			
Elementary school	190 (33.4)	na	na
High school	152 (26.7)	na	na
University education	85 (14.9)	na	na
No formal education	17 (3)	na	na
Missing	125 (22)	na	na

* *na*, not available

Endolimax nana was the most common enteric parasite, present in 216 samples (28.8%) followed by *Entamoeba coli* in 111 samples (14.8%), Complex *Entamoeba histolytica/Entamoeba dispar* in 101 samples (13.5%), *Blastocystis hominis* in 95 samples (12.7%), *Giardia lamblia* in 61 samples (8.1%), *Iodamoeba butschilii* in 33 samples (4.4%), *Strongyloides stercoralis* in 32 samples (4.3%), *Schistosoma mansoni* in 25 samples (3.3%), *Cryptosporidium sp.* in 14 samples (1.9%), *Ascaris lumbricoides* in 12 samples (1.6%), *Cystoisospora belli* in 12 samples (1.6%), *hookworms* in 11 samples (1.5%), *Trichuris trichiura* in 10 samples (1.3%), *Entamoeba hartmani* in 9 samples (1.2%), *Enterobius vermicularis* in 6 samples (0.8%) and *Hymenolepis nana* in one sample (0.1%) (Table 2.4).

Table 2.4. Number of intestinal protozoa and helminths species: monoparasitism and polyparasitism.

Parasites	No (%) (n=749)	Monoparasitism (n=428)	Polyparasitism (n=321)
Protozoa			
*B. hominis****	95 (12.7)	66 (15.4)	29 (9)
Complex *E. histolytica/ E. dispar**	101 (13.5)	50 (11.7)	51 (15.9)
*Cryptosporidium sp.**	14 (1.9)	11 (2.6)	3 (0.9)
*C. belli**	12 (1.6)	11 (2.6)	1 (0.3)
*E. nana***	216 (28.8)	141 (32.9)	75 (23.4)
*E. coli***	111 (14.8)	41 (9.6)	70 (21.8)
*E. hartmani***	9 (1.2)	3 (0.7)	6 (1.9)
*G. lamblia**	61 (8.1)	36 (8.4)	25 (7.8)
*I. butschilii***	33 (4.4)	7 (1.6)	26 (8.1)
Total of protozoa species	652 (87)	366 (85.5)	286 (89)
Helminths			
*A. lumbricoides**	12 (1.6)	4 (0.9)	8 (2.5)
*E. vermicularis**	6 (0.8)	5 (1.2)	1 (0.3)
*H. nana**	1 (0.1)	-	1 (0.3)
Hookworms*	11 (1.5)	6 (1.4)	5 (1.6)
*S. mansoni**	25 (3.3)	20 (4.7)	5 (1.6)
*S. stercoralis**	32 (4.3)	23 (5.4)	9 (2.8)
*T. trichiura**	10 (1.3)	4 (0.9)	6 (1.9)
Total of helminths species	97 (13)	62 (14.5)	35 (11)

* Pathogenic species; ** Non-pathogenic species; *** Non-pathogenic, human pathogen that remain unclear.

The number of samples with one parasite (monoparasitism) is higher (428 positive samples, 57.1%) than those samples with various parasites (polyparasitism) (321 positive samples, 42.9%). Interesting, the frequency of the amoebae (Complex *E.histolytica*/*E.dispar, E. coli* and *E. hartmani)* as well of some geohelminths (*A. lumbricoides* and *T. trichiura*) is higher on samples with various parasites (polyparasitism) (Table 2.4).

We observed a very high frequency of protozoan infections (87%), occupying the first six positions; *E. nana* was the predominant, followed by *E. coli* and Complex *E. histolytica/E. dispar*. The most frequent helminths were *S. stercoralis* and *S. mansoni;* only appearing in seventh position. Of the 16 species of intestinal parasites detected, 11 were pathogenic (Complex *E. histolytica/E. dispar*, *Cryptosporidium sp.*, *C. belli*, *G. lamblia*, *A. lumbricoides*, *E. vermicularis*, *H. nana*, *hookworms*, *S. mansoni*, *S. stercoralis* and *T. trichiura*) and 5 were

non-pathogenic (*B. hominis*, *E. nana*, *E. coli*, *E. hartmani* and *I. butschilii*). The pathogenic species comprises 38.1% of the studied participants (285 of 749), while the non-pathogenic reached 61.9% (464 of 749).

Most of the participants (428 of 569; 75.2%) did not present any co-infection, whereas 141 (24.8%) had two or more parasites simultaneously. Among the multiple infected, 109 individuals were infected with two parasites (19.2%), 26 were infected with three parasites (4.6%), 5 had four parasites (0.9%) and 1 had five (0.1%). Regarding parasitic associations, only 11.8% (67 of 569) were co-parasited by helminths, 84.3% (480 of 569) by protozoa and only 3.9% (22 of 569) by both.

2.5.2. Intestinal parasites risk factors

Age and gender were examined as potential associations for intestinal parasitic infections. A positive association between gender and intestinal parasites (p<0.0001), as well as protozoa (p<0.0001), helminths (p<0.0001) and poliparasitism (p<0.0001) were detected. Male were more likely to be infected with intestinal parasites (OR=1.9; 95%CI of 1.56 to 2.27), protozoa (OR=1.8; 95%CI of 1.50 to 2.20), helminths (OR=2.8; 95%CI of 1.75 to 4.51) and have multiple parasites (OR=3.4; 95% CI of 2.28 to 5.05) compared to female (Table 2.5).

Table 2.5. Associations of intestinal parasites with the gender.

Parasites	Gender		OR (95%CI)	*p*-value
	Male (n=1681) No. (%)	Female (n=1564) No. (%)		
Intestinal parasites	367 (21.8)	202 (12.9)	1.9 (1.56; 2.27)	0.0001
Protozoa	302 (17.9)	182 (11.6)	1.8 (1.50; 2.20)	0.0001
Helminths	65 (3.9)	24 (1.5)	2.8 (1.75; 4.51)	0.0001
Monoparasitism	259 (15.4)	169 (10.8)	1.6 (1.29; 1.96)	0.0001
Polyparasitism	108 (6.4)	33 (2.1)	3.4 (2.28; 5.05)	0.0001

No statistical significant difference was found between intestinal parasites and age (p=0.166). However, when we analyse the parasite species separately we observed that children (0-14 years) were more likely to be infected with *A. lumbricoides* (p=0.031; OR= 8.5; 95% CI= 1.8; 39.4), *E. vermicularis* (p=0.005; OR= 28.2; 95% CI= 4.6; 171.6), *B. hominis* (p=0.002; OR= 3.9; 95% CI= 1.8; 8.4), and *G. lamblia* (p=0.011; OR= 4.1; 95% CI=

1.6; 10.7) as compared to the older participants. Moreover, there were no cases of multiple parasitic infections in children under 5 years old.

2.5.3. Geospatial distribution

The prevalence of intestinal parasites varies by municipalities, most of participants (2847 of 3245; 87.7%) live in metropolitan region and 1748 (53.9%) live in Rio de Janeiro municipality (Tables 2.6 and S2.1). The metropolitan region of Rio de Janeiro had 532 positive cases (16.4%) and the others municipalities had 21 positive cases (0.6%) (Table 2.6). As expected, Rio de Janeiro municipality had a greater number of participants infected with intestinal parasites (332; 10.2%) since it has the larger population (Table S2.1, Figure S2.1). In 16 participants (0.5%) positive for intestinal parasites was not possible to identify the residence.

Table 2.6. Number of positive and negative participants to intestinal parasites by regions.

Regions	Positive participants No. (%)	Negative participants No. (%)	Total of participants No. (%)	Prevalence rates
Rio de Janeiro State				
Metropolitan region	532 (16.4)	2315 (71.3)	2847 (87.7)	18.7
Others municipalities	21 (0.7)	60 (1.8)	81 (2.5)	26.6
Others States of Brazil	-	9 (0.3)	9 (0.3)	-
Missing	16 (0.5)	292 (9)	308 (9.5)	5.2
Total	**569**	**2676**	**3245**	**17.5**

The distribution of parasites species also varied among the municipalities (Table 2.7). The metropolitan region had 93.7% (702 of 749) of the enteric parasites observed: in Rio de Janeiro it was possible to detect 434 enteric parasites (57.9%), Duque de Caxias was the second municipality with 81 (10.8%), followed by Nova Iguaçu (57; 7.6%), Belford Roxo (33; 4.5%), São João de Meriti (25; 3.4%), São Gonçalo (18; 2.4%), Nilópolis (15; 2%), Magé (12; 1.6%), Cachoeira de Macacu (5; 0.7%), Itaboraí (4; 0.5%), Niterói (3; 0.4%), Queimados (3; 0.4%), Itaguaí (2; 0.3%), Maricá (2; 0.3%), Mesquita (2; 0.3%), Seropédica (2; 0.3%), and Japeri (4; 0.5%). We did not have positive samples from participants of Guapimirim, Paracambi, Rio Bonito and Tanguá. Others municipalities amounted 28 (3.7%) enteric parasites, and 19 (2.5%) was not possible to identify the municipality (Table 2.7).

Table 2.7. Distribution of intestinal parasites species by the municipalities of Rio de Janeiro State.

Municipality	Number (%) of intestinal parasites species																Total
	Bh	Eh/Ed	Crypto	Cb	En	Ec	Eh	Gl	Ib	Al	Ev	Hk	Hn	Sm	Ss	Tt	
Metropolitan Region	93 (12.4)	93 (12.4)	14 (1.9)	12 (1.6)	198 (26.4)	104 (13.9)	7 (1.0)	57 (7.6)	32 (4.3)	12 (1.6)	6 (0.8)	10 (1.4)	1 (0.1)	25 (3.4)	30 (4.0)	8 (1.1)	702 (93.7)
Belford Roxo	4 (0.5)	4 (0.5)			8 (1.1)	7 (1.0)	2 (0.3)	2 (0.3)	3 (0.4)			1 (0.1)		2 (0.3)			33 (4.5)
Cachoeira de Macacu	1 (0.1)				2 (0.3)	2 (0.3)											5 (0.7)
Duque de Caxias	13 (1.8)	9 (1.2)	1 (0.1)		19 (2.6)	11 (1.5)	1 (0.1)	12 (1.6)	4 (0.5)	2 (0.3)		1 (0.1)		2 (0.3)	3 (0.4)	3 (0.4)	81 (10.8)
Itaboraí	1 (0.1)				1 (0.1)	1 (0.1)				1 (0.1)							4 (0.5)
Itaguaí	1 (0.1)														1 (0.1)		2 (0.3)
Japeri	1 (0.1)	1 (0.1)			1 (0.1)				1 (0.1)								4 (0.5)
Magé	2 (0.3)	2 (0.3)		1 (0.1)	3 (0.4)	1 (0.1)				1 (0.1)	1 (0.1)					1 (0.1)	12 (1.6)
Maricá					1 (0.1)	1 (0.1)											2 (0.3)
Mesquita				1 (0.1)	1 (0.1)												2 (0.3)
Nilópolis	3 (0.4)	2 (0.3)	1 (0.1)		2 (0.3)	1 (0.1)		2 (0.3)	1 (0.1)	1 (0.1)				1 (0.1)	1 (0.1)		15 (2.0)
Niterói			1 (0.1)		2 (0.3)												3 (0.4)
Nova Iguaçu	6 (0.8)	9 (1.2)	2 (0.3)		14 (1.9)	8 (1.1)	1 (0.1)	6 (0.8)	2 (0.3)	1 (0.1)		1 (0.1)		2 (0.3)	4 (0.5)	1 (0.1)	57 (7.6)
Queimados		1 (0.1)				1 (0.1)								1 (0.1)			3 (0.4)
Rio de Janeiro	58 (7.7)	59 (8.1)	7 (1.0)	10 (1.4)	127 (17.4)	63 (8.6)	3 (0.4)	33 (4.4)	18 (2.5)	5 (0.7)	5 (0.7)	7 (1.0)	1 (0.1)	17 (2.3)	18 (2.5)	3(0.4)	434 (57.9)
São Gonçalo	2 (0.3)	3 (0.4)	1 (0.1)		6 (0.8)	3 (0.4)		1 (0.1)	2 (0.3)								18 (2.4)
São João de Meriti	1 (0.1)	3 (0.4)			11 (1.5)	4 (0.5)		1 (0.1)	1 (0.1)	1 (0.1)					3 (0.4)		25 (3.4)
Seropédica			1 (0.1)			1 (0.1)											2 (0.3)
Guapimirim, Paracambi, Rio Bonito and Tanguá	-	-	-		-	-		-	-	-	-	-	-	-	-	-	-
Others municipalities	2 (0.3)	4 (0.5)			11 (1.5)	4 (0.5)	2 (0.3)	2 (0.3)							2 (0.3)	1 (0.1)	28 (3.8)
Angra dos Reis							1 (0.1)										1 (0.1)

Table 2.7 (*Continued*)

Araruama		1 (0.1)			3 (0.4)	2 (0.3)											6 (0.8)
Barra Mansa		1 (0.1)													1 (0.1)		2 (0.3)
Comendador Levy Gasparian															1 (0.1)		1 (0.1)
Macaé					1 (0.1)												1 (0.1)
Mangaratiba					2 (0.2)												2 (0.3)
Parati	1 (0.1)																1 (0.1)
Santo Antônio de Pádua					1 (0.1)			2 (0.3)									3 (0.4)
São Pedro da Aldeia						1 (0.1)	1 (0.1)										2 (0.3)
Saquarema	1 (0.1)	1 (0.1)			2 (0.3)	1 (0.1)										1 (0.1)	6 (0.8)
Três Rios		1 (0.1)															1 (0.1)
Valença					1 (0.1)												1 (0.1)
Volta Redonda					1 (0.1)												1 (0.1)
Unknown		4 (0.5)			7 (1.0)	3 (0.4)		2 (0.3)	1 (0.1)			1 (0.1)				1 (0.1)	19 (2.5)
Total	**95 (12.7)**	**101 (13.5)**	**14 (1.9)**	**12 (1.6)**	**216 (28.8)**	**111 (14.8)**	**9 (1.2)**	**61 (8.1)**	**33 (4.4)**	**12 (1.6)**	**6 (0.8)**	**11 (1.5)**	**1 (0.1)**	**25 (3.3)**	**32 (4.3)**	**10 (1.3)**	**749 (100)**

Ascaris lumbricoides (Al), *Enterobius vermicularis* (Ev), Hookworms (Hk), *Hymenolepis nana* (Hn), *Schistosoma mansoni* (Sm), *Strongyloides stercoralis* (Ss), *Trichuris trichiura* (Tt), *Blastocystis hominis* (Bh), Complex E*ntamoeba histolytica/Entamoeba díspar* (Eh/Ed), *Cryptosporidium sp.*(Crypto), *Cystoisospora belli* (Cb), *Endolimax nana* (En), *Entamoeba coli* (Ec), *Entamoeba hartmani* (Eh), *Giardia lamblia* (Gl), *Iodamoeba butschilii* (Ib).

2.5.3.1. Metropolitan region of Rio de Janeiro State

Of the 3245 participants, only 2670 informed the zip code that was converted into geographic coordinates. Two thousand six hundred fifty-two (472 infected participants and 2180 participants with negative results) lived in the metropolitan area of Rio de Janeiro State and 1638 (286 infected participants and 1352 participants with negative results) in Rio de Janeiro municipality. The geospatial distribution of participants infected and uninfected by intestinal parasites in the metropolitan region could be observed in Figure 2.2. Based on participants' place of residence we could observe a marked geographical pattern, with a high incidence density near Evandro Chagas National Institute of Infectious Diseases and along Guanabara Bay (Figure 2.2). The geographical distribution of participants with and without intestinal parasites was similar, and we could observe a statistically significant spatial dependency.

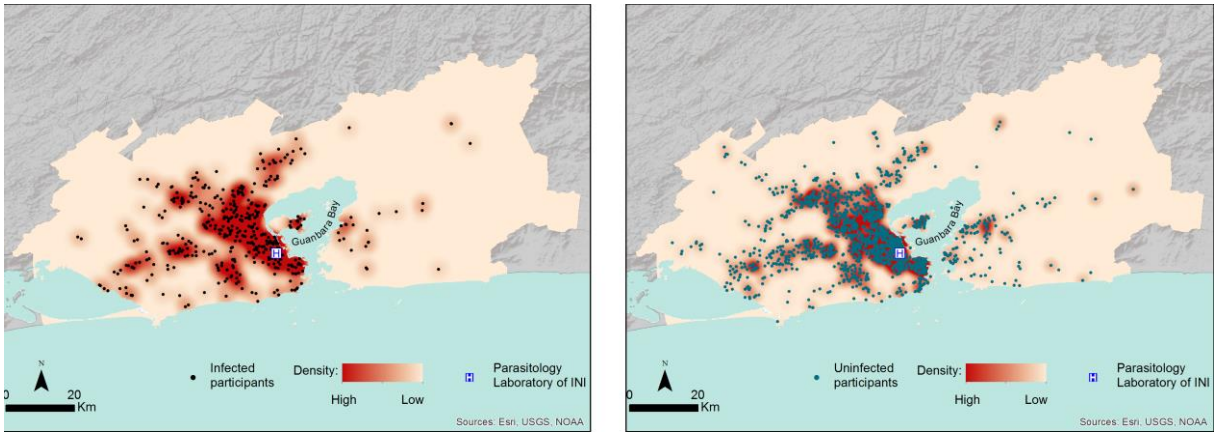

Figure 2.2. Geographical distribution and Gaussian kernel density surface map of participants with and without intestinal parasites. Incidence density in the metropolitan region of Rio de Janeiro State.

Socioeconomic deprived regions were identified in the metropolitan region of Rio de Janeiro State. Lower scores in the deprivation index (q1) represented lower socioeconomic deprivation, and higher scores in the deprivation index (q5) represented higher socioeconomic deprivation (Figure 2.3).

Demographic and socioeconomic characteristics considerably varied among the census tracts (CTs) in metropolitan region of Rio de Janeiro (Figure 2.3). It was possible to observe contiguous CTs with very different material deprivation index (MDIs) (q1 and q5, for example). The number of infected participants was lower in areas with better socioeconomic, educational and sanitary conditions (q1); and a higher number of infected participants were observed in the highest levels of deprivation (q3, q4 and q5). A gradient could be observed

between the quintiles (Table 2.8). Thus, individuals classified in the first deprivation quintile (q1) had less risk of having intestinal parasites than individuals classified in the others deprivation quintiles (Table 2.8).

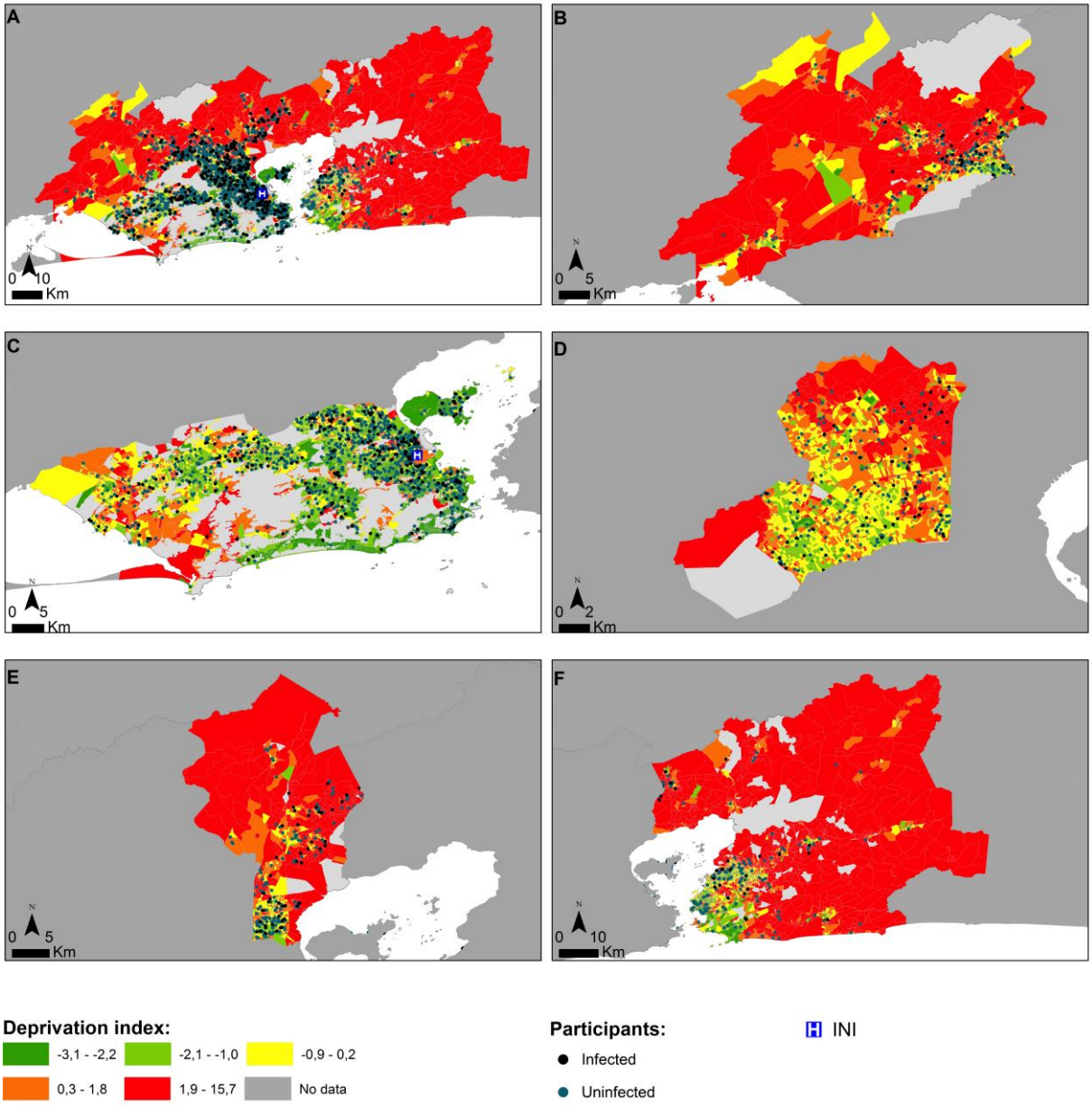

Figure 2.3. Geographical distribution of participants with and without intestinal parasitic infections and the material deprivation index (MDIs). (A) Metropolitan region of Rio de Janeiro; (B) Itaguaí, Japeri, Queimados, Nova Iguaçu, Paracambi and Seropédica municipalities; (C) Rio de Janeiro municipality; (D) Belford Roxo, Mesquita, Nilópolis and São João de Meriti municipalities; (E) Duque de Caxias municipality; (F) Cachoeira de Macacu, Guapimirim, Itaboraí, Magé, Maricá, Niterói, Rio Bonito, São Gonçalo and Tanguá municipalities (green (q1): lower material deprivation; red (q5): higher material deprivation).

Table 2.8. Number of positive and negative participants to intestinal parasites per quintile of deprivation.

Quintiles	Material Deprivation Index (%)			OR (95%CI)	*p*-value
	Positive participants (n=485)	Negative participants (n=2185)	Total of participants (n=2670)		
Q1	40 (6.9)	538 (93.1)	578	[§]	0.001*
Q2	95 (19.7)	388 (80.3)	483	3.3 (2.2; 4.9)	0.001*
Q3	102 (21.4)	374 (78.6)	476	3.7 (2.5; 5.5)	0.001*
Q4	117 (25.6)	340 (74.4)	457	4.6 (3.2; 6.9)	0.001*
Q5	129 (19.7)	526 (80.3)	655	3.3 (2.3; 4.8)	0.001*
ND**	2 (9.5)	19 (90.5)	21	-	-

[§] Reference group

[*] Statistically significant (p<0.05)

[†] CTs that were not possible to calculate the MDI

2.5.3.2. Rio de Janeiro municipality

In Table 2.9 and Figure 2.4 we could observe that 65.8% (1078 of 1638) of participants live more than 100 meters away from slums and that 19.2% (314 of 1638) of participants live within a radius of 100 meters to slums. We could also note that 21.3% (61 of 286) of individuals positive for intestinal parasites live in slums (also called, subnormal agglomerates), with inadequate infrastructure and lack of access to health services. Therefore, participants who live more than 100 meters away from slums had less risk of having intestinal parasites (p=0.001) than participants living in slums (p=0.001).

Table 2.9. Number of positive and negative participants to intestinal parasites according to their distance over the slums in Rio de Janeiro municipality.

Participant´s distance from slums (meters)	Positive participants (n=286)	Negative participants (n=1352)	Total of participants (n=1638)	OR (95%CI)	*p*-value
>100	166 (15.4)	912 (84.6)	1078	[§]	0.001*
50-100	25 (16.7)	125 (83.3)	150	1.1 (0.7; 1.7)	0.084
<50	34 (20.7)	130 (79.3)	164	1.4 (0.9; 2.1)	0.688
Resident	61 (24.8)	185 (75.2)	246	1.8 (1.3; 2.5)	0.001*

[§] Reference group

* Statistically significant (p<0.05)

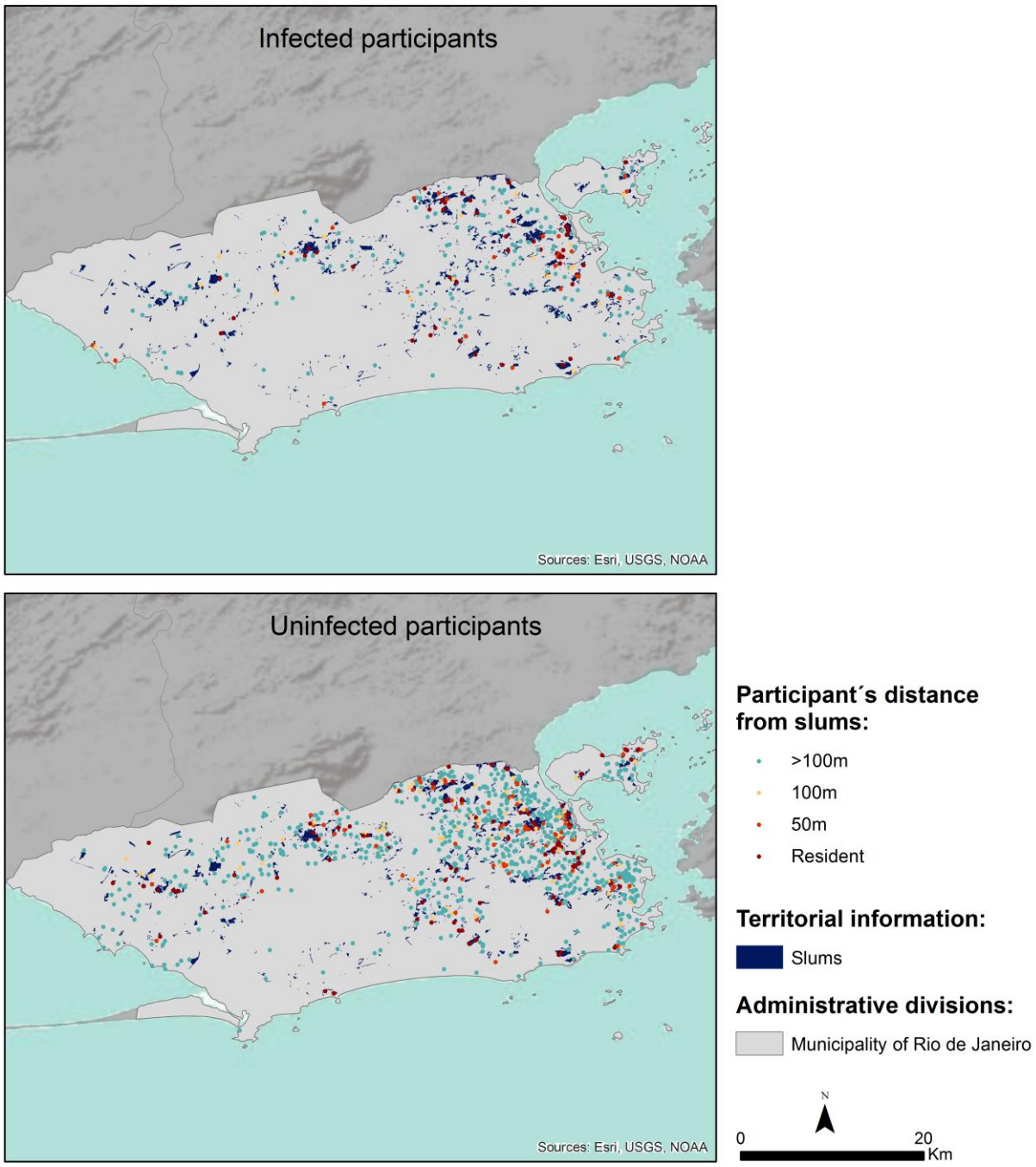

Figure 2.4. Geographical distribution of participants with and without intestinal parasitic infections and their distance over the slums in Rio de Janeiro municipality.

2.6. Discussion

The current study estimated the prevalence of intestinal parasitic infections among individuals from Rio de Janeiro State (Brazil), in addition to evaluating some epidemiological aspects. Spatial analysis was applied for the first time to the case of Rio de Janeiro to describe

the geographical distribution of individuals with enteric parasites infections. The study also looked at socio-economic indicators (social vulnerability indicator) for intestinal infections, in particular family income, education and sanitation (access to safe drinking water). The construction of a material deprivation index allowed us to identify the most vulnerable regions for intestinal parasitic infections in the metropolitan area of Rio de Janeiro State.

The prevalence of intestinal parasitic infections remains high in Rio de Janeiro State (17.5%) and also in the metropolitan region and the municipality (18.7% and 19%, respectively). Previous studies suggest that we may observe a decrease in the prevalence of intestinal parasites in Rio de Janeiro with time. A parasitological survey carried out in 1984 on children from day-care centres detected a prevalence of 35% (Santos *et al*., 1984). Further studies carried out on pregnant women (Macedo and Rey, 1996), children living in low income communities (Costa-Macedo *et al*., 1998) and day-care centres located in slums in the municipality (Uchôa *et al*., 2001) showed a prevalence ranging from 37.6% to 54.5%. A survey made in 2007 in a paediatric hospital (Carvalho-Costa *et al*., 2007) detected values of 18.3%. Although our results indicate that the prevalence is similar to this last study, it should be noted that individuals attending the Evandro Chagas National Institute of Infectious Diseases (INI/FIOCRUZ) were mainly adults, where it was expected that prevalence would be lower when compared to studies on children.

Age is an important risk factor for intestinal parasitic infections. Children are more susceptible to intestinal infectious diseases than adults because of their poor hygiene habits; they are often in contact with contaminated soil and their immune system is immature (Bethony *et al*., 2006; LaBeaud *et al*., 2015). In spite of our small number of samples from young participants, we observed a positive association between infections with *A. lumbricoides*, *E. vermicularis*, *B. hominis* and *G. lamblia* and the younger age.

The distribution of intestinal parasites varied among the municipalities that compose Rio de Janeiro State, with the highest incidence density of intestinal parasites in municipalities with larger population (Rio de Janeiro, Duque de Caxias, Nova Iguaçu, Belford Roxo and São João de Meriti). These results could be explained by the ease of access to the INI hospital, since these areas have the main road corridors of the municipality (Brazil Avenue, Governador Carlos Lacerda Avenue, Presidente João Goulart Avenue and Presidente Dutra highway), and also because many of the infected population lives near INI hospital. Despite São Gonçalo is the second largest municipality, only 2.4% (18 of 749) of the intestinal parasites were detected there. This municipality is located across the Guanabara

Bay, such that access by participants to the INI hospital is probably limited by poor public transportation.

The prevalence of enteric parasites varies between regions of Brazil, and contrasting data are observed: 11.3% in Sergipe (Santos *et al*., 2014); 42% reported from São Paulo (southeast) (Castro *et al*., 2015); 73.5% in Mato Grosso do Sul (midwest) Aguiar *et al*., 2007); 75.3% in Paraná (south) (Buschini *et al*., 2007); 77.2% in Bahia (northeast) (Mariano *et al*., 2015). However, data extracted from previous studies in Brazil should be analysed with some caution, once they were limited, isolated, and usually reflect the results from small towns and/or of restricted groups (day-care centres, schools, indigenous tribes, small hospitals, fishing villages, *etc*.). Attention should also be given to studies conducted in other countries: Argentina (78.3%) in children living in a poor area (Dib *et al*., 2015); Peru (66.3%) in orphanages (Bailey *et al*., 2013); Honduras (43.5%) in school going children (Mejia Torres *et al*., 2014); Pakistan (52.8%) in children residing in slum areas (Mehraj *et al*., 2008); and India (68%) in school going children (Greenland *et al*.,2015).

In the present work, the most common pathogenic species detected were Complex *E. histolytica/E. dispar* (13.5%) and *G. lamblia* (8.1%). These two parasites are frequently found in Brazil (Aguiar *et al*., 2007; Seixas *et al*., 2011; David *et al*., 2015; Mariano *et al*., 2015). However, detection *of G. lamblia* cysts is particularly alarming since these are resistant to conventional routine disinfectants, and are frequently found in sewage effluent and surface water (Hatam-Nahavandi *et al*., 2015). In addition, individuals infected with *G. lamblia* are largely asymptomatic, and can spread the infection, contributing to high epidemic rates. Similarly, concern should also be given to the presence of *B. hominis* (12.7%), since its pathogenicity is still controversial (Basak *et al*., 2014). In Minas Gerais (Brazil), Cabrine-Santos and colleagues (2015) observed that 8% of participants with diarrhoea had only *Blastocystis* spp. (monoparasitism); suggesting that the parasite may have a pathogenic character.

Although soil-transmitted helminths (*A. lumbricoides, T. trichiura*, hookworms and *S. stercoralis*) are the most frequent parasites found in many countries (Mejia Torres *et al*., 2014; LaBeaud *et al*., 2015), they were not the predominant enteric parasites in this study. Probably these parasites cannot complete their life cycles due the absence of an adequate soil environment or the presence of road/sidewalk paving or a high construction index (Barreto *et al*., 2010). The low prevalence of *S. mansoni* infections was also observed. The transmission of *S. mansoni* is dependent on the presence of a water and an intermediate host snail, which may be not available in the areas of this study. According to the Brazilian Ministry of Health

(MS, 2012), the positive rate of *S. mansoni* in Rio de Janeiro State is 1.56%, making it the State with the lowest number of confirmed cases.

We noticed a positive association between intestinal parasites and the male gender. Similar results are observed in Brazil (Cabrine-Santos *et al*., 2015) and Iran (Arani *et al*., 2008; Sadeghi *et al*., 2015), with a slightly higher prevalence of intestinal parasites in males than females. This association could be due to hygienic behaviours, specific occupations or even sexual activities, particularly among homosexuals, that may result in faecal/oral contact that subsequently leads to transmission of these agents (Schmerin *et al*., 1978; Escobedo *et al*., 2014).

Chemotherapy is one of the intervention strategies that reduce the incidence of intestinal diseases. Regular deworming with the drugs albendazole and mebendazole is the current global control strategy to reduce the prevalence of helminths, and is implemented in Brazil (MS, 2012). However, the deworming programmes are not effective against protozoa infections. In this study we clearly observed that the frequency of protozoan infections (87%) was much higher than that of helminths (13%). It is worth mentioning that nitazoxanide is an antiparasitic drug with a broad-spectrum activity against a variety of intestinal parasites (including protozoa and helminths). However, this product is not included in the list of pharmaceutical care products of the Unified Health System (SUS) in Brazil.

A number of individuals (141; 24.8%) were infected by multiple enteroparasites: 3.5% (5 of 141) of participants were infected with helminths, 80.9% (114 of 141) were infected with protozoa and 15.6% (22 of 141) by both. Polyparasitism remains persistent in the country: 18.4% of such cases were reported in São Paulo (Castro *et al*., 2015), 49.2% in Mato Grosso do Sul (Aguiar *et al*., 2007), 26.7% in Paraná (Buschini *et al*., 2007), 51.2% in Bahia (Mariano *et al*., 2015). These works all showed the high frequency of protozoa. Polyparasitism had been observed in many countries (Mehraj *et al*., 2008; Fletcher *et al*., 2014; Shiferaw *et al*., 2015); for example, in Kenya, 7% of the study population was infected with multiple parasites (LaBeaud *et al*., 2015), and Mejia Torres *et al.* (2014) observed that 14.6% of children in Honduras were infected with more than one parasite.

This study confirms that the population has a high frequency of intestinal parasites, principally protozoa. Although the majority of parasites (62%) were non-pathogenic (*B. hominis*, *E. coli*, *E. hartmani*, *E. nana* and *I. butschilii*), it is important to note that these species have the same transmission path as other pathogenic protozoa, such as Complex *E. histolytica/E. dispar* and *G. lamblia*, indicating exposure to faecal contamination. The frequency of these parasites added to the high frequency of polyparasitism can be used as

indicators of transmission through the faecal/oral route, thereby pointing to in the transmission of intestinal parasites via the supply of water for human consumption, or the ingestion of contaminated food.

Several authors have demonstrated the vulnerability of drinking water supply systems due contamination, which can lead to problems, such as the deterioration of water quality, which lead to the proliferation of pathogens, and, therefore, increase the risk of waterborne diseases (Dotorelo *et al*., 2013; Nescerecka *et al*., 2014). Water for the citizens of the metropolitan region of Rio de Janeiro is provided by two principal supply systems, called Guandu-Piraí and Imunana-Laranjal. Both of these undergo the conventional treatment process, including coagulation, flocculation, filtration (granulated active carbon), fluoridation and chlorination (ANA, 2010). Two companies carry out the operation and management of the water systems, one of which is public (State Company of Water and Sewage - CEDAE) and the other is a concession (Niterói Water). The Niterói Water Company only operates on the distribution of treated water, which is supplied by CEDAE from the water collected in the Imunana-Laranjal system. Although both systems operate satisfactorily, in agreement with Brazilian standards of technical quality and health (MS, 2004), water distribution generally has problems inherent in the characteristics of the use and occupation of urban land in the metropolitan region of Rio de Janeiro, particularly in the municipalities and neighbourhoods with higher levels of social and economic inequality. In these areas, lack of access to collection services and sewage treatment leads to the contamination of the water supply network through cross connections and low pressure zones, thereby leading to the entry of sewage and rainwater into the system. This situation is exacerbated in neighbourhoods and slums located in higher areas, where the pressure in the network is insufficient to maintain a constant water flow, and, according to the Brazilian Standard, drinkability (Barcellos *et al*., 1998; Freitas *et al*., 2001).

Although we did not directly investigate this matter, we know that in developing countries, such as Brazil, access to clean water, sanitation facilities and health infrastructure does not follow the population growth. Research conducted in two low-income communities of Campos dos Goytacazes (north of Rio de Janeiro State/Brazil) confirmed by water analysis that the entire underground water of the study area was contaminated and a high faecal contamination was detected in well water. The authors concluded that possibly inadequate sanitation, with sewage discharged directly into the soil in some points, visible leakage, along with inadequate, and negligent routine maintenance in some septic tank systems could

certainly have contributed to the dissemination of diseases caused by parasites (Moraes Neto *et al*., 2010).

The high prevalence of intestinal parasitic infections is also closely related to the low level of education, the low household incomes family and improper hygienic practices (Ostan *et al*., 2007; Mehraj *et al*., 2008; Moraes Neto *et al*., 2010). This study evaluated the socio and economic conditions of the Rio de Janeiro population using an index of material deprivation (MDI) composed of three indicators (sanitation, income and education).

The Rio de Janeiro metropolitan area is comprised of many census tracts (CTs), very close together and with very different MDIs, resulting in the highly heterogeneous character of the Rio de Janeiro territory. For example, while the INI hospital was classified in the first deprivation quintile (q1), a large part of the resident population in its surroundings live in slums or very poor neighbourhoods and was classified in the last deprivation quintiles (q4 or q5). Such proximity of participants to slums makes them more likely to be infected with intestinal parasites. Clearly, the geospatial distribution of the detected intestinal parasitic infections was not random or homogeneous, but was influenced by the MDI and the proximity to INI. Discrepancy of the MDIs among the closest CTs reveals the need for a horizontal decision-making process, not only in the poorest areas of the municipality, but throughout their surrounding areas.

Improvements in sanitation systems, deworming and the creation of poverty reduction programmes (Bolsa Família and Favela Bairro Program) in Brazil have helped greatly to reduce the prevalence of intestinal parasites over the years, but much obviously remains to be done. Safe drinking water is a defining aspect of a developed country, and even today it is still a significant challenge to public health worldwide. Additionally, the lack of access to health services near their home forces individuals to travel great distances to demand medical treatment, and, in many cases, the lack or deficiencies in public transport prevents these people from accessing the medical units.

Access to medical care, preventative chemotherapy and improvements in water supply and sanitation are matters of urgency, and also require a massive education campaign for low and middle-income families. Water of good microbial quality must be continuously supplied to the households (avoiding storage, which is another factor for contamination), and thus preventing its theft. Diseases are not distributed occasionally or randomly, the existence of risk factors determines their distribution, so that constant and continuous monitoring is required. Efforts directed to build a health surveillance system are urgent for Rio de Janeiro, and require strategies based on: sanitary conditions, water supply, population vulnerability,

socio-demographic and environmental factors such as age, gender, education, household characteristics and income. Knowing the geographical distribution of intestinal parasites in Rio de Janeiro population is an important first step that will assist in the decision-making process necessary to design effective preventive and control programs; however, more epidemiological studies are imperative. The ability to readily identify and reach individuals at highest risk of infection is an important aspect of parasitic disease control programmes.

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2.8. Supplementary data

Table S2. Number of positive and negative participants to intestinal parasites by regions.

Regions	Positive participants (n=569)		Negative participants (n=2676)		Total (n=3245)
	N	%	N	%	
RIO DE JANEIRO STATE					
Metropolitan region	532	93.5	2315	86.5	2847
Belford Roxo	25	4.4	98	3.7	123
Cachoeira de Macacu	3	0.5	3	0.1	6
Duque de Caxias	56	9.8	197	7.4	253
Guapimirim	0	0	11	0.4	11
Itaboraí	4	0.7	17	0.6	21
Itaguaí	2	0.4	11	0.4	13
Japeri	2	0.4	8	0.3	10
Magé	7	1.2	25	0.9	32
Maricá	1	0.2	24	0.9	25
Mesquita	2	0.4	20	0.7	22
Nilópolis	13	2.3	32	1.2	45
Niterói	3	0.5	38	1.4	41
Nova Iguaçu	41	7.2	203	7.6	244
Paracambi	0	0	8	0.3	8
Queimados	2	0.4	13	0.5	15
Rio Bonito	0	0	7	0.3	7
Rio de Janeiro	332	58.3	1416	52.9	1748
São Gonçalo	15	2.6	87	3.3	102
São João de Meriti	22	3.9	85	3.2	107
Seropédica	2	0.4	6	0.2	8
Tanguá	0	0	6	0.2	6
Others municipalities	21	3.7	60	2.2	81
Angra dos Reis	1	0.2	1	0	2
Araruama	3	0.5	7	0.3	10
Barra do Piraí	0	0	1	0	1
Barra Mansa	2	0.4	2	0.1	4
Cabo Frio	0	0	1	0	1
Campo dos Goytacazes	0	0	1	0	1
Comendador Levy Gasparian	1	0.2	0	0	1
Duas Barras	0	0	1	0	1
Iguaba Grande	0	0	2	0.1	2
Macaé	1	0.2	4	0.1	5
Mangaratiba	2	0.4	5	0.2	7
Miguel Pereira	0	0	1	0	1
Nova Friburgo	0	0	2	0.1	2
Parati	1	0.2	0	0	1
Paty de Alferes	0	0	1	0	1
Petrópolis	0	0	3	0.1	3
Pinheiral	0	0	2	0.1	2
Quissama	0	0	1	0	1
Resende	0	0	1	0	1
Rio Claro	0	0	1	0	1
Rio das Ostras	0	0	5	0.2	5
Santa Maria Madalena	0	0	1	0	1
Santo Antônio de Pádua	3	0.5	0	0	3
São Fidélis	0	0	1	0	1
São Francisco de Ibapoana	0	0	1	0	1
São José do Vale do Rio Preto	0	0	2	0.1	2
São Pedro da Aldeia	1	0.2	2	0.1	3
Saquarema	3	0.5	4	0.1	7
Teresópolis	0	0	1	0	1
Trajano Morais	0	0	2	0.1	2
Três Rios	1	0.2	0	0	1
Valença	1	0.2	0	0	1
Vassouras	0	0	1	0	1
Volta Redonda	1	0.2	3	0.1	4
OTHERS STATES OF BRAZIL	0	0	9	0.3	9
UNKNOWN	16	2.8	292	10.9	308

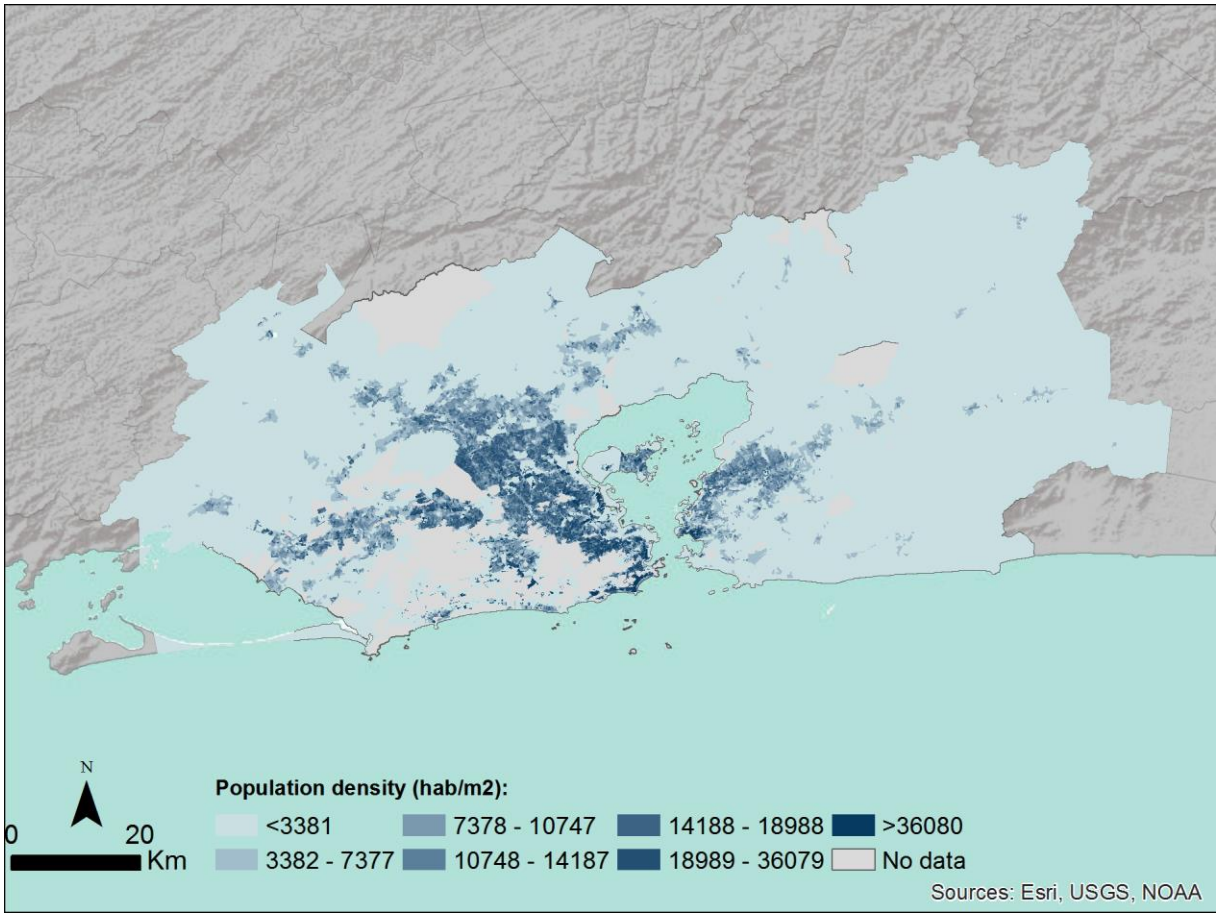

Figure S2.1. Population density of Rio de Janeiro State.

Chapter 3

Molecular characterization of *Giardia lamblia*: first report of assemblage B in human isolates from Rio de Janeiro (Brazil)

Clarissa P Faria, Graziela M Zanini, Gisele S Dias, Sidnei Silva, Maria C Sousa (**2016**), "Molecular Characterization of *Giardia lamblia*: First Report of Assemblage B in Human Isolates from Rio de Janeiro (Brazil)", Published in PLOS One 11(8): e0160762.

3.1. Abstract

Despite the high prevalence of giardiasis, the genetic characterization of *Giardia lamblia* has been poorly documented in Brazil and molecular epidemiology research has only been conducted in the last few years. The aim of this study was to determine the prevalence of different *G. lamblia* assemblages and detect mixed infections among patients with giardiasis. The cross-section survey was conducted among patients attending the FIOCRUZ in Rio de Janeiro. In order to discriminate the genetic assemblages/sub-assemblages, *G. lamblia* isolates were characterized by PCR-RFLP and qPCR using four loci genes (*bg*, *gdh*, *tpi* and *orf*C4). Of the 65 positive samples, 41 (63.1%) were successfully amplified by nested-PCR of *bg* and *gdh* genes. Among them, 16 were typed as sub-assemblage AII, 7 as BIII, 4 as BIV and 8 as a mixture of BIII and BIV. After the analysis by qPCR assay, a total of 55 (84.6%) samples were amplified using at least one locus: *bg* gene was amplified in 38 (58.5%) samples, *gdh* in 41 (63.1%), *tpi* in 39 (60%), and *orf*C4 in 39 (60%). Multilocus genotyping results showed that 29 (52.7%) samples belonged to assemblage A and 26 (47.3%) samples belonged to assemblage B. In 2011 and 2012, 20 (74.1%) samples belonged to assemblage A and 7 (25.9%) belonged to assemblage B. In subsequent years (2013-2015) there was a predominance of assemblage B, 19 (67.9%) *versus* 9 (32.1%) assemblage A. This is the first time that assemblage B of *G. lamblia* was reported in human clinical samples from Rio de Janeiro (Brazil) and is the first report about genetic characterization using four genes. The qPCR assemblage-specific showed no mixed infections by assemblages A and B. A switch in genetic profile over the years was observed, firstly predominance of assemblage A and lastly of assemblage B.

3.2. Introduction

Giardia lamblia (syn. *G. intestinalis*, *G. duodenalis*) is one of the most frequent human intestinal protozoa reported worldwide, with estimated prevalence rates of 20-30% in developing countries and 2-5% in industrialized countries (Feng and Xiao, 2011). Giardiasis has been included in the Neglected Diseases Initiative of the World Health Organization (WHO) since 2004 due its impact on health (Savioli *et al*., 2006).

G. lamblia is considered a species complex, whose members show little variation in their morphology but presents a remarkable genetic variability. This species is divided into eight distinct genetic assemblages (A-H), but only assemblages A and B are known to infect humans. The remaining assemblages are likely to be host specific, as assemblages C and D predominantly occur in dogs and other canids, assemblage E in hoofed livestock, assemblage F in cats, assemblage G in rats and assemblage H in marine mammals (Monis *et al*., 2009; Lasek-Nesselquist *et al*., 2010). Assemblage A was subdivided into three sub-assemblages: AI is preferentially found in animals; AII is commonly found in humans, although it was reported in a few studies in animals; and sub-assemblage AIII is exclusively found in animals. The host distribution of assemblage B, which was subdivided into two sub-assemblages BIII and BIV, is predominantly human and much less common in animals (Sprong *et al*., 2009; Ryan and Cacciò, 2013).

Mixtures of assemblages in individual isolates have often been observed, and the frequency of mixed infections may be underestimated by the use of a single marker (Ryan and Cacciò, 2013). The application of assemblage-specific primers coupled with the use of more than one molecular marker has been employed to assess, more accurately, the occurrence of mixed infections in clinical samples and to improve the detection of assemblages (Geurden *et al*., 2008; Almeida *et al*., 2010; Feng and Xiao, 2011). Until now the molecular analysis of *G. lamblia* samples at the β-giardin (*bg*), glutamate dehydrogenase (*gdh*) and triose phosphate isomerase (*tpi*) genes confirmed the high genetic variability within assemblages A and B (Wielinga and Thompson, 2007).

Assemblages A and B have been considered genetic variants of the same species. However, the latest studies suggest that the genomic differences between assemblages A and B are sufficient to classify them into two different species (Franzén *et al*., 2009; Adam *et al*., 2013). Certainly, the understanding of the epidemiology of giardiasis is committed by the uncertainty of taxonomy.

In Brazil the prevalence of giardiasis varies dramatically between different regions of the country mainly due to its enormous expanse of territory (David *et al*., 2015; Mariano *et al*., 2015). Despite the high prevalence of the infection, the genetic characterization of the parasite has been poorly documented and molecular epidemiology research has been conducted in the last few years (Souza *et al*., 2007; Kohli *et al*., 2008; Santos *et al*., 2012; Durigan *et al*., 2014; Colli *et al*., 2015; David *et al*., 2015; Oliveira-Arbex *et al*., 2015). Most of the epidemiological studies detected *G. lamblia* on the basis of microscopic examination (Carvalho *et al*., 2006; Santos *et al*., 2014; Castro *et al*., 2015; Mariano *et al*., 2015). So far only one study was performed with Rio de Janeiro samples (Volotão *et al*., 2007), consequently the *G. lamblia* assemblages in this city are poorly known. The objective of this study was to determine the prevalence of different *G. lamblia* assemblages and sub-assemblages among patients with giardiasis attending a referral hospital in Rio de Janeiro, therefore providing additional information on the molecular epidemiology of this parasite in the country. The study also aimed to determine the occurrence of mixed infections using primers targeting *gdh*, *tpi* and *orf*C4 genes specific for assemblages A and B.

3.3. Methods

3.3.1. Stool samples collection and laboratory methods

The cross-section survey was carried out from January 2011 to February 2015 in Evandro Chagas National Institute of Infectious Diseases (INI/FIOCRUZ), a referral hospital in infectious diseases in Brazil, located in Rio de Janeiro. This hospital receives patients from all municipalities, mainly the metropolitan area. According to the last census conducted in 2010, Rio de Janeiro had a population of 6.320.446 inhabitants and the metropolitan region, which is composed of 21 municipalities and is the second largest metropolitan area in Brazil, had 11.812.482 inhabitants (IBGE, 2010).

Stool samples were collected by the patient in plastic disposable flasks with and without preservatives and maintained at 4^{o}C until laboratory analysis on the same day. Flasks were labeled with the name, collection date and the hospital number. The medical request came along with the sample. The parasitological tests were conducted at the Parasitology Laboratory of INI by experienced laboratory technologists. This laboratory is certified by the College of American Pathologists.

For laboratory diagnosis of *G. lamblia*, the fresh specimens were analyzed by means of centrifugation sedimentation (de Carli, 2001) and centrifugal flotation in zinc sulphate

solution (Faust *et al*., 1938). Specimens preserved in MIF solution (mertiolate-iodine-formaldehyde) were processed by the centrifugation sedimentation method (de Carli, 2001). The slides were then observed under the microscope (Nikon Eclipse E200, magnification of 10 and 40X). In order to improve the detection of positive samples, a total of 262 randomly selected microscopy negative fecal samples were screened using the *Giardia lamblia* Antigen ELISA kit (Genway Biotech Inc., USA) according to the manufacturer's instructions. All patients attending INI/FIOCRUZ are dewormed when diagnosed (drugs are provided by the institution itself).

3.3.2. Molecular study

3.3.2.1. DNA extraction

The molecular analysis was performed only on samples without preservatives (n=65). Approximately 5g of fecal sample, positive for *G. lamblia,* was washed with distilled water, filtered through doubled gauze and then centrifuged (1000 x *g* for two minutes). These procedures were repeated two times. The concentrated cysts were stored at -20°C until DNA extraction. Samples collected in 2011 and 2012 were subjected to DNA extraction in 2013, whereas samples collected from 2013 were extracted regularly within one month of collection. DNA extraction was performed using the QIAamp DNA Stool mini Kit (Qiagen, Germany) according to the manufacturer's instructions. For PCR negative samples, a modified DNA extraction was implemented with minor modifications. In the first step, glass pearls and polyvinylpyrrolidone 10% solution was added and the incubation time was increased to one hour at 95°C; in the final steps, glycogen was added for DNA precipitation.

3.3.2.2. Nested-PCR

Extracted DNA was analyzed by nested-PCR using three *G. lamblia* gene loci: small-subunit ribosomal RNA (*ssu rRNA*) (Appelbee *et al*., 2003), β-giardin (*bg*) (Cacciò *et al*., 2002; Lalle *et al*., 2005) and glutamate dehydrogenase (*gdh*) (Read *et al*., 2004). Amplification of the *ssu rRNA* gene was performed with primers Gia2029 and Gia2150c in the primary PCR, and with RH11 and RH4 primers in the secondary reaction, generating a 292bp fragment (Appelbee *et al*., 2003) (Table 3.1). After an initial denaturation of 96°C for 4min, a set of 35 cycles was run, each consisting of 45s at 96°C, 30s of annealing (55°C for the primary reaction, 59°C for the second), and 45s at 72°C, followed by a final extension step of 4min at 72°C. The amplification of the *bg* gene was performed using a semi-nested PCR and a nested PCR protocols. The first amplification reaction was common to both PCR

protocols generating a 753bp fragment using the primer pair G7 and G759 (Cacciò *et al*., 2002). In the semi-nested PCR reaction, a fragment of 384bp was amplified using the primer pair G376 and G759 (Cacciò *et al*., 2002) and in the nested PCR a fragment of 511bp was amplified using the primer pair 2005F and 2005R (Lalle *et al*., 2005) (Table 3.1). The primary and the semi-nested amplifications were carried out as follows: 1 cycle of 94^{o}C for 5min, followed by 35 cycles of 94^{o}C for 30s, 65^{o}C for 30s and 72^{o}C for 1min. A final extension of 72^{o}C for 7min and a 4^{o}C hold was used. The nested PCR was performed with the following amplification conditions: 1 cycle of 95^{o}C for 15min, followed by 35 cycles of 95^{o}C for 30s, 65^{o}C for 30s and 72^{o}C for 1min, and a final extension of 72^{o}C for 7min. For the amplification of the 432pb fragment of *gdh* gene, a semi-nested PCR was done as described by Read *et al*. (2004). Primary PCR was run using the forward primer GDHeF and reverse primer GDHiR (Table 3.1). For secondary PCR, forward primer GDHiF and reverse primer GDHiR were used. The primary and the secondary reactions were performed under the following conditions: 1 cycle of 94^{o}C for 2min, 56^{o}C for 1min and 72^{o}C for 2min, followed by 55 cycles of 94^{o}C for 30s, 56^{o}C for 20s and 72^{o}C for 45s, and a final extension of 72^{o}C for 7min.

All reactions contained 12.5µL of NZYTaq 2x Green Master Mix (Nzytech, Portugal), 1µL of each primer (10pmol/µL), 1µL of extracted DNA and 8.5µL of sterile water, performing a final volume of 25µL. PCR was carried out on the MJ Mini™ Thermal Cycler (BioRad). In all PCR reactions, *Giardia*-positive DNA sample (strain WB, clone 6 [ATCC 30957]) and nuclease-free distilled water were used as positive and negative controls, respectively. The PCR products were analyzed on 1.5% agarose gels stained with ethidium bromide and visualized using a gel documentation system (Uvitec, UK).

Table 3.1. List and sequence of primers.

Locus	Primer	Sequence (5´-3´)	Reference
ssu rRNA*	Gia2029	AAGTGTGGTGCAGACGGACTC	Appelbee *et al*., 2003
	Gia2150c	CTGCTGCCGTCCTTGGATGT	
	RH11	CATCCGGTCGATCCTGCC	
	RH4	AGTCGAACCCTGATTCTCCGCCAGG	
bg*	G7	AAGCCCGACGACCTCACCCGCAGTGC	Cacciò *et al*., 2002
	G759	GAGGCCGCCCTGGATCTTCGAGACGAC	
	G376	CATAACGACGCCATCGCGGCTCTCAGGAA	
	2005F	GAACGAACGAGATCGAGGTCCG	Lalle *et al*., 2005
	2005R	CTCGACGAGCTTCGTGTT	
gdh*	GDHeF	TCAACGTYAAYCGYGGYTTCCGT	Read *et al*., 2004
	GDHiF	CAGTACAACTCYGCTCTCGG	
	GDHiR	GTTRTCCTTGCACATCTCC	
gdh*	gdhA_F	CCGGCAACGTTGCCCAGTTT	Almeida *et al*., 2010
	gdhA_R	ACTTGTCCTTGAACTCGGA	
	gdhB_F	CGTATTGGCGTCGGCGGT	
	gdhB_R	TGTGGCCTCTGGTCTGATAG	
tpi*	tpiA_F	TCGTCATTGCCCCTTCCGCC	Almeida *et al*., 2010
	tpiA_R	CGCTGCTATCCTCAACTG	
	tpiB_F	GATGAACGCAAGGCCAATAA	
	tpiB_R	GATTCTCCAATCTCCTTCTT	
orf*C4*	orfC4A_F	CTGTAGACAGGGCCCAGGCC	Almeida *et al*., 2010
	orfC4A_R	ATGATGTCCCCTGCCCTTAAT	
	orfC4B_F	ACTGTCCATTTCTATCTGAG	
	orfC4B_R	GGATTCCATTGGCCTCCACCT	

* Primers used in the nested-PCR; ** Primers used in the qPCR.

3.3.2.3. qPCR

The extracted DNA was also analyzed by real-time quantitative assay (qPCR) using *gdh*, *tpi* and *orf*C4 (open reading frame C4) genes as previously described (Almeida *et al*., 2010). Amplification reactions contained 12.5µL of Sso Fast™ EvaGreen SuperMix (BioRad, USA), 1µL of each primer (10pmol/µL), 1µL of extracted DNA and 9.5µL of sterile water, for a final volume of 25µL. All reactions were performed in triplicate; positive and negative controls were also included in each PCR. Cycle threshold (Ct) values of >36 were considered to reflect limited reproducibility due to low copy numbers.

qPCR assays were performed on CFX 96™ Real Time PCR Detection System (BioRad) and the primers used are listed in Table 3.1. A minor modification was done in the thermal

profile. All reactions started with a denaturation step at 98°C for 2min, followed by 40 cycles of denaturation (98°C for 5s) and annealing (59°C for 5s). The melting curve program was performed at the end of each reaction and consisted of 95°C for 5s, 65°C for 1min, and heating to 95°C with continuous acquisition (5 acquisitions per degree Celsius).

3.3.2.4. Genetic characterization of *G. lamblia*

The *bg* and *gdh* positive nested-PCR samples were subsequently analyzed by polymerase chain reaction-restriction fragment length polymorphisms (PCR-RFLP), in order to discriminate the *G. lamblia* genetic assemblages.

PCR-RFLP analysis was carried out by digesting the secondary PCR products of the nested-PCR of *bg* and *gdh* genes. The *bg* gene PCR products were digested with ten units of the endonucleases *Hha*I (384pb fragment) (Cacciò *et al*., 2002) and *Hae*III (511pb fragment) (Lalle *et al*., 2005) (New England Biolabs Inc., USA) in a final volume of 20µL for 4h at 37^{o}C. The amplified products of *gdh* gene were digested with two units of the endonucleases *Nla*IV or *Rsa*I (New England Biolabs Inc., USA) in a final volume of 20µL for 3h at 37^{o}C (Read *et al*., 2004). Profiles were analyzed on 3% agarose gels stained with ethidium bromide and visualized using a gel documentation system (Uvitec, UK).

Additionally, *G. lamblia* A and B assemblage-specific primers targeting the *gdh*, *tpi* and *orf*C4 genes (Table 3.1) were used to genotype *G. lamblia* assemblages and to detect mixed infections of assemblage A and assemblage B, in qPCR assay (Almeida *et al*., 2010).

3.3.3. Statistical analysis

The data entry was carried out using Excel software and analyzed using Statistical Package for the Social Sciences (SPSS) version 16. Percentages were used to perform the exploratory analysis of the categorical variables and quantitative variables are presented as mean ± standard deviation (SD). Pearson´s chi-squared and Fisher's Exact Test were used for categorical data. The level of statistical significance was set as $p<0.05$.

3.3.4. Ethical statement

The Research Ethics Committee Evandro Chagas National Institute of Infectious Diseases (INI/FIOCRUZ) approved the use of the patients stool samples (protocol number 127.542). This project was in accordance with the Brazilian Ethical Resolutions, especially Resolution CNS 196/1996 and its complementary and the Code of Medical Ethics of 1988 (articles 122 - 1307). Written informed consent was obtained from all patients or legal

guardians of patients younger than 18 years, prior to sample collection. The informed consent was provided after a detailed explanation of the objectives of the work. A term of privacy and confidentiality was signed by the researches for patients for whom it was not possible to obtain informed consent beforehand.

3.4. Results

3.4.1. Characteristics of study participants

During the study period, a total of 5179 patients were initially screened for the presence of intestinal parasites (helminths and protozoa) by direct microscopy. The detection of *G. lamblia* coproantigen was performed by ELISA on 262 patients out of 5081 microscopy negative samples. All *Giardia* positive samples (microscopically or ELISA) were submitted to molecular methods**.** The prevalence of enteroparasites found in this study was described previously by our team (Faria *et al*., unpublished data). *G. lamblia* cysts were identified in 32 of the 1554 (2.1%) patients attending the hospital in 2011; 29 of 1374 (2.1%) patients in 2012; 17 of 1190 (1.4%) in 2013; 19 of 938 (2%) in 2014, and 1 of 123 (0.8%) in 2015. Overall, 98 patients were infected with *G. lamblia*, of whom 65 stool sample (without preservative) were included in further molecular analysis. The majority of the patients were adults (69.2%) with an average of 32.54±13.69 years of age (Mean±SD; median=32). The highest *Giardia* prevalence occurred between 30-39 years (33.3%), and there were more male than female infected (69.2% *versus* 30.8%). Seventy-two percent of patients were educated above the primary; most of them (95.4%) live in metropolitan region and 61.5% live in Rio de Janeiro municipality.

3.4.2. Coproantigen ELISA

Among the 262 *Giardia* negative samples on microscopy analysis only one (0.4%) was positive for *Giardia* coproantigen ELISA.

3.4.3. Nested-PCR

Out of the 65 positive stool samples for *G. lamblia*, 13 (20%) were collected in 2011, 22 (33.8%) in 2012, 11 (16.9%) in 2013, 18 (27.7%) in 2014, and 1 (1.5%) in 2015 (Table 3.2). Among them, 60 (92.3%) were successfully amplified at the *ssu rRNA* gene, a multi copy gene, which largely confirms the microscopy results (Table 3.2). In relation to *bg* and

gdh, single copy genes, 41 (63.1%) samples were successfully amplified by nested-PCR: 38 (58.5%) at the *bg* locus and 32 (49.2%) in *gdh* locus (Table 3.2).

We could observe that nested-PCR efficiency was dependent on the sampling years. Amplification were less effective in samples collected in 2011 and 2012, only 31 samples of 35 (88.6%) were PCR-positive, whereas 29 samples of 30 (96.7%) were PCR-positive in subsequent years (Table 3.2).

Twenty-nine samples (44.6%) were amplified in three loci, 12 were amplified in two loci (18.5%) and 19 (29.2%) were only amplified in one locus (Table S3.1).

3.4.4. qPCR

After the analysis by qPCR assay, a total of 54 (83.1%) samples were amplified using at least one locus (Table 3.2). This technique was more sensitive than nested-PCR of *bg* and *gdh* detecting more 13 samples (20%). All positive samples in qPCR showed a Ct value less than 35 (ranged from 24 to 35) (Table S3.2). The *tpi* product was amplified in 48 samples (73.8%), followed by the *orf*C4 gene in 43 (66.2%) and the *gdh* gene in 37 samples (56.9%) (Table 3.2). As with nested-PCR, qPCR efficiency of the samples varied according to the collection year and time interval between collection and DNA extraction. Samples collected in 2011 and 2012 showed a lower efficiency (26 of 35, 74.3%) compared with samples from 2013 (28 of 30, 93.3%) (Table 3.2).

The three loci were amplified in 33 samples (50.8%), two loci in 8 (12.3%) samples and one locus in 13 samples (20%). The qPCR was negative in eleven samples (Table S3.1).

Table 3.2. Summary of the results of nested-PCR and qPCR in *Giardia lamblia* positive samples.

Year	Samples No. (%)	Nested-PCR				qPCR				Amplified samples (nested-PCR + qPCR) No. (%)	Not amplified samples No. (%)
		ssu rRNA	*bg*	*gdh*	*bg+gdh*	*tpi*	*orf*C4	*gdh*	*gdh+tpi+orf*C4		
2011	13 (20)	11 (16.9)	6 (9.2)	2 (3.1)	7 (10.8)	7 (10.8)	6 (9.2)	3 (4.7)	7 (10.8)	11 (16.9)	2 (3.1)
2012	22 (33.8)	20 (30.8)	9 (13.8)	7 (10.8)	10 (15.4)	16 (24.6)	12 (18.5)	10 (15.4)	19 (29.2)	20 (30.8)	2 (3.1)
2013	11 (16.9)	11 (16.9)	9 (13.8)	8 (12.3)	9 (13.8)	10 (15.4)	9 (13.8)	9 (13.8)	10 (15.4)	11 (16.9)	0
2014	18 (27.7)	17 (26.2)	13 (20)	14 (21.5)	14 (21.5)	15 (23.1)	15 (23.1)	15 (23.1)	17 (26.2)	17 (26.2)	1 (1.5)
2015	1 (1.5)	1 (1.5)	1 (1.5)	1 (1.5)	1 (1.5)	0	1 (1.5)	0	1 (1.5)	1 (1.5)	0
Total	65 (100)	60 (92.3)	38 (58.5)	32 (49.2)	41 (63.1)	48 (73.8)	43 (66.2)	37 (56.9)	54 (83.1)	60 (92.3)	5 (7.7)

ssu rRNA, multi copy gene*; bg, gdh, tpi* and *orfC4,* single copy genes.

3.4.5. Nested-PCR and qPCR

Nested-PCR and qPCR techniques successfully amplified 60 (92.3%) samples using at least one locus (Table 3.2). The amplification rates of the five markers used were 92.3%, 58.5%, 64.6%, 73.8%, 66.2% for the *ssu rRNA*, *bg*, *gdh*, *tpi* and *orf*C4 genes, respectively. The *ssu rRNA* gene, a multi copy gene, showed a better amplification rate by nested-PCR. In relation to the single copy genes, *tpi* showed better results by qPCR (Table 3.2). Regarding *gdh* gene, we could detect that 32 (49.2%) samples were successfully amplified in conventional PCR, 37 (56.9%) in qPCR, totaling 42 (64.6%) amplified samples.

All five loci were amplified in 26 (40%) samples, four loci in 15 (23.1%), three loci in 10 (15.4%), two loci in 4 (6.2%), and a locus gene in 5 (7.7%). Six samples (9.2%) were only amplified using nested-PCR (INI 6, 29, 37, 38, 42, and 46), 54 (83.1%) were amplified by both techniques (PCR + qPCR) and 5 (7.7%) samples were negative in all five loci despite repeated trials (INI 5, 22, 36, 40, 63) (Table S3.1). Most of the negative samples were collected between the years 2011 (2 samples) and 2012 (2 samples) (Table 3.2).

3.4.6. Genotyping analysis of *G. lamblia*

3.4.6.1. PCR-RFLP

The *G. lamblia* assemblage was successfully determined on 41 samples by PCR-RFLP at the *bg* and *gdh* locus gene: assemblage A was found in 21 samples (51.2%) and assemblage B in 20 samples (48.8%) (Table 3.3). Among the 38 samples amplified by *bg* gene, assemblage A was identified in 21 (55.3%) patients and assemblage B in 17 (44.7%). At the *gdh* locus, assemblage A was observed in 13 (40.6%) samples and assemblage B in 19 (59.4%) (Table 3.3).

Restriction patterns showed that 16 samples are sub-assemblage AII, and no sub-assemblage AI was identified; 7 were classified as BIII, 4 as BIV and 8 mixed infection (BIII + BIV); no mixed infection with the two assemblages A and B was identified (Table 3.4).

Table 3.3. Genotypic characterization of *Giardia lamblia* by PCR-RFLP and assemblage A- and B-specific qPCR.

Assemblage	PCR-RFLP			qPCR				Genotyped samples (PCR-RFLP + qPCR) No. (%)
	bg	*gdh*	Total (*bg*+*gdh*)	*gdh*	*tpi*	*orf*C4	Total (*gdh*+*tpi*+*orf*C4)	
A	21 (55.3)	13 (40.6)	21 (51.2)	20 (54.1)	27 (56.3)	22 (51.2)	28 (51.9)	29 (52.7)
B	17 (44.7)	19 (59.4)	20 (48.8)	17 (45.9)	21 (43.7)	21 (43.7)	26 (48.1)	26 (47.3)
Total	38	32	41	37	48	43	54	55

Table 3.4. Genotypic characterization of *Giardia lamblia* sub-assemblages by PCR-RFLP.

Sub-assemblage	PCR-RFLP		
	bg	***gdh***	**Total (*bg*+*gdh*)**
AII	16	13	16
BIII	-	7	7
BIV	-	4	4
BIII + BIV	-	8	8

3.4.6.2. qPCR assemblage-specific

The qPCR was used to allow a more accurate detection of mixed infections by the two assemblages (A + B) in clinical samples. At the *gdh* locus, assemblage A was observed in 20 (54.1%) patients and assemblage B in 17 (45.9%). At the *tpi* gene, 27 (56.3%) samples belonged to assemblage A and 21 (43.7%) belonged to assemblage B. Similar results were observed at *orf*C4 locus, where assemblage A was identified in 22 (51.2%) samples and assemblage B in 21 (48.8%). Regarding the three markers (*gdh*, *tpi*, *orfC4*), 28 (51.9%) samples belonged to assemblage A, 26 (48.1%) to assemblage B and no mixed infection (A + B) was detected (Table 3.3).

3.4.6.3. PCR-RFLP and qPCR assemblage-specific

The PCR-RFLP and qPCR results showed 100% concordance in typing *G. lamblia* isolates (Table 3.3). Although more sensitive, qPCR was unable to subtyping samples. Twenty-nine assemblage A and twenty-six assemblage B samples were identified by PCR-RFLP and qPCR, totalizing 55 (84.6%) genotyped samples (Table 3.3). At the *gdh* locus, 42 samples were amplified using the nested-PCR and qPCR: assemblage A was observed in 20 patients and assemblage B in 22, being the gene that detected a larger number of samples belonging to assemblage B (Table S3.1).

Due to amplification difficulties, not all samples were genotyped at the sub-assemblages level; 5 samples (INI 14, 15, 30, 32, 37) belonged to assemblage A and one (INI 34) to assemblage B (Table S3.1).

Analyzing the assemblages prevalences over the four years of this study, it is possible to observe in 2011 and 2012 the assemblage A was predominant (Table S3.1). In 2011 six samples belonged to assemblage A and two belonged to assemblage B, and in 2012, 14 samples were assemblage A and 5 were assemblage B. However, in subsequent years there was a predominance of assemblage B: in 2013 three samples belonged to assemblage A and

seven belonged to assemblage B; in 2014 six samples were assemblage A and 11 were assemblage B; in 2015 the sample belongs to assemblage B.

3.4.7. Socio-demographic profile of genetic assemblages

There were no statistical differences between assemblages A and B regarding residence, gender and age distribution ($p>0.05$) (Table 3.5). However, assemblage B had a slight prevalence between 30-39 year of age; probably due to a larger number of patients in this age group. As expected, Rio de Janeiro municipality had a greater number of patients (40 samples; 61.5%) infected with *G. lamblia* due to its larger population.

Table 3.5. Distribution of *Giardia lamblia* assemblages according to age groups, sex, housing municipality and sampling year.

Characteristic	Assemblage A	Assemblage B	Total
	No. (%)	No. (%)	
Age (years old)			
0-9	1 (3.5)	4 (15.4)	5
10-19	3 (10.3)	1 (3.8)	4
20-29	8 (27.6)	5 (19.2)	15
30-39	7 (24.1)	12 (46.2)	21
40-49	6 (20.7)	3 (11.5)	12
50-59	3 (10.3)	1 (3.8)	6
Missing	1 (3.5)	-	2
Gender			
Female	9 (31)	7 (26.9)	20
Male	20 (69)	19 (73.1)	45
Municipality			
Belford Roxo	1 (3.5)	-	1
Caxias	4 (13.8)	4 (15.4)	9
Nilópolis	-	1 (3.8)	1
Nova Iguaçu	3 (10.3)	5 (19.2)	9
Rio de Janeiro	18 (62.1)	15 (57.7)	40
Santo Antônio de Pádua	-	1 (3.8)	1
São Gonçalo	1 (3.5)	0	1
São João de Meriti	1 (3.5)	0	1
Missing	1 (3.5)	0	2
Year of collection			
2011	6 (20.7)	2 (7.7)	13
2012	14 (48.3)	5 (19.2)	22
2013	3 (10.3)	7 (26.9)	11
2014	6 (20.7)	11 (42.4)	18
2015	0	1 (3.8)	1

3.5. Discussion

Despite the high prevalence of giardiasis in Brazil, there is a lack of information on the genetic diversity of *G. lamblia*. So far only one study was performed with human samples in Rio de Janeiro and the genetic data was based just on the *bg* locus gene. The purpose of this study was to determine the prevalence of *G. lamblia* assemblages in patients attending the Evandro Chagas National Institute of Infectious Diseases (FIOCRUZ, Rio de Janeiro), using four different molecular markers.

Our study detected the presence of assemblages A (52.7%) and B (47.3%), with a slight predominance of the first. It is the first time that assemblage B was observed in human clinical samples in Rio de Janeiro. The previous study performed in Rio de Janeiro with human isolates reported only the presence of assemblage A (Volotão *et al*., 2007). The distribution of assemblage A and assemblage B varies greatly from one country to another and sometimes within the same country. Surely, due to the enormous expanse of territory of Brazil, prevalence of the assemblages varies between regions and contrasting data are observed. The majority of *G. lamblia* infections identified in Fortaleza (Kohli *et al*., 2008), Minas Gerais (Santos *et al*., 2012), Paraná (Colli *et al*., 2015) and São Paulo (Oliveira-Arbex *et al*., 2015) were assemblage B. Conversely, a study from São Paulo observed that assemblage A was more prevalent (Souza *et al*., 2007). Recently, David *et al*. (2015) and Durigan *et al*. (2014) showed no significant difference between assemblages distribution, being similar to findings by our group. In Latin America, Ramírez *et al*. (2015) reported that assemblage B was predominant in Colombia, while in México Torres-Romero *et al.* (2014) note predominance of assemblage A and in Cuba Puebla *et al*. (2014) found that assemblages A and B were detected at equal frequencies.

In our study, a change in the genetic profile over the years could be observed as well. Assemblage A was most prevalent until the year 2012, while in the subsequent years (2013-2015) there was an increased number of cases of assemblage B. Kohli *et al*. (2008) reported that children with assemblage B shed significantly more cysts than children infected with assemblage A. This coupled with fecal-oral transmission, may contribute to the higher prevalence and dispersion of assemblage B. We observed a predominance of assemblage B in adults between the ages 30 to 39 years, although this may be due to a greater number of patients in this age group. A study conducted in England (Minetti *et al*., 2015) also detected a higher prevalence of assemblage B in adults in their 30s and 40s and a predominance of assemblage A in older people (≥70 years old. Though we cannot draw any conclusions with

the results obtained here, further research is needed in order to evaluate the possible aforementioned association.

Regarding assemblage A, the samples that were genotyped at the sub-assemblage level (16 of 29; 55.2%) were characterized as sub-assemblage AII, an anthroponotic genotype (Ryan and Cacciò, 2013). Similarly, previous works about human giardiasis in Brazil showed that sub-assemblage AII was identified more often than sub-assemblage AI (Souza *et al*., 2007; Durigan *et al*., 2014, Colli *et al*., 2015; David *et al*., 2015; Oliveira-Arbex *et al*., 2015). Nevertheless, these results contrast once more with the data obtained in Rio de Janeiro (Volotão *et al*., 2007), where 97% of the samples were identified as sub-assemblage AI, a genotype commonly found in animals. Besides changes in frequency of infections with different assemblages over time, we could also observe a switchover in the sub-assemblages, where sub-assemblage AI was not detected and sub-assemblage AII became more frequent. Among assemblage B, our study observed a greater number of patients with sub-assemblage BIII, which is not in agreement with previous results from Brazil, where sub-assemblage BIV was identified more often (Durigan *et al*., 2014; Colli *et al*., 2015; Oliveira-Arbex *et al*., 2015). The detection of sub-assemblages AII, BIII and BIV in the clinical samples of patients from Rio de Janeiro suggest that transmission of giardiasis may be mainly person-to-person (directly or indirectly by water or food), since both assemblages are predominant in humans. This hypothesis is also supported by the absence of sub-assemblage AI, mostly found infecting companion animals and livestock (Sprong *et al*., 2009), in patients of our study.

The prevalence of each assemblage varies from region to region, but assemblage B seems to be more common in Brazil and in other countries than assemblage A (Sprong *et al*., 2009; Feng and Xiao, 2011; Ryan and Cacciò, 2013). *G. lamblia* assemblages are not evenly distributed, and the reasons are still unclear. Possibly, the distribution is not geographically associated and may be explained by parasite factors (such as the rate of multiplication, variable surface proteins expressed, resistance to pharmaceuticals, and ability to invade immune response), host factors (such as immune status, history of exposure, diet, and concomitant intestinal microbiota), different transmission routes and infection sources (Cotton *et al*., 2011; Feng and Xiao, 2011). In addition, the preferential amplification of one particular locus gene could also account for the differences in prevalence of assemblages (Sprong *et al*., 2009; Almeida *et al*., 2010).

Even though no significant statistical difference was observed between assemblages distribution (52.7% assemblage A and 47.3% assemblage B), mixed infections with both assemblages (A + B) was not detected. However, intra-assemblage mixed infections were

observed in assemblage B (BIII+BIV). The presence of more than one assemblage infecting one patient has been previously recorded in Brazil (Kohli *et al*., 2008; Durigan *et al*., 2014) and in other countries (Feng and Xiao, 2011). Sometimes the failure to detect these mixtures is not due to the absence of the mixed infection, but because one assemblage can be preferentially amplified over another at one locus (Ryan and Cacciò, 2013). To prevent such thing we used qPCR assemblage-specific (Almeida *et al*., 2010), and confirmed that mixed infection were not present in the studied samples. The qPCR technique also improved the *G. lamblia* detection, in relation to nested-PCR of *bg* and *gdh* genes. The increased specificity and sensitivity, as well as simultaneous detection of assemblages A and B are benefits that qPCR provided in the present study.

Additionally, genotyping by PCR-RFLP and qPCR were identical and no divergence or inconsistencies in the assemblages were found among the four different loci. It is important to highlight that none out of 49 samples, simultaneously positive at two, three or four loci, had incongruent genotyping results (each other). So we are very confident that the assemblage's profile of the samples successfully characterized represents the genetic population of *G. lamblia* in Rio de Janeiro. These results are in contrast with previous reports where incongruent genotyping results have been reported (Cacciò *et al*., 2008; Sprong *et al*., 2009; Huey *et al*., 2013). However, the use of several molecular markers (MLG) allowed us to considerably increase the detection of *G. lamblia* assemblages and to determine, in a reliable manner, the distribution of assemblages across the population.

The *ssu rRNA* gene is strongly conserved and due their multicopy nature, the PCR that targets this locus has a high sensitivity (Wielinga and Thompson, 2007; Thompson and Ash, 2016). Thus, to ensure the presence or absence of *G. lamblia* DNA, the *ssu rRNA* gene was used. Among the 65 positive samples, we could detect *G. lamblia* DNA in 60 samples, largely confirming the microscopy results.

Nevertheless, our molecular approach did not amplify five samples (7.7%) (false-negatives). Therefore we concluded that DNA degradation, insufficient DNA or inhibition of PCR could have limited the success of the amplification. The stool samples were stored without preservatives at -20°C before DNA extraction. Probably the freezing for several months (or years) may have led to degradation of the cysts/DNA of *G. lamblia*. Minetti *et al*. (2015) had similar results, where the overall PCR amplification success of the samples varied accordingly to the year of collection and the time from collection to DNA extraction. Specimens collected recently (extracted within one month) showed a higher amplification success rate compared with amplification of older specimens. Many researchers have reported

the difficulty of typing assemblages, even in the microscopically positive samples. Typically, the failure in amplifying is related to the nature of the biological sample used, DNA ineffective extraction and DNA degradation. Stool samples contain PCR inhibitors, such as bilirubin, bile salts, hemoglobin, phenolic compounds, and complex polysaccharides, which are co-purified together during DNA extraction. It also has enormous quantity of non-specific DNA and the potential for low numbers of cysts. Prolonged storage of the stool samples also take into consideration, old samples may possible have degradation of genetic material (Wilke and Robertson, 2009; Kuk *et al*., 2012).

Moreover, the DNA region between the PCR primers and the genomic sequences could be degraded or there was nucleotide mismatches, that may cause a strong reduction or even a lack of amplification, specially taking into consideration that *bg*, *gdh*, *tpi* and *orf*C4 genes were single copy (Broglia *et al*., 2013). In negative samples, new DNA extraction was performed with minor modifications: addition of glass beads and a solution of polyvinylpyrrolidone (PVP) in the first step; increasing the incubation time (5 minutes for 1 hour at 95^{o}C); and addition of glycogen for DNA precipitation. Despite this procedure the samples remained negative.

The detection of *G. lamblia* by microscopy has been demonstrated to be less sensitive compared to ELISA (Feng and Xiao, 2011). According to Sommer *et al*. (2015), *Giardia* cysts are eliminated along with the feces intermittently, which make the coproantigen ELISA the most reliable method for detection of an infection with this protozoan parasite. Conversely, a recent study comparing rapid diagnostics methods observed ELISA false-positive for *G. lamblia* (den Bossche *et al*., 2015). In our study, among the 262 *Giardia* negative samples analyzed by microscopy, only one (0.4%) was positive for *Giardia* coproantigen ELISA and was not amplified by molecular methods (false-positive). Perhaps the patient's immune system eliminated the parasite, having only the release of antigens in feces. These results allowed us to conclude that collecting three fecal specimens on alternate days, the use of cysts concentration technique and microscopy observation by experienced laboratory technologists is an appropriate approach for *G. lamblia* detection.

This is the first description of genetic characterization of *G. lamblia* in Brazil using *bg*, *gdh*, *tpi* and *orf*C4 genes, and it is also the first time that assemblage B of *G. lamblia* was reported in human clinical samples from Rio de Janeiro. A switch in genetic profile over the years was observed, firstly predominance of assemblage A and lastly of assemblage B. The frequency of sub-assemblages has changed completely, where AI disappeared and mixed infection within assemblage B was detected. Obviously, the detection of anthroponotic sub-

assemblages (AII, BIII and BIV) does not exclude the investigation of the role of animals in the dynamic of transmission, especially because information on *G. lamblia* isolated from animals of Rio de Janeiro (and others States in Brazil) are scarce. Few studies have been performed in Brazil regarding assemblages of *G. lamblia* in samples obtained from humans and animals. Further studies using accurate molecular typing are imperative for unraveling the intricate epidemiology of giardiasis.

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3.7. Supplementary data

Table S3.1. Genotyping of 65 *G. lamblia* positive samples based on *ssu rRNA*, *bg*, *gdh*, *tpi* and *orf*C4 genes.

Year	Isolates (ID)	Genes					
		ssu rRNA	***bg***	***gdh***		***tpi***	***orf*C4**
		Nested-PCR	**PCR-RFLP**	**PCR-RFLP**	**qPCR**	**qPCR**	**qPCR**
2011	INI 30	**Pos**	**A**	Neg	**A**	**A**	**A**
	INI 31	**Pos**	Neg	Neg	Neg	**B**	**B**
	INI 32	**Pos**	**A**	Neg	Neg	**A**	**A**
	INI 33	**Pos**	Neg	**BIII**	**B**	**B**	**B**
	INI 35	**Pos**	**AII**	Neg	Neg	**A**	Neg
	INI 37	**Pos**	**A**	Neg	Neg	Neg	Neg
	INI 39	**Pos**	**AII**	Neg	Neg	**A**	**A**
	INI 41	**Pos**	**AII**	**AII**	**A**	**A**	**A**
	INI 29, 38, 42	**Pos**	Neg	Neg	Neg	Neg	Neg
	INI 36, 40	Neg	Neg	Neg	Neg	Neg	Neg
2012	INI 1, 7, 8, 12	**Pos**	Neg	Neg	**A**	**A**	**A**
	INI 2, 3	**Pos**	Neg	Neg	Neg	**A**	Neg
	INI 4	**Pos**	Neg	Neg	**A**	Neg	Neg
	INI 10	**Pos**	**B**	**BIII + BIV**	**B**	**B**	**B**
	INI 11, 17, 19, 23	**Pos**	**AII**	**AII**	**A**	**A**	**A**
	INI 13	**Pos**	Neg	**BIII**	Neg	Neg	**B**
	INI 14, 15	**Pos**	**A**	Neg	Neg	**A**	Neg
	INI 21	**Pos**	**B**	**BIV**	Neg	Neg	**B**
	INI 24	**Pos**	Neg	Neg	Neg	**B**	Neg
	INI 25	**Pos**	Neg	Neg	Neg	**A**	**A**
	INI 34	**Pos**	**B**	Neg	Neg	**B**	Neg
	INI 5, 22	Neg	Neg	Neg	Neg	Neg	Neg
	INI 6	**Pos**	Neg	Neg	Neg	Neg	Neg
2013	INI 26	**Pos**	Neg	Neg	**B**	**B**	**B**
	INI 27	**Pos**	**B**	**BIV**	**B**	**B**	**B**
	INI 28, 43	**Pos**	**B**	**BIII**	**B**	**B**	**B**
	INI 44	**Pos**	**AII**	Neg	**A**	**A**	**A**
	INI 45, 47	**Pos**	**AII**	**AII**	**A**	**A**	**A**
	INI 48	**Pos**	**B**	**BIV**	**B**	**B**	Neg
	INI 49	**Pos**	**B**	**BIII**	Neg	**B**	**B**
	INI 50	**Pos**	**B**	**BIII + BIV**	**B**	**B**	**B**
	INI 46	**Pos**	Neg	Neg	Neg	Neg	Neg

Table S3.1 (*Continued*)

2014	INI 51, 52, 58, 59, 60, 66	**Pos**	**AII**	**AII**	**A**	**A**	**A**
	INI 53, 57	**Pos**	**B**	**BIII**	**B**	**B**	**B**
	INI 54, 61, 67	**Pos**	**B**	**BIII + BIV**	**B**	**B**	**B**
	INI 55, 62	**Pos**	Neg	Neg	**B**	**B**	**B**
	INI 56	**Pos**	Neg	Neg	Neg	Neg	**B**
	INI 64	**Pos**	**B**	**BIII + BIV**	Neg	**B**	Neg
	INI 65	**Pos**	Neg	**BIV**	**B**	**B**	Neg
	INI 68	**Pos**	**B**	**BIII + BIV**	**B**	Neg	**B**
	INI 63	Neg	Neg	Neg	Neg	Neg	Neg
2015	INI 69	**Pos**	**B**	**BIII + BIV**	Neg	Neg	**B**

Pos, positive

Neg, negative

Table S3.2. Threshold cycle values.

Isolates (ID)	Genes		
	gdh	***tpi***	***orfC4***
INI 1	34	31	30
INI 2		33	
INI 3		31	
INI 4	29		
INI 7	33	33	31
INI 8	33	32	33
INI 10	32	32	30
INI 11	32	30	31
INI 12	33	32	33
INI 13			34
INI 14		33	
INI 15		33	
INI 17	27	26	27
INI 19	26	28	26
INI 21			32
INI 23	31	30	30
INI 24		32	
INI 25		32	34
INI 26	32	32	34
INI 27	30	30	27
INI 28	32	31	30
INI 30	33	31	31
INI 31		33	32
INI 32		33	34
INI 33	31	33	33
INI 34		35	
INI 35		31	
INI 39		29	30
INI 41	31	30	30
INI 43	32	28	27
INI 44	29	28	29
INI 45	30	29	30
INI 47	32	30	30
INI 48	33	33	
INI 49		34	34
INI 50	34	35	32
INI 51	27	24	26
INI 52	26	24	25
INI 53	32	31	28
INI 54	33	33	33
INI 55	34	35	34
INI 56			35
INI 57	32	32	30
INI 58	31	27	31
INI 59	30	28	29
INI 60	30	28	32

Table S3.2 (*Continued*)

INI 61	29	28	27
INI 62	31	32	31
INI 64		35	
INI 65	33	33	
INI 66	30	28	29
INI 67	32	35	33
INI 68	32		33
INI 69			32

Chapter 4

New multilocus genotypes of *Giardia lamblia* human isolates

Clarissa P Faria, Graziela M Zanini, Gisele S Dias, Sidnei Silva, Maria C Sousa (**2016**), "New multilocus genotypes of *Giardia lamblia* human isolates", submitted to Infection, Genetics and Evolution.

4.1. Abstract

Giardia lamblia is considered a species complex, whose members show little variation in their morphology, but have a remarkable genetic variability. The aim of this study was to identify inter- and intra-assemblage genetic variation in *G. lamblia* among patients in Rio de Janeiro. The parasitological study was performed on faeces and DNA was extracted from the positive samples for *G. lamblia*. The genetic assemblages and subtypes were determined via multilocus sequence typing (MLST) using β-giardin, triose phosphate isomerase and glutamate dehydrogenase gene loci. Fourteen assemblage A samples were successfully genotyped at the three MLST loci (*bg*/*tpi*/*gdh*). Two previously identified multilocus genotypes were found (AII-1 and AII-4), and two novel multilocus genotypes are proposed (AII-8, profile A2/A2/A4; AII-9, profile A3/A2/A2). Sequence analysis showed that assemblage B isolates have a higher nucleotide variation than assemblage A isolates. Novel assemblage B sequences were described and most (66.7%) had heterogeneous nucleotides, which prevented the definition of multilocus genotypes. It is the first time that MLST has been used to characterise *G. lamblia* isolates in human clinical samples from Rio de Janeiro. Additionally, MLST enabled the detection of novel subtypes in both assemblages and the description of two novel multilocus genotypes in assemblage A. This study provides new insight into the genetic diversity of assemblage A and shows that MLST should be used to characterise *G. lamblia* in Brazil and around the world.

4.2. Introduction

Giardia lamblia (syn. *Giardia intestinalis*, *Giardia duodenalis*) is an intestinal protozoa found in a wide range of hosts including humans, and domestic and wild animals. This parasite is considered a species complex with at least eight distinct assemblages (labelled A to H), but only assemblages A and B have been detected in both humans and a wide range of other mammalian hosts. The other assemblages are likely to be host-specific: C and D are mainly found in dogs and other canids, E in hoofed livestock, F in cats, G in rodents and H in marine mammals (Monis *et al*., 1999, 2003; Thompson, 2004; Lasek-Nesselquist *et al*., 2010). Molecular genetic analyses have shown that *G. lamblia* assemblage A is divided into three sub-assemblages: AI, mainly zoonotic; AII, commonly anthroponotic, although it has been reported in a few studies on animals, and AIII, found only in animals. Assemblage B, which is divided into two sub-assemblages, BIII and BIV, is predominantly found in humans and much less commonly in animals (Sprong *et al*., 2009; Ryan and Cacciò, 2013).

PCR-based molecular analysis techniques have been used to investigate the genetic diversity of *G. lamblia*. Initially, most of the molecular epidemiological studies were based on the analysis of a single locus, but parasite assemblages are currently distinguished by multilocus genotyping (MLG) tools. Small subunit ribosomal RNA (*ssu rRNA*) (Hopkins *et al*., 1997; Appelbee *et al*., 2003), β-giardin (*bg*) (Cacciò *et al*., 2002; Lalle *et al*., 2005), glutamate dehydrogenase (*gdh*) (Read *et al*., 2004; Cacciò *et al*., 2008) and triose phosphate isomerase (*tpi*) (Sulaiman *et al*., 2003; Bertrand *et al*., 2005) genes are the four genetic markers commonly used in genotyping and subtyping *G. lamblia* in many host species and geographical locations. As the amount of sequence information increased, incongruent genotyping results (lack of concordance in the assignment of isolates to a specific assemblage) began to be reported. Thus, the analysis of a single locus was not considered sufficient to reliably determine the distribution of assemblages across the population (Cacciò *et al*., 2008; Sprong *et al*., 2009; Almeida *et al*., 2010; Huey *et al*., 2013). Therefore, the use of MLG tools became imperative.

Based on MLG analysis of the *bg*, *gdh*, and *tpi* genes, Cacciò *et al*. (2008) proposed a subtype nomenclature system with 10 different MLGs for assemblage A isolates (AI-1, AI-2, AII-1, AII-2, AII-3, AII-4, AII-5, AII-6, AII-7, AIII-1). The same was not done for assemblage B because of the greater diversity encountered between and within these isolates. A precise nomenclature for the MLGs of assemblage B isolates was impossible to be

proposed due the presence of heterogeneous templates (overlapping nucleotide peaks at specific positions in the sequence).

The distribution of assemblages A and B varies greatly in Brazil, largely because of the enormous expanse of territory involved. While earlier reports demonstrated the prevalence of assemblage A (Souza *et al*., 2007; Volotão *et al*., 2007), recent works have found a prevalence of assemblage B infection (Kohli *et al*., 2008; Santos *et al*., 2012; Colli *et al*., 2015; Oliveira-Arbex *et al*., 2015), and others have found no significant difference between the distribution of the assemblages (Durigan *et al*., 2014; David *et al*., 2015; Nunes *et al*., 2016; Faria *et al*., 2016).

The aim of this study was to identify inter- and intra-assemblage genetic variation in *G. lamblia* based on multilocus sequence typing (MLST) of the *bg*, *tpi* and *gdh* genes and to define MLGs for assemblages A and B in patients with giardiasis attending a referral hospital in Rio de Janeiro, Brazil.

4.3. Materials and methods

4.3.1. Population study

A total of 65 faecal samples positive for *G. lamblia* were collected between January 2011 and February 2015 from patients attended in Evandro Chagas National Institute of Infectious Diseases (INI/FIOCRUZ), a referral hospital in infectious diseases in Rio de Janeiro, Brazil.

The Research Ethics Committee Evandro Chagas National Institute of Infectious Diseases (INI/FIOCRUZ) approved the study (protocol number: 127.542). This project was in accordance with the Brazilian Ethical Resolutions, especially Resolution CNS 196/1996 and its complementary and the Code of Medical Ethics of 1988 (articles 122 - 1307). Written informed consent was obtained from all patients or legal guardians of patients younger than 18 years, prior to sample collection. The informed consent was provided after a detailed explanation of the objectives of the work. A term of privacy and confidentiality was signed by the researches for patients for whom it was not possible to obtain informed consent beforehand.

4.3.2. DNA extraction

The molecular analysis of *G. lamblia* was performed only on faecal samples without preservatives. Approximately 5g of faecal sample was washed with distilled water, filtered through doubled gauze, and then centrifuged (1000 x *g* for two minutes). These steps were repeated two more times. The concentrated cysts were stored at -20°C until DNA extraction, which was performed using the QIAamp DNA Stool mini Kit (Qiagen) according to the manufacturer's instructions. A new DNA extraction was performed in PCR-negative samples with minor modifications. In the first step, glass pearls and polyvinylpyrrolidone 10% solution was added and the incubation time was increased to one hour at 95°C, and in the finals steps glycogen was added for DNA precipitation. Genomic DNA was preserved at -20^{o}C until analysis.

4.3.3. PCR analysis

Extracted DNA was subsequently analysed by PCR using three *G. lamblia* gene loci: *bg*, *tpi* and *gdh* (Table 4.1). The amplification of the *bg* gene was performed using a semi-nested PCR protocol (Cacciò *et al*., 2002). The primer pair G7 and G759 was used in the primary PCR and a 753bp fragment was generated. In the semi-nested PCR reaction, a fragment of 384bp was amplified using the primer pair G376 and G759. Nested PCR amplification of fragments of the *tpi* (530 bp) gene was done using primers AL3543 and AL3546 in the first round, and AL3544 and AL3545 in the second round, as described by Sulaiman *et al*. (2003). For the amplification of the *gdh* gene, a semi-nested PCR and a nested PCR were done as described by Read *et al*. (2004) and Cacciò *et al*. (2008), respectively. The forward primer GDHeF and reverse primer GDHiR were used for the semi-nested PCR in the primary reaction. For secondary PCR, forward primer GDHiF and reverse primer GDHiR were used and a fragment of 432bp was generated. For the nested PCR reaction, a fragment of 530bp was amplified using the primers Ghd1 and Gdh2 for the primary amplification, and Gdh3 and Gdh4 for the nested amplification. The cycling conditions (primary and secondary reactions of nested-PCR) for the *bg*, *tpi*, and *gdh* genes were performed as follows: an initial denaturation step of 94^{o}C for 5min, followed by 35 cycles of 30s at 94^{o}C, annealing for either 30s (65^{o}C for *bg*, and 50^{o}C for *gdh*) or 45s (50^{o}C for *tpi* gene), 1min at 72^{o}C, and a final extension of 72^{o}C for 7min. The primary and the secondary reactions of *gdh* semi-nested PCR were performed under the following conditions: 1 cycle of 94^{o}C for 2min, 56^{o}C for 1min and 72^{o}C for 2min, followed by 55 cycles of 94^{o}C for 30s, 56^{o}C for 20s and 72^{o}C for 45s, and a final extension of 72^{o}C for 7min.

Table 4.1. List and sequence of primers used for the molecular amplification of *Giardia lamblia* isolates.

Locus	Primer	Sequence (5´-3´)	Reference
bg	G7	AAGCCCGACGACCTCACCCGCAGTGC	Cacciò *et al*., 2002
	G759	GAGGCCGCCCTGGATCTTCGAGACGAC	
	G376	CATAACGACGCCATCGCGGCTCTCAGGAA	
tpi	AL3543	AAATIATGCCTGCTCGTCG	Sulaiman *et al*., 2003
	AL3544	CCCTTCATCGGIGGTAACTT	
	AL3545	GTGGCCACCACICCCGTGCC	
	AL3546	CAAACCTTITCCGCAAACC	
gdh	GDHeF	TCAACGTYAAYCGYGGYTTCCGT	Read *et al*., 2004
	GDHiF	CAGTACAACTCYGCTCTCGG	
	GDHiR	GTTRTCCTTGCACATCTCC	
gdh	Gdh1	TTCCGTRTYCAGTACAACTC	Cacciò *et al*., 2008
	Gdh2	ACCTCGTTCTGRGTGGCGCA	
	Gdh3	ATGACYGAGCTYCAGAGGCACGT	
	Gdh4	GTGGCGCARGGCATGATGCA	

All reactions contained 12.5µL of NZYTaq 2x Green Master Mix (Nzytech), 1µL of each primer (10pmol/µL), 1µL of extracted DNA and 8.5 µL of sterile water, performing a final volume of 25µL. PCR was carried out on the MJ Mini™ Thermal Cycler (BioRad). In all PCR reactions, *Giardia*-positive DNA sample (strain WB, clone 6 [ATCC 30957]) and nuclease-free distilled water were used as positive and negative controls, respectively. The PCR products were analysed on 1.5% agarose gels stained with ethidium bromide and visualized using a gel documentation system (Uvisave, Uvitec).

4.3.4. Multilocus sequencing and phylogenetic analysis

Positive secondary PCR products were purified using QIAquick PCR Purification kit (Qiagen), quantified (ND1000, NanoDrop) and sequenced with secondary PCR primers in both directions using the respective forward and reverse primers with an Applied Biosystems 3730 xL DNA Analyser (Applied Biosystems). Chromatograms and nucleotide sequences were analysed, edited using BioEdit Sequence Alignment Editor Programme (http://www.mbio.ncsu.edu/bioedit/bioedit. html), and were aligned with reference sequences retrieved from the GenBank using Clustal W. In order to determine the *Giardia* assemblage, sub-assemblage and subtype, BLAST software (http://www.ncbi.nlm.nih.gov/blast/) was used for comparison of the nucleotide sequences obtained in the present work with sequences

available at GenBank. The phylogenetic analysis was performed in MEGA software v.6 (www.megasoftware.net) using neighbour-joining (NJ) and maximum likelihood (ML) algorithms with evolutionary distances calculated by Tamura-Nei method. Bootstrap analysis was applied to evaluate the reliability of clusters by using 1000 replicates. The sequences from the three loci (*bg*, *tpi* and *gdh*) were also concatenated. The accession numbers and the corresponding assemblages of the sequences obtained from the GenBank database are displayed in Table S4.1. The novel sequences without ambiguous positions from this study were deposited in the GenBank under accession numbers KX085486-KX085495.

4.4. Results

Of the 65 samples positive for *G. lamblia*, 39 were successfully amplified and sequenced (Table 4.2). Sequence results showed that 18 samples belonged to assemblage A, 21 samples belonged to assemblage B and no mixed infections (A + B) were detected. Thirty-one (79.5%) samples were amplified at the *bg* locus, 32 (82.1%) at *gdh* locus and 37 (94.9%) at *tpi* locus. Twenty-nine samples were amplified in all loci, three were amplified in two loci and seven were only amplified at one locus (one were amplified at *bg* locus, one at *gdh*, and five at *tpi*). Among the 32 samples amplified in more than one locus, no incongruent assignment to assemblages ("assemblage swapping") was detected.

Table 4.2. Genotyping results of the 39 positive stool samples for *Giardia lamblia* based on three different loci using sequence analysis.

Assemblage	1 locus			2 loci		3 loci	Total
	bg	***tpi***	***gdh***	***bg* + *tpi***	***tpi* + *gdh***	***bg* + *tpi* + *gdh***	
A	1	3	-	-	-	14	18
B	-	2	1	1	2	15	21
Total	1	5	1	1	2	29	39

4.4.1. Assemblage A sequences

All 18 samples classified as assemblage A corresponded to sub-assemblage AII. Among the 15 samples amplified at the *bg* locus, nine showed complete sequence identity with the previously described subtype A2 (KC8, accession number AY072723) and five were identical to subtype A3 (ISSGF7, accession number AY072724) (Table 4.3). One sample (INI 17) showed overlapping nucleotides (C - T / Y) at the positions 421 and 429, which could classify the sample as subtype A2 and/or A3 (Table 4.3).

Of the 17 samples successfully amplified at the *tpi* locus, all sequences showed 100% sequence identity with subtype A2 (JH, accession number U57897) except the sample INI 44 where a single nucleotide polymorphism (SNP) was observed (G - A at position 349) (Table 4.3). This sample showed complete identity with two previously described sequences, KF922901 and KF922892 from São Paulo/Brazil (Durigan *et al*., 2014) (Table 4.3), and is distinct from the six subtypes (A1-A6) described by Cacciò *et al*. (2008) and thus represents a new subtype. No overlapping nucleotides were observed at *tpi* sequences.

Fourteen samples were amplified at *gdh* locus, seven being identical to subtype A2 (Bris136, accession number AY178737) and the others were identical to subtype A4 (ECUST 2196, accession number JX994237) (Table 4.3). No sequences with novel substitutions or overlapping nucleotides, at any position, were observed at *gdh* sequences.

Table 4.3. Polymorphisms at the β-giardin, triose phosphate isomerase and glutamate dehydrogenase loci of assemblage A samples compared to reference sequences obtained from GenBank.

Sub-assemblage	**Subtype**	**Isolate (ID)**	**Nucleotide position**			
β-giardin			421	429	567	690
AI	A1	**WB**	C	T	C	A
AII	A2	**KC8**, INI 19, 44, 47, 51, 52, 58, 59, 60, 66			T	G
AII	A3	**ISSGF7**, INI 11, 23, 39, 41, 45	T	C	T	G
AII	A2 and/or A3	INI 17	Y	Y	T	G
Triose phosphate isomerase			129	349		399
AI	A1	**WB**	T	G		C
AII	A2	**JH**, INI 7, 11, 19, 20, 23, 25, 38, 41, 45, 47, 51,	C			T
AII	Novel A2	INI 44[§]	C	A		T
Glutamate dehydrogenase			699	753	807	831
AI	A1	**Portland1**	T	C	C	C
AII	A2	**Bris136**, INI 11, 41, 45, 47, 51, 58, 60	C	T	T	T
AII	A4	**ECUST 2196**, INI 19, 20, 23, 44, 52, 59, 66		T	T	T

Nucleotide substitutions are numbered from the ATG codon of each gene, empty spaces indicate identity to the A1 reference sequence (GenBank accession numbers: AY258617 for *bg*, L02120 for *tpi* and M84604 for *gdh*). Isolates in bold are reference sequences from the GenBank.

[§]Sequence previously observed by Durigan *et al*., 2014 (GenBank acc. No. KF922901 and KF922892)

4.4.2. Assemblage B sequences

At the *bg* locus, 12 (75%) of 16 assemblage B sequenced samples exhibited no ambiguous nucleotides at any position in the chromatogram and six subtypes could be identified, one had already been described and five were novel (Table 4.4). Three samples (INI 54, 61 and 64) showed complete identity with a previously described sequence (KF736104, sub-assemblage BIII) (Table 4.4). The sequences of four samples (INI 27, 48, 50 and 67) were classified as new variants of BIII reference sequence (Ld18, AY072726) and the remaining five samples (INI 21, 49, 53, 57 and 68) were classified as new variants of BIV reference sequence (ISSGF4, AY072728). These nine samples showed novel substitutions at positions 393, 396, 456, 477, 525, 570, 606, 615, 714 and 717, along *bg* sequence and prevented the assigned to a specific subtype. All of them showed 99% identity with previous published sequences.

At the *tpi* locus, 12 (60%) of 20 samples displayed unambiguous nucleotides (Table 4.4). Six different subtypes were identified: nine samples corresponded to three previously reported subtypes and three samples were novel subtypes. The sequence of five samples (INI 33, 43, 49, 53 and 56) showed 100% identity with the sub-assemblage BIII reference sequence (Bah12, AF069561) (Table 4.4). One sample (INI 50) showed complete identity with the published sequence AY368165 (BIII / isolate 2434) and three samples (INI 48, 65 and 69) had sequences identical to BIV isolate Ad-19 (AF069560). The sequences of INI 21 and INI 55 showed 99% identity with the BIV isolate GS/M (L02116) and Ad19, respectively. The sequence of sample INI 64 corresponded to a mixture of sub-assemblages BIII and BIV, and seven SNPs were observed along the sequence.

Among the 18 sequences obtained at the *gdh* locus, only five (27.8%) exhibited no double peaks and four different subtypes were identified (Table 4.4). One sample (INI 26) was identical to BIII isolate Bah 12c14 (EF685684) and other sample (INI 33) showed complete sequence identity with BIII isolate FCQ 21 (AY178756) (Table 4.4). INI 48 sequence was identical to sub-assemblage BIV reference sequence (Ad28, AY178738) or to JX972186 (which was found in a horse in Colombia). Two samples (INI 27 and 65) showed 99% identity with the BIV reference sequence (Ad28, AY178738), with only one SNP (G - A at position 354). Moreover, the two latter isolates showed 100% identity with two previously described sequences (KT334248 and KT334251), however with just 52% of coverage.

Table 4.4. Polymorphisms at the β-giardin, triose phosphate isomerase and glutamate dehydrogenase loci of assemblage B samples compared to reference sequences obtained from GenBank.

Isolate (ID)	***bg***									
	393	396	456	477	525	570	606	615	714	717
BIII (Ld18)	A	C	C	T	T	C	C	C	C	C
BIV (ISSGF4)					C	T	T	T	-	-
BIII (KF736104), INI 54, 61, 64	G	T								
INI 21, 68	G	T			C	T	T	T	T	T
INI 27, 48				C						
INI 50	G	T							T	T
INI 49, 53, 57	G	T			C	T	T		T	T
INI 67	G	T	T							

	tpi																
	39	45	91	165	168	210	217	271	280	297	304	393	402	429	438	483	504
BIII (Bah12), INI 33, 43, 49, 53, 56	-	T	C	C	C	G	G	C	A	A	A	C	A	G	T	A	C
BIV (Ad19), INI 48, 65, 69	A		T	T	T	A								A			
BIV (GS/M)	A		T	T	T	A											
INI 21	A		T	T	T												T
BIII (isolate 2434), INI 50	G										G	T					
INI 55	A		T	T	T	A	A							A			
INI 64	G	C	T	T	T			T	G	G			G		C	G	

	gdh													
	309	354	429	447	519	540	561	612	699	723	807	825	867	921
BIII (Bah12c14), INI 26	-	-	T	T	C	C	C	G	T	C	G	A	C	G
BIII (FCQ21), INI 33	Y	G			Y				C				Y	
BIV (Ad28), INI 48	T	G	C	C	C	T	T	A	C	T	T	G		A
INI 27, 65	T	A	C	C	C	T	T	A	C	T	T	G		A

Nucleotide substitutions are numbered from the ATG codon of each gene, empty spaces indicate identity with the BIII reference sequence (GenBank accession numbers: AY072726 [Ld18] for *bg*, AF069561 [Bah12] for *tpi* and EF685684 [Bah12c14] for *gdh*). Isolates in bold are reference sequences from the GenBank. The dash (-) indicates deletion.

4.4.3. Multilocus analysis

A total of 14 assemblage A samples were successfully genotyped at the three MLST loci (*bg*/*tpi*/*gdh*) (Table 4.5). Two previously identified MLGs were found: AII-1, profile A2/A2/A2, in four samples (INI 47, 51, 58, 60); AII-4, profile A3/A2/A4, in one sample (INI 23). Two novel MLGs were identified: AII-8 (proposed), profile A2/A2/A4, in four samples (INI 19, 52, 59, 66); AII-9 (proposed), profile A3/A2/A2, in three samples (INI 11, 41, 45). One sample (INI 17), with overlapping nucleotides, could be identified as AII-4 and/or on the new proposed MLG, AII-8. One novel assemblage A MLG was observed (INI 44), and could not be classified according to Cacciò *et al*. (2008) nomenclature proposal.

Table 4.5. Multilocus genotyping results of assemblages A and B from 15 samples based on sequencing data from the β-giardin, triose phosphate isomerase and glutamate dehydrogenase loci.

Assemblage	Isolate (ID)	Subtype			MLG
		bg	***tpi***	***gdh***	
A	INI 47, 51, 58, 60	A2	A2	A2	AII-1
	INI 23	A3	A2	A4	AII-4
	INI 19, 52, 59, 66	A2	A2	A4	AII-8 (proposed)
	INI 17	A2 or A3	A2	A4	AII-4 or AII-8
	INI 11, 41, 45	A3	A2	A2	AII-9 (proposed)
	INI 44	A2	Novel A2	A4	Novel AII
B	INI 48	Novel BIII	BIV	BIV	Novel B

A total of 15 assemblage B samples were sequenced at all three genes. Due the high number of heterogeneous nucleotides at *gdh* gene (13 isolates), only one isolate (INI 48) had no double peak at any position and were classified as a novel assemblage B MLG (Table 4.5).

The classification of the 21 assemblage B isolates into sub-assemblages is shown in Table 4.6. Mixed infection within the sub-assemblages BIII + BIV was identified in one sample (INI 64) at *tpi* locus. Incongruent genotyping results were observed in an intra-assemblage level, five isolates (INI 21, 27, 48, 64 and 69) were classified BIII at *bg* locus and as sub-assemblage BIV at the others loci.

Table 4.6. Multilocus sequencing typing of *Giardia lamblia* assemblage B isolates.

Isolate (ID)	Sequencing		
	bg	***tpi***	***gdh***
INI 10	BIII	N/A	N/A
INI 21	BIII	BIV	
INI 26		BIII	
INI 27	BIII	N/A	BIV
INI 28	N/A	N/A	N/A
INI 33		BIII	BIII
INI 43	N/A	BIII	N/A
INI 48	BIII	BIV	BIV
INI 49, 53	BIII	BIII	N/A
INI 50	BIII	BIII	N/A
INI 54, 61, 67	BIII	N/A	N/A
INI 55		BIV	
INI 56			BIII
INI 57	BIII	BIII*	BIII*
INI 64	BIII	BIII + BIV	N/A
INI 65		BIV	BIV
INI 68	BIII	N/A	N/A
INI 69	BIII*	BIV	N/A

Empty spaces indicate isolates that were not sequenced; N/A indicate isolates that could not be assigned to a specific sub-assemblage; and asterisks (*) indicate isolates containing heterogeneous nucleotides.

4.4.4. Heterogeneous sequencing profile in assemblage B

Regarding the assemblage B isolates, the number of sequences showing heterogeneous nucleotides was much higher than among assemblage A samples. Additionally, the percentage of samples with double peaks varied among the three different genes, being higher in *gdh* locus (13 of 18; 72.2%), followed by *tpi* (8 of 20; 40%) and *bg* (4 of 16; 25%) loci (Table 4.7).

At the *bg* gene, double peaks occurred randomly at eight positions (C or T / Y). Two samples (INI 10 and INI 69) were classified as new variants of BIII. Nonetheless two isolates (INI 28 and 43) could not be classified in sub-assemblage due to the great number of heterogeneous nucleotides (Table 4.7).

Overlapping nucleotides were observed at 25 positions in the *tpi* gene and the majority occurred at positions 39, 91, 165, 168, 210 and 429 (positions used to differentiate BIII and BIV). Forty-five positions presented double peaks at *gdh* gene, most of them occurred at

positions 309, 357, 360, 429, 447, 519, 540, 546, 612, 699, 723, 807, 825, 834, 867 and 933 (nine of these positions have been proposed for sub-assemblage differentiation). The majority of samples could not be assigned to a specific sub-assemblage at the *tpi* (INI 10, 27, 28, 54, 61, 67 and 68) and *gdh* (INI 10, 28, 43, 49, 50, 53, 54, 61, 64, 67, 68 and 69) loci due the presence of ambiguous nucleotides in specific positions. Only one sequence (INI 57) can be classified as BIII sub-assemblage in both loci (Table 4.7).

4.4.5. Phylogenetic analysis

The tree obtained from the concatenated *bg*, *tpi* and *gdh* sequences confirmed the monophyletic status of assemblages A and B (Figure 4.1), and presented similar results in comparison with the phylogenetic analysis of individual genes (Figures S4.1, S4.2 and S4.3). The sequences distinctly grouped together into the expected assemblages A and B. Dendrograms were able to differentiate *G. lamblia* assemblages, sub-assemblages and subtypes, with high bootstrap support.

The maximum likelihood and neighbour joining trees placed all sequences classified as assemblage A in one cluster with sub-assemblage AII sequences references, with high bootstrap values. Additionally, the analyses showed that the MLGs identified in our samples were identical or very closely related to previously described MLGs (AII-1 and AII-4) in human isolates from Brazil and Europe. The proposed MLGs (AII-8 and AII-9), as well as the new MLG (INI 44, novel AII), clustered with MLGs previously described (Cacciò *et al*., 2008). The only assemblage B isolate (INI 48) in which the MLG can be performed has been grouped with isolates from assemblage BIV and was classified as a new MLG.

Table 4.7. List of heterogeneous positions in β-giardin, triose phosphate isomerase and glutamate dehydrogenase genes sequences of assemblage B.

Isolate (ID)	***bg***																										
	393			396			477			525			570			606			615			636			714			717		
BIII (Ld18)*	A			C			T			T			C			C			C			C			C			C		
BIV (ISSGF4)*										C			T			T			T			C			-			-		
INI 10	G			T						**Y**																				
INI 28										C			**Y**			**Y**			**Y**			**Y**			**Y**			**Y**		
INI 43										C			T			**Y**			**Y**						**Y**			**Y**		
INI 69	G			T			**Y**																							
	tpi																													
				1	1	1	1	1	2	2	2	2	2	2	2	2	3	3	3	3	4	4	4	4	4	5	5			
	3	4	9	4	6	6	6	6	1	1	1	5	6	7	8	9	0	1	8	9	0	2	7	8	8	0	1			
	9	1	1	1	0	2	5	8	0	6	7	6	4	1	0	7	4	2	1	3	2	9	1	3	6	4	0			
BIII (Bah12)*	-	-	C	C	G	G	C	C	G	C	G	G	G	C	A	A	A	C	C	C	A	G	A	A	G	C	G			
BIV (Ad19)*	A	G	T				T	T	A													A								
INI 10	**R**	G	**Y**				**Y**	**Y**		**Y**													**M**							
INI 27	A	G	**Y**	**Y**		**R**	**Y**	**Y**	**R**													**R**								
INI 28	G	**R**	**Y**				**Y**	**Y**			**R**		**R**										**M**		**R**					
INI 54	**R**	G	**Y**		**R**		**Y**	**Y**	**R**													**R**				**Y**				
INI 57	G	G															**R**			**Y**										
INI 61	**R**	G	**Y**				**Y**	**Y**				**R**						**Y**					**M**							
INI 67	A	G	**Y**				**Y**	**Y**						**Y**	**R**	**R**			**Y**		**R**		C	G			A			
INI 68	**R**	G	**Y**				**Y**	**Y**	**R**													**R**								

	gdh																																								
	2	3	3	3	3	3	3	3	4	4	4	4	4	4	5	5	5	5	5	5	5	5	6	6	6	6	6	6	6	7	7	7	7	7	8	8	8	8	8	8	8	8	9	9
	9	0	5	5	5	6	7	9	0	2	4	8	9	9	1	4	4	6	7	7	8	9	1	1	2	6	8	9	9	0	0	2	3	5	0	1	2	3	4	4	6	9	9	2	3
	7	9	3	4	7	0	0	0	2	9	7	6	2	8	9	0	6	1	0	6	2	7	2	5	4	0	7	0	9	2	5	3	8	6	7	5	5	4	1	3	7	4	7	1	3
BIII (Bah12c14)*	-	-	-	-	-	-	-	-	-	T	T	T	C	T	C	C	C	C	C	G	G	C	G	A	A	C	C	G	T	C	T	C	G	T	G	A	A	C	C	C	C	C	C	G	T
BIV (Ad28)*	C	T	C	G	T	G	G	C	C	C	C					T		T					A						C			T			T		G							A	C
INI 10	**Y**	C	C	G	**Y**	**R**	**R**	C	C						**Y**	**Y**	**Y**	**Y**											**Y**			**Y**		**Y**	**K**		**R**								C
INI 28	C	**Y**	C	G	**Y**	**R**	G	C	C						T		**Y**										**Y**		**Y**			**Y**	**R**		**K**			**Y**			**Y**				**Y**
INI 43	C	**Y**	C	G	**Y**	**R**	G	C	C						T		**Y**												C						**K**	**R**		T			**Y**				C
INI 49	C	**Y**	C	G	**Y**	**R**	G	C	C	**Y**					T		**Y**												**Y**				**R**		**K**			**Y**			**Y**				C
INI 50	C	T	C	G	C	**R**	G	**Y**	C	**Y**	**Y**				**Y**	**Y**	**Y**												**Y**						T			T			T				C
INI 53	C	**Y**	**Y**	G	**Y**	**R**	G	C	**Y**	**Y**	**Y**	**Y**		**Y**	**Y**		**Y**	**Y**			**R**		**R**						C			**Y**			**K**			**Y**			**Y**				**Y**
INI 54	C	**Y**	C	G	**Y**	G	G	C	C	**Y**	**Y**				**Y**	**Y**			**Y**			**Y**	**R**					**R**	C	**Y**	**Y**	T		C	**K**		**R**		**Y**					**Y**
INI 57	C	C	C	G	T	G	G	C	C						T		**Y**												C						**K**	G		T			T				C
INI 61	**Y**	**Y**	C	**R**	**Y**	**R**	G	C	C	**Y**	**Y**				**Y**	**Y**	**Y**	**Y**	**Y**			**Y**	**R**						C			**Y**		**Y**	**K**		**R**						**R**	**Y**
INI 64	**Y**	**Y**	C	G	T	G	G	C	C	**Y**	**Y**		**Y**		**Y**	**Y**	**Y**						**R**												T			T							C
INI 67	C	C	C	G	T	G	G	C	C	**Y**	C				**Y**	**Y**				**R**				**R**	**R**	**Y**						**Y**			T		**R**	**Y**			**Y**	**Y**	**R**	**Y**
INI 68	C	T	C	G	C	**R**	G	C	C	**Y**	**Y**				**Y**	**Y**	**Y**		**Y**			**Y**	**R**					**R**	**Y**		**Y**	**Y**	**R**	**Y**	T		**R**	**Y**			**Y**				C
INI 69	**Y**	**Y**	C	**R**	**Y**	**R**	G	C	C	**Y**	**Y**				**Y**	**Y**		**Y**					A												T		G			**Y**				**R**	**Y**

Nucleotide substitutions are numbered from the ATG codon of each gene, empty spaces indicate identity with the BIII reference sequence (GenBank accession numbers: AY072726 [Ld18] for *bg*, AF069561 [Bah12] for *tpi*, and EF685684 [Bah12c14] for *gdh*). Asterisks (*) indicates reference sequences obtained from GenBank. Heterogeneous positions are in bold and the dash (-) indicates deletion.

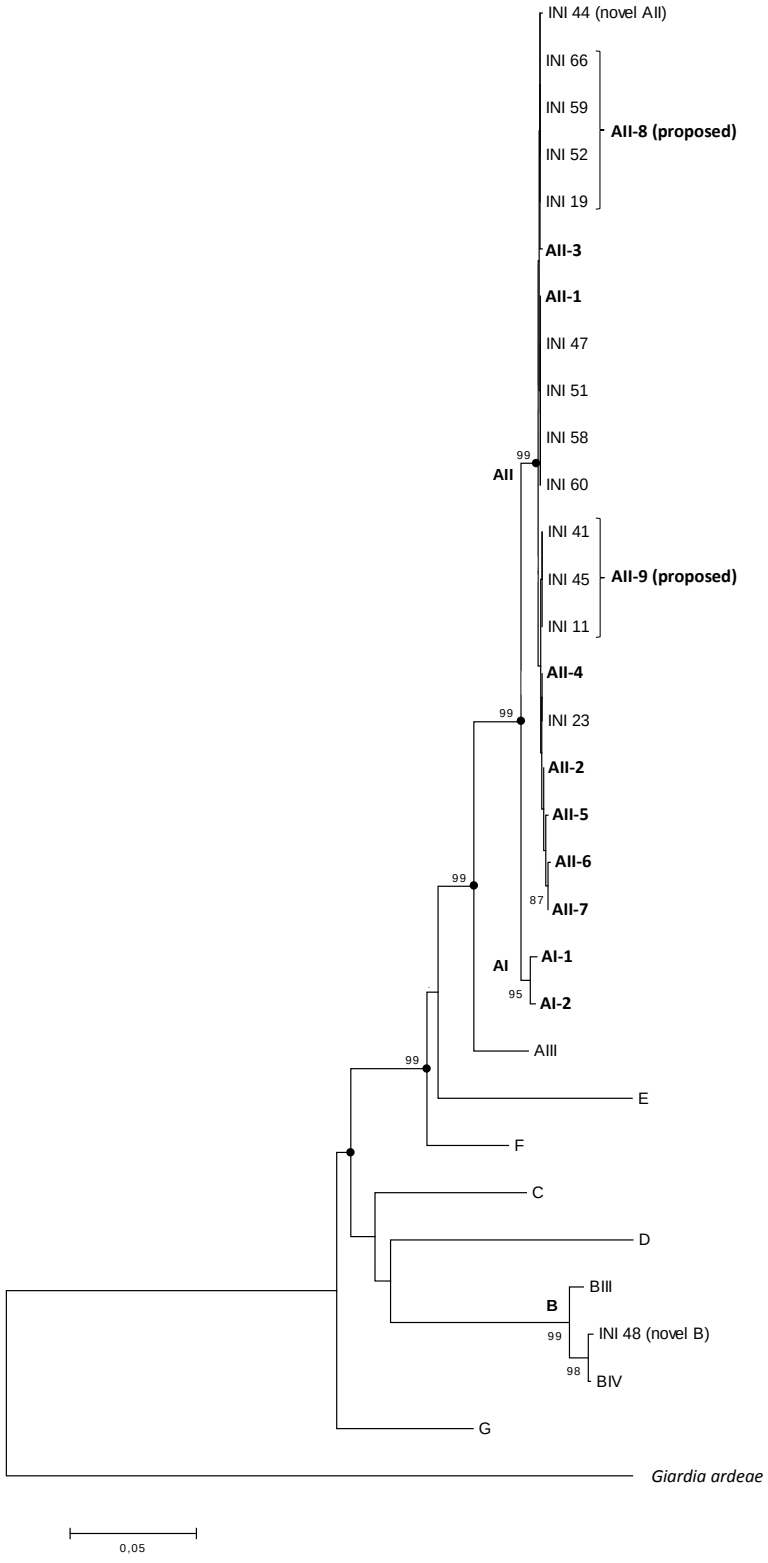

Figure 4.1. Phylogenetic relationship of *Giardia lamblia* isolates constructed by neighbour-joining analysis of concatenated *bg*, *tpi* and *gdh* nucleotide sequences. Multilocus genotypes from a previous study (Cacciò *et al*., 2008) are indicated in bold. Symbol (●) indicates nodes branches conserved when the tree was reconstructed using maximum likelihood. Optimal nucleotide substitution model was Tamura-Nei, and only bootstraps values >80% are shown.

4.5. Discussion

This work is the first study to use multilocus sequence typing (MLST) of the *bg*, *tpi* and *gdh* genes to characterise *G. lamblia* in Rio de Janeiro/Brazil. We have looked at the genetic variability of *G. lamblia* isolates, identified the inter- and intra-assemblage level of genetic variation and described two novel MLGs in assemblage A.

Sequence analysis showed that assemblage B isolates have a higher genetic polymorphism than assemblage A isolates, with heterogeneous nucleotides found in fourteen of twenty-one isolates. The presence of double peaks hindered the classification of sub-assemblages and subtypes and in most cases prevented the unambiguous identification of MLGs. For instance, a considerably higher genetic variability was observed in *gdh* gene, with the majority of sequences having double peaks along the chromatogram that often coincided with positions used to differentiate the sub-assemblages. Considerable genetic variability was also observed in *tpi* and *bg* genes. Moreover, SNPs were observed in the sequence of twelve assemblage B isolates, at positions that affect the identification of sub-assemblages and subtypes within the assemblage. These results showed that the sequenced isolates displayed mainly novel polymorphisms and that assemblage B isolates exhibited many more subtypes than assemblage A, which is in agreement with several works from other research groups (Cacciò *et al*., 2008; Lebbad *et al*., 2008; Minetti *et al*., 2015). The highest number of subtypes was identified in the *bg* (six) and *tpi* (six) genes, followed by *gdh* (four). We identified five new subtypes at the *bg* locus, and one subtype previously described in isolates from a roe deer and a red deer in Poland (Stojecki *et al*., 2015). At the *tpi* gene, three novel subtypes were detected and three subtypes have been reported before (Monis *et al*., 1999; Sulaiman *et al*., 2004). At *gdh* locus, four different subtypes have been identified, one new and three that have previously been described, one of which was detected in a horse from Colombia (Santín *et al*., 2013).

Assemblage A sequences across the three studied loci showed 100% homology with previous published sequences. Ambiguous nucleotides in the chromatogram and sequences containing SNPs were observed in two isolates (one at the *bg* locus and other at *tpi*) (Durigan *et al*., 2014; Lebbad *et al*., 2011). The sequence of isolate INI 44 was classified as a novel variant of subtype A2 (substitution at position 349 in the *tpi* gene) since a single point mutation suffices for the description of a new subtype (Cacciò and Ryan, 2008; Monis *et al*., 2009; Sprong *et al*., 2009). Furthermore, an SNP at position 699 (C - T) of the *gdh* gene were detected in two samples (BfR156 and BfR166) and was enough to classify the sequences as

new (novel AII) (Broglia *et al*., 2013).

In 2008, Cacciò *et al*. identified 10 different MLGs for assemblage A on the basis of the combination of three genetic markers (*bg*, *tpi* and *gdh*) and proposed the standardization of the nomenclature. The sequences/subtypes found in our work had already been reported but the combinations of them at the three MLST loci were different from those described before. We detected four assemblage A MLGs: two previously described MLGs (AII-1 and AII-4) and two novel MLGs. Therefore, our group suggested following the numbering of MLGs proposed by Cacciò *et al*. (2008), and named the novel combinations as AII-8 and AII-9. Several authors have also observed new MLGs with different combinations of *bg*, *tpi* and *gdh* (Geurden *et al*., 2009; Lebbad *et al*., 2010, 2011; Broglia *et al*., 2013), including two previous surveys (Minetti *et al*., 2015; Soba *et al*., 2015) reporting the same combinations of our proposed MLGs (AII-8 and AII-9).

Consistent with previous studies conducted in Brazil, samples classified as assemblage A were all typed as AII (Souza *et al*., 2007; Santos *et al*., 2012; Durigan *et al*., 2014; David *et al*., 2015; Colli *et al*., 2015; Oliveira-Arbex *et al*., 2015; Faria *et al*., 2016). Nevertheless, our results contrast with the data obtained in Rio de Janeiro (Volotão *et al*., 2007), where 97% of the samples were identified as sub-assemblage AI and assemblage B was not detected. These findings should be analysed carefully since genotyping data were based on a single marker (*bg*). Some studies have suggested that assemblage A sequences might have been preferentially amplified by the *bg* gene and sequences classified as assemblage B are not amplified or are less amplified in this locus (Broglia *et al*., 2013; Durigan *et al*., 2014). Recently, our group detected the presence of assemblage B and observed a change in the genetic profile over the years in Rio de Janeiro city, with assemblage A being prevalent until 2012 whereas from 2013-2015 there was an increased number of cases of assemblage B (Faria *et al*., 2016). Besides changes in the frequency of infections with different assemblages over time, we also noted a switchover in the sub-assemblages, where sub-assemblage AI was not detected and sub-assemblage AII became more frequent. These findings could explain the discrepancy between our results and those reported in Rio de Janeiro (Volotão *et al*., 2007). Certainly, the use of several molecular markers (MLGs) allowed us to considerably increase the characterization of *G. lamblia* isolates and to reliably determine the distribution of assemblages in the population of Rio de Janeiro. Additionally, our results suggest that zoonotic transmission is low or absent in Rio de Janeiro, at least in relation to assemblage A, where sub-assemblage AI was not detected.

MLG approaches improved our understanding of transmission dynamics of human

giardiasis, determined possible associations between symptoms and assemblages and helped the study about zoonotic potential transmission (Huey *et al*., 2013; Ryan and Cacciò, 2013; Minetti *et al*., 2015; Thompson and Ash, 2016). However, most of the studies in Brazil were based on the analysis of a single marker and it is only in the last few years that the MLST protocol has been applied (Durigan *et al*., 2014; Colli *et al*., 2015; David *et al*., 2015; Oliveira-Arbex *et al*., 2015). For example, Colli *et al*. (2015) determined the prevalence of *G. lamblia* assemblages in Paraná (Southern Brazil) and reported the connection among different links in the epidemiology chain using the MLG tools.

Comparing our results with other Brazilian works, we can see that samples from Paraná (Colli *et al*., 2015) and São Paulo (David *et al*., 2015; Oliveira-Arbex *et al*., 2015) had low levels of polymorphism and no heterogeneous nucleotides were detected, which was significantly different from the profile of our sequences. In contrast, another study conducted in São Paulo reported a high degree of genetic diversity, with the detection of mixed assemblages (A+B) in 25% of the samples, the observation of overlapping nucleotides and the identification of assemblage C in five human clinical samples (Durigan *et al*., 2014). Discrepancies were certain to be found between the different studies accomplished in Brazil, mainly because the country is so vast and the cultural, behavioural and social diversity of the population so varied.

The successful amplifications of the three studied loci were performed differently. The *tpi* and *gdh* genes were amplified in 94.9% and 82.1% of samples, respectively, and the *bg* gene had the lowest rate of amplification (79.5%). Indeed, some studies have observed that the *tpi* gene had the highest amplification success, followed by *gdh* and *bg* (Huey *et al*., 2013; Durigan *et al*., 2014; Oliveira-Arbex *et al*., 2015). Differences in the amplification rate have also been reported for different genetic loci by other groups (David *et al*., 2015; Minetti *et al*., 2015). Several factors may influence the PCR efficiency such as the quality and quantity of DNA templates and the presence of inhibitors (Wilke and Robertson, 2009). Perhaps the DNA region between the PCR primers and the genomic sequences were degraded or there were nucleotide mismatches, which may cause a strong reduction or even a lack of amplification, especially considering that the three genes used were single copy (Broglia *et al*., 2013; Thompson and Ash, 2016).

Congruent genotyping results were obtained at the three genetic loci investigated, which is in contrast with some previous reports (Cacciò *et al*., 2008; Sprong *et al*., 2009; Huey *et al*., 2013). However, discordant intra-assemblage assignment was observed in assemblage B isolates (INI 21, 27, 48, 64 and 69): the *bg* locus was not consistent with the

other two loci (*tpi* and *gdh*). When these results were compared with data described previously by our team (Faria *et al*., 2016) inconsistent results were also observed at sub-assemblage level. Six samples (INI 10, 50, 54, 61, 67 and 68) that were genotyped by PCR-RFLP as mixtures of sub-assemblages BIII and BIV were classified as sub-assemblage BIII by sequencing analysis in this study. Mixed patterns of alleles have been observed before, which may be the result of recombinations between different assemblage B subtypes (Lebbad *et al*., 2011).

Two principal mechanisms can explain the lack of concordance in the assignment to assemblages in different loci as well as the occurrence of DNA sequences with overlapping nucleotides: allelic sequence heterozygosity (ASH) and mixed infections. *Giardia* has two diploid nuclei which may accumulate specific mutations independently and this could generate ASH, while mixed infections are the presence of genetically different cysts in the same sample and can occur at the inter- and intra-assemblage levels. The occurrence of ASH between the two nuclei of a single cyst and also between different cysts has been demonstrated, but the reasons for different ASH levels between assemblages A and B is not yet understood (Ankarklev *et al*., 2012). According to the literature, the assemblage A genome displays a very low level of ASH compared with assemblage B (Cacciò and Sprong, 2010; Ankarklev *et al*., 2012). The occurrence of ASH complicates the assignment of specific subtypes, especially for assemblage B, and it is very difficult to distinguish between ASH and mixed infections. Another possible explanation for the presence of double peaks is the occurrence of meiotic recombination, which suggests the potential for sexual reproduction in *Giardia*, but the evidence of sex still elusive (Birky, 2010; Cacciò and Sprong, 2010).

It is important to emphasize that we did not find any mixed infections of assemblages A and B. Sometimes the failure to detect mixed infections does not mean that one is not present, but that one assemblage or sub-assemblage has been preferentially amplified over another at one locus (Ryan and Cacciò, 2013). To prevent this, three different assemblage-specific primers (*tpi*, *gdh* and *orf*C4) were used (Faria *et al*., 2016), and it was confirmed that mixed infections were not present in the studied samples. It therefore seems that the occurrence of double peaks is not the result of mixed infections of assemblages A and B, but may be caused by there being multiple subtypes in the same sample or the occurrence of ASH.

In fact, there has recently been an increase in sequence information and the description of new sequences/subtypes has been reported in the past few years (Ryan and Cacciò, 2013; Durigan *et al*., 2014; Minetti *et al*., 2015; Nunes *et al*., 2016). Even isolates classified as

assemblage A (for example, INI 17 and INI 44), that show less variability than B, could not be classified under the previous MLST nomenclature proposal (Cacciò *et al*., 2008). MLG is a useful subtyping tool for assemblage A but less valuable for assemblage B because of the high frequency of heterogeneous positions in the chromatogram. As suggested before, a re-evaluation of the current molecular epidemiological methods and the development of new target regions of the genome with lower substitution rates may facilitate the study of assemblage B (Ankarklev *et al*., 2012; Wielinga *et al*., 2015).

This study is the first report to use MLST to genetically characterise *G. lamblia* human isolates in Rio de Janeiro, Brazil. The identification of the novel subtypes in both assemblages and the description of novel assemblage A MLGs reflect the genetic distribution of the parasite. Our work has provided a new insight into the genetic diversity of parasite and improved our understanding of the epidemiology of the disease by elucidating the dynamics of giardiasis in the population of Rio de Janeiro. It has shown that MLST should be applied in the characterisation of *G. lamblia* in Brazil and elsewhere. Further studies on the molecular epidemiology are imperative since the parasite was found to vary widely in Brazilian isolates and this knowledge is crucial for both veterinary and public health researchers.

4.6. References

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4.7. Supplementary data

Table S4.1. Accession number for gene sequences obtained from GenBank used for *G. lamblia* sub-assemblage identification.

Assemblages	*β-giardin*	*Triose phosphate isomerase*	*Glutamate dehydrogenase*
AI	AY258617 (A1)	L02120 (A1)	M84604 (A1)
AII	AY072723 (A2)	U57897 (A2)	AY178737 (A2)
	AY072724 (A3)	-	JX994237 (A4)
AIII	EU216429	EU781002	EU637582
BIII	AY072726	AF069561	EF685684
	AY072725		
BIV	AY072728	AF069560	AY178738
C	AY545646	AF069563	U60982
	-		EF507635
D	AY545647	DQ246216	U60986
		-	EF507629
E	AY072729	EF654686	AY178740
		-	AY178741
F	AY647264	AF069558	AF069057
	-	-	EF507596
G	EU769221	AY228640	AY178745
	-	-	AY178746

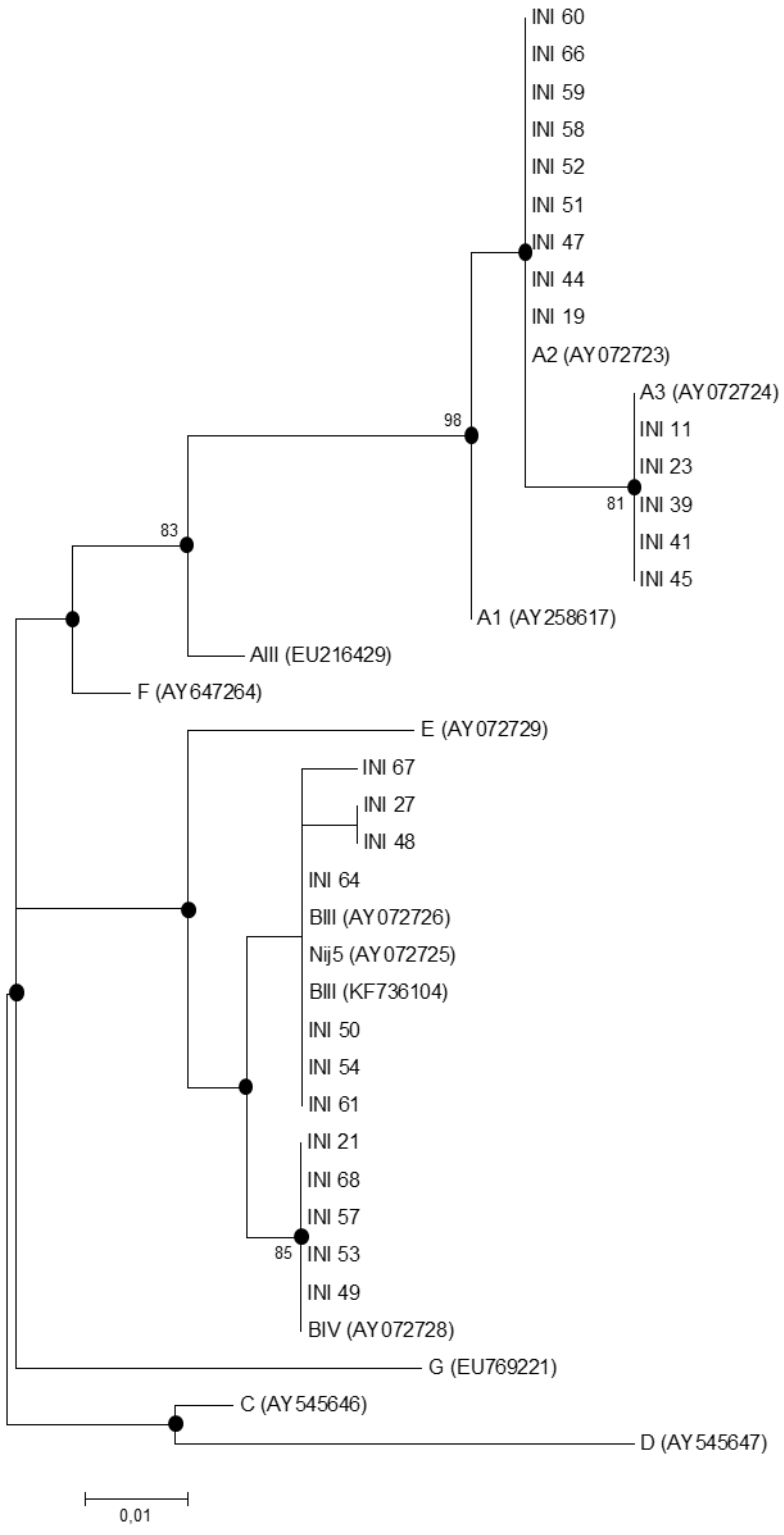

Figure S4.2. Phylogenetic relationship of *G. lamblia* constructed by maximum likelihood analysis, based on nucleotide sequences of *bg* gene retrieved from this study compared with reference sequences of known assemblages from GenBank.

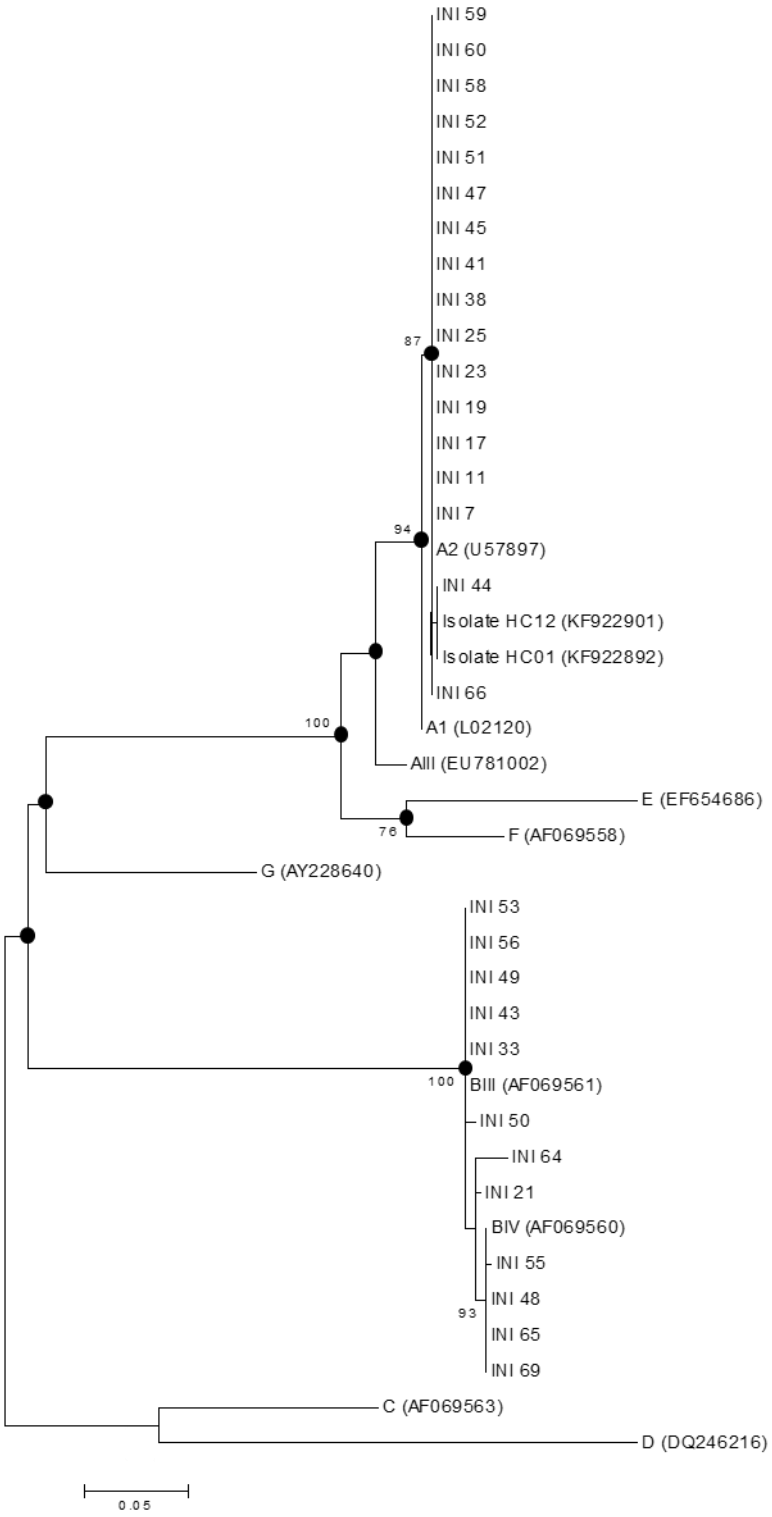

Figure S4.3. Phylogenetic relationship of *G. lamblia* constructed by maximum likelihood analysis, based on nucleotide sequences of *tpi* gene retrieved from this study compared with reference sequences of known assemblages from GenBank.

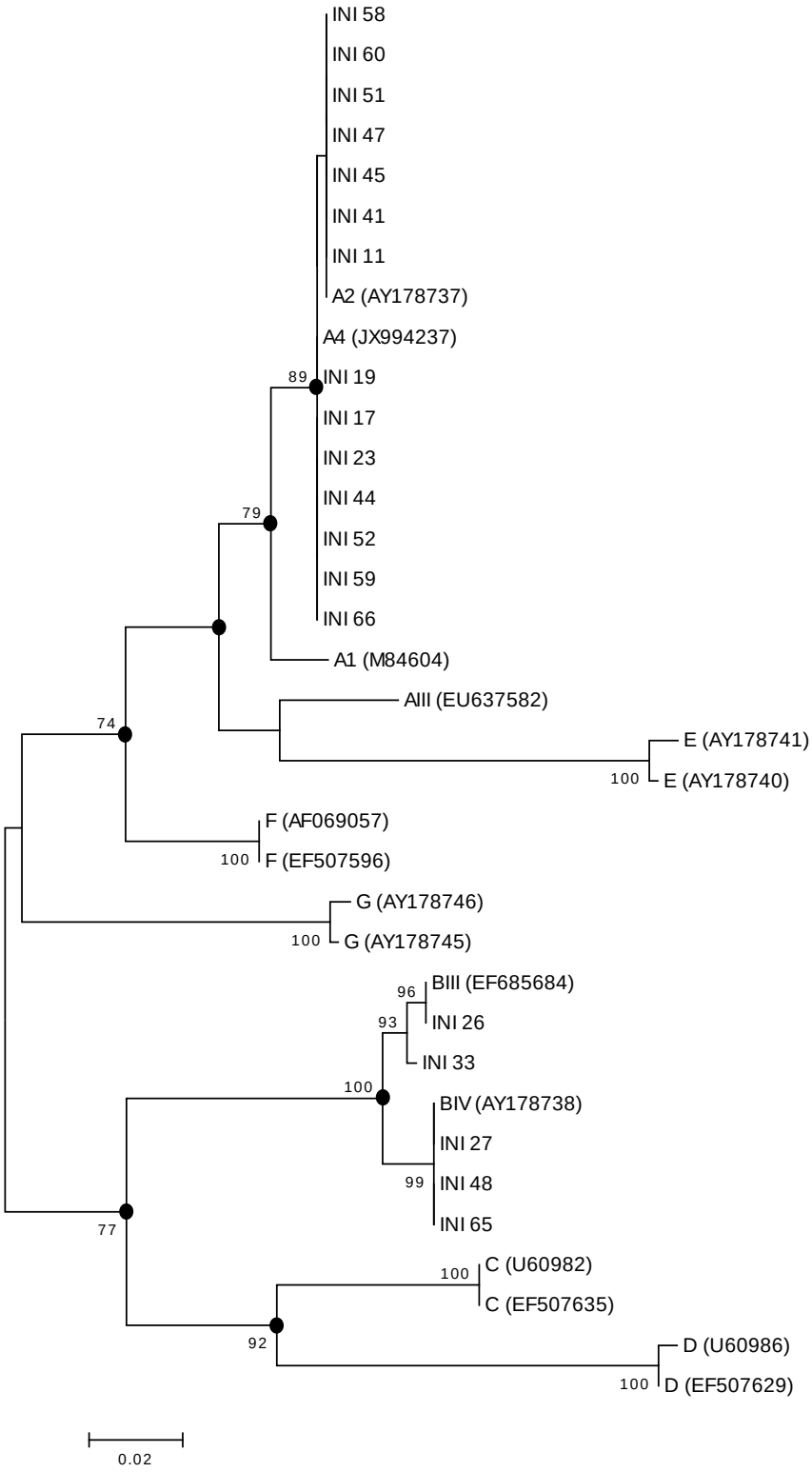

Figure S4.4. Phylogenetic relationship of *G. lamblia* constructed by maximum likelihood analysis, based on nucleotide sequences of *gdh* gene retrieved from this study compared with reference sequences of known assemblages from GenBank.

Chapter 5

Associations of *Giardia lamblia* assemblages with HIV infections and symptomatology: HIV virus and assemblage B were they born to each other?

Clarissa P Faria, Graziela M Zanini, Gisele S Dias, Maria C Sousa (**2016**), "Associations of *Giardia lamblia* assemblages with HIV infections and symptomatology: HIV virus and assemblage B were they born to each other?", submitted to Acta Tropica.

5.1. Abstract

Giardia lamblia is an intestinal parasite that has an extensive genetic variation among isolates. This species is divided into eight different assemblages (A to H), but only assemblages A and B have been associated with human infections. Studies on the associations of *G. lamblia* assemblages and symptoms have been done but were inconclusive. The aim of this study was to correlate *G. lamblia* assemblages with symptoms in patients with and without HIV/AIDS and its association with the CD4 T cell count. The cross-sectional survey was conducted among patients attending the Evandro Chagas National Institute of Infectious Diseases (INI/FIOCRUZ) in Rio de Janeiro from January 2011 to February 2015. Thirty-eight of 65 microscopically positive stool samples for *G. lamblia* were from HIV positive patients and 27 were from HIV negative patients. Of the HIV infected patients, 19 (55.9%) were genotyped as assemblage B of which 9 (47.4%) had a CD4 T cell count below 200 cells/mm^3. In addition, we found a greater number of samples belonging to assemblage B in symptomatic cases (11 of 19; 57.9%). Our data suggest that assemblage B is very likely to be found in HIV infected patients and probably the lower CD4 T count gives advantages for assemblage B replication. Furthermore, assemblage B seems to be associated with symptomatology, particularly abdominal pain, asthenia, diarrhea, fever, headache and myalgia. This study provides information on *G. lamblia* assemblages and symptoms in patients with and without HIV/AIDS virus and their association with CD4 T cell counts.

5.2. Introduction

Giardia lamblia (syn. *G. intestinalis* and *G. duodenalis*) is considered one of the leading causative agents of diarrhea and is seen as an important waterborne disease pathogen that infects animals and humans worldwide (Feng and Xiao, 2011). The spectrum of clinical manifestations of giardiasis is quite variable, ranging from asymptomatic infections to acute or chronic diarrhea. The clinical manifestations of giardiasis are self-limiting in most cases, with transient intestinal complications that are usually solved completely, but because of the potential for chronic or intermittent symptoms, treatment is recommended (Eckmann, 2003; Robertson *et al*., 2010). The clinical signs of infection include diarrhea, bloating, vomiting, dehydration, abdominal pain, flatulence, and nausea. There is no appearance of blood in the stool since *Giardia* is a non-invasive parasite and few virulence factors have been identified (Ankarklev *et al*., 2010; Eckmann, 2003; Read *et al*., 2002).

A considerable amount of data has shown that *G. lamblia* is a species complex whose members show little variation in their morphology but present a remarkable genetic variability (Cacciò and Ryan, 2008; Thompson, 2004). Due to its invariant morphology, investigations of aspects such as biology, host specificity and transmission patterns require the direct genetic characterization of parasites from fecal samples. Actually *G. lamblia* is divided into at least eight distinct genetic assemblages (A-H), however only assemblages A and B are known to infect humans. The others are likely to be host specific, as assemblages C and D occur mostly in dogs and other canids, assemblage E in hoofed livestock, assemblage F in cats, assemblage G in rats and assemblage H in marine mammals (Lasek-Nesselquist *et al*., 2010; Monis *et al*., 1999; Thompson, 2004).

G. lamblia presents a heterogeneous clinical manifestations and one hypothesis is that the parasite assemblages could play a part in the development of symptoms. Molecular analyses have suggested that the genomic differences between assemblages A and B are sufficient to classify them into two different species (Adam *et al*., 2013; Franzén *et al*., 2009). Some authors believe that the genomic differences between strains WB (assemblage A) and GS (assemblage B) may explain some of the phenotypic differences *et al*., 2010; Xu *et al*., 2012). Studies trying to associate *G. lamblia* assemblages with symptoms had been done all over the world (Haque *et al*., 2005; Minetti *et al*., 2015; Puebla *et al*., 2014). However, results from these studies have been controversial, with some authors correlating symptoms with assemblage A (Breathnach *et al*., 2010; Haque *et al*., 2005; Pestechian *et al*., 2014; Read *et al*., 2002; Sahagún *et al*., 2008), others with assemblage B (Gelanew *et al*., 2007; Homan and

Mank, 2001; Lebbad *et al*., 2011; Minetti *et al*., 2015; Puebla *et al*., 2014), while others did not find any correlation (Kohli *et al*., 2008; Lebbad *et al*., 2008; Pelayo *et al*., 2008). Additionally, there have been very few studies about the correlations of *G. lamblia* assemblages with clinical manifestation in patients with the human-acquired immunodeficiency virus (HIV), and, again, conclusive results were not achieved (Lim *et al*., 2011; Maikai *et al*., 2012).

Immunocompromised patients are one of the high-risk groups for infection. Several studies have reported that individuals with immune deficiencies such as HIV/AIDS were more likely to have *Giardia* infection (Adamu *et al*., 2013; Boaitey *et al*., 2012; Sanyaolu *et al*., 2011). Indeed, patients with hypogammaglobinemia have been linked to a predisposition for chronic giardiasis (Oksenhendler *et al*., 2008). Hence, the aim of this study was to correlate *G. lamblia* assemblages with symptoms in patients with and without HIV/AIDS and its association with the CD4 T cell count.

5.3. Materials and methods

5.3.1. Population study

A total of 65 fecal samples positive to *G. lamblia* were collected between January 2011 and February 2015 from patients attending the Evandro Chagas National Institute of Infectious Diseases (INI/FIOCRUZ), a referral hospital in infectious diseases in Brazil, located in Rio de Janeiro. This hospital receives patients from all municipalities, mainly the metropolitan area. According to the last census conducted in 2010, Rio de Janeiro municipality had a population of 6.320.446 inhabitants and the metropolitan region (which is composed of 21 municipalities and is the second largest metropolitan area in Brazil) had 11.812.482 inhabitants (IBGE, 2010).

5.3.2. Data collection and laboratory procedures

Stool samples were collected by the patient in plastic disposable flasks without preservatives and maintained at 4^{o}C until laboratory analysis on the same day. The flasks were labeled with the name, collection date and the hospital number. The parasitological tests were conducted at the Parasitology Laboratory of INI by experienced laboratory technologists. This laboratory is certified by the College of American Pathologists. For laboratory diagnosis of *G. lamblia*, the fresh specimens were analyzed by means of centrifugation sedimentation (de Carli, 2001) and centrifugal flotation in zinc sulphate

solution (Faust *et al*., 1938). The slides were then observed under the microscope (Nikon Eclipse E200, magnification of 10 and 40X).

In addition to parasitological tests, clinical and epidemiological data (age, educational level, gender and residence place) were collected from the hospital´s database. The patients were classified as asymptomatic or symptomatic (abdominal pain, asthenia, cough, diarrhea, fever, headache, myalgia, rapid weight loss, vertigo and/or vomiting). According to World Health Organization (WHO) criteria diarrhea is defined as the passage of three or more unformed stools per day or more frequent passage than is normal for the individual (WHO, 2013).

The hematological, biochemical (lipidogramme and proteinogramme) and/or immunologic results were collected from the patient´s clinical records, as well as the information about the CD4 T cell counts (cells/mm^3) and the adherence (or not) to the antiretroviral therapy (ART) in the HIV infected patients.

All patients attending INI/FIOCRUZ are dewormed when diagnosed and HIV infected patients received antiretroviral therapy (ART) according to Brazilian Ministry of Health's consensus recommendations (drugs are provided by the institution itself).

5.3.3. Genotyping of *G. lamblia*

DNA extraction was performed using the QIAamp DNA Stool mini Kit (Qiagen, Germany) according to the instructions of the manufacturer. The parasite assemblage and sub-assemblage were determined using PCR-RFLP amplification, and real time quantitative PCR (qPCR) (Faria *et al*., 2016).

5.3.4. Statistical analysis

The data entry was carried out using Excel software and analyzed using Statistical Package for the Social Sciences (SPSS) version 16. Percentages were used to perform the exploratory analysis of the categorical variables and quantitative variables are presented as mean ± standard deviation (SD). Pearson´s chi-squared and Fisher's Exact Test were used for categorical data. The level of statistical significance was set as $p<0.05$.

5.3.5. Ethical considerations

The Research Ethics Committee Evandro Chagas National Institute of Infectious Diseases (INI/FIOCRUZ) approved the study (protocol number: 127.542). This project was in accordance with the Brazilian Ethical Resolutions, especially Resolution CNS 196/1996 and

its complementary and the Code of Medical Ethics of 1988 (articles 122 - 1307). Written informed consent was obtained from all patients or legal guardians of patients younger than 18 years, prior to sample collection. The informed consent was provided after a detailed explanation of the objectives of the work. A term of privacy and confidentiality was signed by the researches for patients for whom it was not possible to obtain informed consent beforehand.

5.4. Results

5.4.1. Characteristics of the study population

Among the 65 patients positive for *G. lamblia*, the majority were adults (69.2%) with an average of 32.54±13.69 years (Mean±SD; median=32) (Table 5.1). The highest prevalence occurred between 30-39 years (33.3%) and there were more male than female patients (69.2% *versus* 30.8%). Seventy-two percent of patients were literate; most of them (53.8%) live in Rio de Janeiro municipality and 83.1% live in metropolitan region (Table 5.1).

Of these positive patients for *G. lamblia*, 38 were HIV infected patients and 27 were non-HIV-infected patients. In HIV infected patients, 14 (36.8%) had CD4 T cell counts higher than 500 cells/mm^3, 2 (5.3%) had between 350 and 500 cells/mm^3, 6 (15.8%) had between 200 and 350 cells/mm^3, and 16 (42.1%) had less than 200 cells/mm^3. As aforementioned all HIV infected patients were included in the ART programs, however, only 24 (63.2%) adhere to the therapy.

Table 5.1. Characteristics of the patients infected with *Giardia lamblia* according to HIV status.

Characteristics	Overall (n=65)		HIV+ (n=38; 58.5%)		HIV- (n=27; 41.5%)	
	No.	%	No.	%	No.	%
Gender						
Female	20	30.8	9	23.7	11	40.7
Male	45	69.2	29	76.3	16	59.3
Age group (years)						
0-14	6	9.2	1	2.6	5	18.5
15-25	12	18.5	6	15.8	6	22.2
26-65	45	69.2	31	81.6	14	51.9
Missing	2	3.1	-	-	2	7.4
Educational status						
Elementary school	17	26.2	12	31.6	5	18.5
High school	19	29.2	14	36.8	5	18.5
University education	11	16.9	8	21.1	3	11.1
No formal education	3	4.6	1	2.6	2	7.4
Missing	15	23.1	3	7.9	12	44.4
Place of residence						
RJ[a] municipality	35	53.8	19	50	16	59.3
Metropolitan area of RJ[a]	54	83.1	17	44.7	2	7.4
Others States of Brazil	1	1.5	-	1	1	3.7
Missing	10	15.4	2	5.3	8	29.6
CD4 count (cell/mm^3)						
>500			14	36.8		
350-500			2	5.3		
200-349			6	15.8		
< 200			16	42.1		
ART[b]						
Yes			24	63.2		
No			14	36.8		

[a] *RJ* Rio de Janeiro

[b] *ART* Antiretroviral therapy

5.4.2. Patients clinical status and presence of co-infections

Evandro Chagas National Institute of Infectious Diseases attended individuals with HIV/AIDS, HTLV, sexually transmitted diseases (STDs), Chagas disease, toxoplasmosis, leishmaniasis, mycoses, tuberculosis, and acute febrile diseases (dengue, malaria, influenza, chicken pox, leptospirosis, among others). Of the 65 patients positive for *G. lamblia*, 21 (32.3%) showed clinical symptoms while 44 (67.7%) were asymptomatic (Tables 5.2 and S5.1). The majority of symptomatic patients were HIV positive (n=16, p=0.045), being diarrhea the most common symptom.

Additionally, the association between presence or absence of co-infections and the HIV status was also analyzed. Most of the patients (48 of 65; 73.8%) positive for *G. lamblia* infection did not present any co-infection with parasite (monoparasitism), whereas 17 (26.2%) had two or more intestinal parasites simultaneously (polyparasitism). Among the multiple infected, 15 patients had two parasites and the others had more than two parasites. Regarding parasitic associations, 17.6% (3 of 17) of the patients were co-infected by helminths and 82.4% (14 of 17) by protozoa. The only three patients co-infected with helminths were all HIV negative, contrasting with the remaining co-infected patients. For example, patients co-infected with *Cryptosporidium* sp. or *E. coli* were HIV positive (100% and 66.7%, respectively) and all of them were symptomatic (Tables 5.2 and S5.1). In HIV infected patients were not observed association between the CD4 T cell count and the presence or absence of polyparasitism.

Twenty patients (30.8%) also have other infections, besides intestinal parasites and HIV virus (Table 5.2). The bacteria that cause tuberculosis and syphilis were more often found in HIV infected patients (83.3% and 100%, respectively), although our results were not statistically significant. Opposite results were observed with the parasites that cause leishmaniasis and Chagas disease, being more frequent in patients HIV negative.

Of the patients who had hematology and biochemical tests requested, no association were reported with the studied parameters like complete blood count, lipidogramme and proteinogramme (Table S5.2). However, among the four patients co-infected with *Leishmania*, three had increased concentration of eosinophil in the blood (p=0.014) (Table S5.1).

Table 5.2. Clinical information of *Giardia lamblia* infected patients according to HIV status and parasitic co-infections.

Characteristics	Overall (n=65)		HIV+ (n=38; 58.5%)		HIV- (n=27; 41.5%)	
	No.	%	No.	%	No.	%
*Symptoms**						
Yes	21	32.3	16	42.1	5	18.5
No	44	67.7	22	57.9	22	81.5
Clinical symptoms						
Abdominal pain	7	10.8	4	10.5	3	11.1
Asthenia	1	1.5	1	1.5	-	-
Cough	7	10.8	6	15.8	1	3.7
Diarrhea	15	23.1	12	31.6	3	11.1
Fever	8	12.3	6	15.8	2	7.4
Headache	3	4.6	1	1.5	2	7.4
Myalgia	3	4.6	2	5.3	1	3.7
Rapid weight loss	8	12.3	6	15.8	2	7.4
Vertigo	1	1.5	-	-	1	3.7
Vomiting	5	7.7	2	5.3	3	11.1
Intestinal parasitic infection						
Monoparasitism (*G. lamblia*)	48	73.8	31	81.6	17	63
Polyparasitism						
G. lamblia + *A. lumbricoides*	1	1.5	-	-	1	3.7
G. lamblia + *H. nana*	1	1.5	-	-	1	3.7
G. lamblia + *S. mansoni* + *T. trichiura* + hookworms	1	1.5	-	-	1	3.7
G. lamblia + *B. hominis*	3	4.6	2	5.3	1	3.7
G. lamblia + *Cryptosporidium* sp.	2	3.1	2	5.3	-	-
G. lamblia + *E. coli*	3	4.6	2	5.3	1	3.7
G. lamblia + *E. histolytica*	1	1.5	-	-	1	3.7
G. lamblia + *E. nana*	4	6.2	1	2.6	3	11.1
G. lamblia + *B. hominis* + *E. histolytica*	1	1.5	-	-	1	3.7
Total of polyparasitism	17	26.2	7	18.4	10	37
Others diseases						
Chagas disease	2	3.1	-	-	2	7.4
Leishmaniasis	4	6.2	-	-	4	14.8
Mycoses	4	6.2	2	5.3	2	7.4
Syphilis	4	6.2	4	10.5	-	-
Tuberculosis	6	9.2	5	13.2	1	3.7

* Statistically significant association among HIV infected patients with symptoms (p=0.045).

5.4.3. Associations of *G. lamblia* assemblages with HIV infections and symptomatology

Of the 65 samples microscopy-positive for *G. lamblia*, 60 (92.3%) were successfully amplified in the multilocus genotyping using at least one locus (*bg*, *gdh*, *orf*C4, *tpi* and/or *ssu rRNA*) (Faria *et al*., 2016). Thirty-two (53.3%) samples belonged to assemblage A and 28 (46.7%) to assemblage B (Table S5.1).

In the HIV infected patients, 15 (44.1%) were genotyped as assemblage A and 19 (55.9%) as assemblage B (Table 5.3). It is noteworthy that among the 28 samples genotyped as assemblage B, 67.9% (19 samples) were HIV infected patients. Despite the observed results are not statistically significant, we could observe an increased prevalence of assemblage B in HIV infected patients.

Table 5.3. Distribution of the *Giardia lamblia* assemblages among patients over the years.

Assemblage	2011-2012		2013-2015		Total	
	A	B	A	B	A	B
HIV+ (n=34)	10	6	5	13	15	19
HIV- (n=26)	13	2	4	7	17	9
Total (n=60)	23	8	9	20	32	28

Regarding the CD4 T cell counts, we could observe that patients with a higher CD4 T cell count (>200 cell/mm^3) have an equal distribution among the assemblages, 10 (50%) samples belonged to A and 10 (50%) samples to B (Table 5.4). However, the same was not detected when the CD4 T cell count was below 200 cell/mm^3. In this case, it was observed a greater number of patients (9; 64.3%) harboring assemblage B.

Table 5.4. Distribution of *Giardia lamblia* assemblages according to CD4 T cell counts in HIV infected patients.

Assemblage	CD4 T cell count (cell/mm^3)				Total
	> 500	350-500	200-349	< 200	
A	5	2	3	5	15
B	7	-	3	9	19
Total	12	2	6	14	34

When we studied the association of the symptoms with assemblages, no statistical association was observed (Tables 5.5). Nevertheless, we could observe a greater number of samples belonging to assemblage B in symptomatic cases, 11 (57.9%) *versus* 8 (42.1%)

belonging to assemblage A. Conversely, assemblage A was more frequently observed in asymptomatic cases (24 of 41; 58.5%). The most common symptoms in patients with assemblage B were abdominal pain, asthenia, diarrhea, fever, headache and myalgia (Table 5.5). Rapid weight loss, vertigo and vomiting were more frequent in assemblage A; whereas cough had similar frequence between the assemblages. No association was reported between assemblages and co-infections (Table S5.1).

Table 5.5. Association between the *Giardia lamblia* assemblages and symptomatology.

Characteristics	Assemblage		Total
	A (%)	B (%)	
Symptoms			
Yes	8 (42.1)	11 (57.9)	19
No	24 (58.5)	17 (41.5)	41
Clinical symptoms			
Abdominal pain	2 (33.3)	4 (66.7)	6
Asthenia	-	1 (100)	1
Cough	3 (50)	3 (50)	6
Diarrhea	6 (42.9)	8 (57.1)	14
Fever	1 (14.3)	6 (85.7)	7
Headache	1 (33.3)	2 (66.7)	3
Myalgia	-	3 (100)	3
Rapid weight loss	4 (57.1)	3 (42.9)	7
Vertigo	1 (100)	-	1
Vomiting	3 (75)	1 (25)	4

The distribution of *G. lamblia* assemblages among the symptomatic and asymptomatic patients according to their HIV status not showed statistically differences (Table 5.6).

Table 5.6. Association between *Giardia lamblia* assemblages and symptoms according to HIV status.

Assemblage	HIV +		HIV -		Total	
	A	B	A	B	A	B
Symptomatic	5	9	3	2	8	11
Asymptomatic	10	10	14	7	24	17
Total	15	19	17	9	32	28

5.5. Discussion

Previous studies have documented the prevalence of *G. lamblia* among HIV infected patients (Kiros *et al*., 2015; Marley *et al*., 2016; Sanyaolu *et al*., 2011; Tian *et al*., 2012). However, to the best of our knowledge, this is the first study to provide information on the *G. lamblia* assemblages and symptoms in patients with and without HIV/AIDS virus and the association with CD4 T cell counts worldwide.

There was a positive association between the HIV infected patients and the symptomatology, and diarrhea was the most frequent clinical sign. It is well documented that HIV infected patients have a weakened immune system due to the depletion of CD4 T cells and this makes them more susceptible to a range of infections. Gastrointestinal infections are very common and diarrhea is the hallmark symptom (Akinbo and Omoregie, 2011; Wiwanitkit, 2001). Several studies reported that HIV infected patients were more likely to have intestinal parasitic infection (Nkenfou *et al*., 2013; Sanyaolu *et al*., 2011), particularly patients with a CD4 T count below 200 cell/mm^3 (Mehta *et al*., 2013). Of the non-opportunistic parasites, *G. lamblia* is one of those most commonly found in HIV infected patients (Boaitey *et al*., 2012; Kiros *et al*., 2015; Mehta *et al*., 2013; Nkenfou *et al*., 2013).

In relation to parasite infections, the majority of the patients seen at INI were infected only with *G. lamblia* (73.8%) and the rest (26.2%) were mainly co-infected by protozoa, which agrees with other studies conducted in Brazil (Castro *et al*., 2015; Mariano *et al*., 2015). The low prevalence of helminths observed in our study is probably due to the chemotherapy used in the parasite control programs. Regular deworming with the drugs albendazole and mebendazole is the current control strategy to reduce the prevalence of helminths in Brazil (MS, 2012). However, these programs are not effective against protozoa infections.

Co-infections with helminths were only detected in non-HIV-infected patients. Conversely, patients co-infected with *Cryptosporidium* sp. were only observed in symptomatic HIV infected patients, a finding consistent with previous studies that also detected a high prevalence of this opportunistic intestinal parasite in symptomatic HIV patients (Alemu *et al*., 2011; Sanyaolu *et al*., 2011). Similarly, we found that *E. coli* a non-pathogenic parasite that is usually seen more frequently in HIV infected patients (Sanyaolu *et al*., 2011), had a prevalence of 66% in HIV infected patients.

With regard to other co-infections, tuberculosis and syphilis were more frequent in HIV infected patients (83.3% and 100%, respectively). HIV infection is a risk factor for

tuberculosis infection and to its progression to an active disease (Getahun *et al*., 2010; Pawlowski *et al*., 2012) and syphilis facilitates the transmission and acquisition of HIV infection (Zetola and Klausner, 2007). Leishmaniasis and Chagas disease, however, were only detected in non-HIV-infected patients. While HIV, syphilis and tuberculosis infections are homogeneously distributed in the population of Rio de Janeiro and have a high prevalence (MS, 2014; MS, 2015a; MS, 2015b), leishmaniasis and Chagas disease are not. The reported cases are usually autochthonous, imported from endemic areas of Brazil, attributed to blood transfusion, or travel history, and rarely vertical transmission via the placenta (mother to child), or accidentally in laboratories (Kawa and Sabroza, 2002; Lyra *et al*., 2015; Sangenis *et al*., 2015).

No association was found between the socio-demographic variables such as age, gender, place of residence and level of education with the HIV status. Most of the hematology and biochemical parameters between HIV-infected *versus* non-HIV-infected patients were not statistically significant. However, association between *Leishmania* infection and the increased level of eosinophils was detected. This result was unexpected since *Leishmania* infection is associated with Th1 response and macrophage activation (Azeredo-Coutinho *et al*., 2016; Oliveira *et al*., 2014).

There was an increased prevalence of assemblage B in HIV infected patients, even though there was no significant statistical association. Analyzing the assemblage's prevalence over the years, we continue to confirm these results. In 2011 and 2012 there was a predominance of assemblage A, with most patients being non-HIV-infected. In the next three years (2013-2015), there was a switch in genetic profile, with an increasing number of patients having assemblage B and most of them being HIV positive. Taking into consideration the CD4 T cell count, a larger number of HIV positive patients with less than 200 cell/mm^3 and *G. lamblia* assemblage B co-infection has been found.

The genetic characterization of *G. lamblia* in HIV infected patients was previously reported in Malaysia, but the relationship between the clinical manifestation and assemblages could not be done because of the small number of positive samples (Lim *et al*., 2011). One year later, a similar study was conducted in Nigeria and again the small number of positive samples prevented the association being made between the genetic characterization of the parasite and HIV-infected patients (Maikai *et al*., 2012). Few studies have been conducted on this issue up to now, and, even with the small number of samples analyzed, we can assume that assemblage B is more frequently detected in HIV-infected patients. Corroborating our results, recently, a cross-sectional study of *G. lamblia* infection positively correlated

assemblage B with HIV infection (Matey *et al*., 2016).

Furthermore, we observed a greater number of samples belonging to assemblage B with symptoms (57.9%), particularly abdominal pain, asthenia, diarrhea, fever, headache and myalgia. A correlation between infection with assemblage B and the presence of symptoms has been reported before (Al-Mohammed, 2011; Puebla *et al*., 2014). Recently, a study conducted in England showed that people infected with assemblage B had more symptoms and greater frequency of vomiting, abdominal pain, swollen stomach, and loss of appetite than people infected with assemblage A (Minetti *et al*., 2015). In contrast, other studies correlated assemblage A with symptoms (Breathnach *et al*., 2010; Pestechian *et al*., 2014; Sahagún *et al*., 2008).

Some authors believe that the genomic differences between assemblages A and B are sufficient to classify them as separate species, and this could explain some of the phenotypic differences (Adam *et al*., 2013; Franzén *et al*., 2009). It has been suggested that different symptom spectra were apparently associated with different assemblages in different populations (Robertson *et al*., 2010). As aforesaid, several studies were carried out in different countries with different research groups, reporting correlations between assemblages and symptoms. But despite the effort, there is still a lack of concordance on this issue and many questions remain to be answered.

Overall, our results suggest that assemblage B has a good chance of being found in HIV-infected patients and probably the lower CD4 T count is advantageous for assemblage B replication. Moreover, patients harboring assemblage B were more likely to have symptomatic infections than patients with *G. lamblia* assemblage A isolates. It would be particularly interesting proceed the studies about relationship between clinical symptoms, CD4 T cell counts and assemblages of *G. lamblia* in HIV-infected patients. As previously mentioned, HIV infection increases the risk of having intestinal parasitic infections, including *G. lamblia*. The detection and treatment of infections are important measures to improve the quality of life of HIV-infected patients.

5.6. References

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Table S5.1. Characteristics of the studied population.

ID isolates	Symptoms[§]	HIV status[§]	CD4	ART[§]	Polyparasitism	Other diseases	Eosinophilia[§]	Assemblages[#]
INI 1	N	N			*G. lamblia* + *E. histolytica*		Y	A
INI 2	N	N					N	A
INI 3	N	N					N	A
INI 4	N	N					N	A
INI 5	N	Y	> 500	N	*G. lamblia* + *E. nana*		N	NA
INI 6	N	Y	> 500	N			Y	A
INI 7	N	N			*G. lamblia* + *B. hominis*		N	A
INI 8	Y [d, h]	Y	< 200	N		Syphilis	N	A
INI10	N	Y	> 500	Y			N	B
INI 11	N	N					N	A
INI 12	N	N			*G. lamblia* + *E. nana*		N	A
INI 13	Y [a, b, c, h]	Y	< 200	N		Tuberculosis	N	B
INI 14	Y [c, d]	Y	< 200	Y			N	A
INI 15	N	Y	500-350	N			N	A
INI 17	Y [c, h, i]	N				Mycoses	N	A
INI 19	N	Y	> 500	N		Mycoses	N	A
INI 21	N	Y	< 200	N	*G. lamblia* + B. *hominis*		N	B
INI 22	N	N				Tuberculosis	Y	NA
INI 23	Y [a, d]	Y	> 500	Y			N	A
INI 24	N	N				Leishmaniasis	Y	B
INI 25	N	N			*G. lamblia* + *A. lumbricoides*	Leishmaniasis	Y	A
INI 26	Y [d, e, h]	Y	< 200	Y	*G. lamblia* + *Cryptosporidium* sp.		N	B

Table S5.1 (*Continued*)

INI 27	N	Y	< 200	Y			N	B
INI 28	Y [d, e]	Y	> 500	Y		Syphilis	N	B
INI 29	Y [d]	Y	< 200	Y		Mycoses	N	B
INI 30	N	N				Chagas diseases	N	A
INI 31	N	Y	< 200	N		Tuberculosis	N	B
INI 32	Y [a, d, e, f, j]	N					N	A
INI 33	N	Y	349-200	Y			Y	B
INI 34	N	N					N	B
INI 35	N	Y	> 500	Y			N	A
INI 36	N	Y	< 200	Y			N	NA
INI 37	N	Y	< 200	Y			N	A
INI 38	N	N			*G. lamblia + E. nana*		N	A
INI 39	N	N					N	A
INI 40	Y [c, e, h, j]	Y	< 200	N		Tuberculosis	Y	NA
INI 41	Y [d, j]	Y	> 500	N			N	A
INI 42	N	Y	500-350	Y		Tuberculosis	N	A
INI 43	Y [d, e, g]	Y	349-200	Y		Syphilis	N	B
INI 44	N	Y	< 200	Y			N	A
INI 45	N	Y	349-200	Y			N	A
INI 46	N	N				Leishmaniasis	N	B
INI 47	Y [c, d, h]	Y	< 200	N			N	A
INI 48	N	Y	> 500	Y			N	B
INI 49	Y [a, d]	N			*G. lamblia + E. histolytica + B. hominis*		N	B
INI 50	Y [c, e, f, g]	Y	349-200	Y	*G. lamblia + E. coli*		N	B
INI 51	N	Y	349-200	N			N	A

Table S5.1 (*Continued*)

INI 52	N	N					N	A
INI 53	N	Y	> 500	Y			N	B
INI 54	N	Y	< 200	Y			N	B
INI 55	N	N				Leishmaniasis	Y	B
INI 56	N	N			*G. lamblia* + *H. nana*	Chagas diseases	Y	B
INI 57	Y [a, d]	Y	> 500	Y	*G. lamblia* + *E. coli*		N	B
INI 58	N	N				Mycoses	N	A
INI 59	N	N			*G. lamblia* + *E. nana*		N	A
INI 60	Y [h, j]	N			*G. lamblia* + hookworm + *S. mansoni* + *T. trichiura*		Y	A
INI 61	Y [c, e, h]	Y	> 500	Y		Tuberculosis	N	B
INI 62	N	Y	< 200	N			Y	B
INI 63	Y [a, d]	Y	> 500	Y			N	NA
INI 64	Y [d]	Y	< 200	Y	*G. lamblia* + *Cryptosporidium* sp.		N	B
INI 65	N	N					N	B
INI 66	N	Y	349-200	N	*G. lamblia* + *B. hominis*		N	A
INI 67	N	N					N	B
INI 68	N	Y	> 500	Y		Syphilis	N	B
INI 69	Y [a, d, e, f, g, j]	N			*G. lamblia* + *E. coli*		N	B

[§] Y, yes; N, no

[#] NA, not amplified

[a] abdominal pain, [b] asthenia, [c] cough, [d] diarrhea, [e] fever, [f] headache, [g] myalgia, [h] rapid weight loss, [i] vertigo, [j] vomiting

Table S5.2. Discrimination of the blood tests according to HIV status.

Blood tests	Overall	HIV+	HIV-
Complete Blood Count			
RBC[a]			
Increased	2	0	2
Decreased	11	8	3
Haemoglobin			
Increased	0	-	-
Decreased	13	10	3
Haematocrit			
Increased	0	-	-
Decreased	18	12	6
MCV[b]			
Increased	3	3	0
Decreased	7	4	3
MHC[c]			
Increased	2	2	0
Decreased	11	6	5
MCHC[d]			
Increased	1	1	0
Decreased	7	6	1
Leucocytes			
Increased	6	2	4
Decreased	7	5	2
Metamyelocytes			
Increased	1	1	0
Decreased	0	-	-
Band neutrophils			
Increased	4	4	0
Decreased	2	2	2
Segmented neutrophils			
Increased	6	3	3
Decreased	3	2	1
Lymphocytes			
Increased	4	3	1
Decreased	5	3	2
Monocytes			
Increased	4	4	0
Decreased	4	2	2
Platelets			
Increased	1	1	0
Decreased	2	2	0
Eosinophil			
Increased	11	4	7
Decreased	5	5	0
Iron binding capacity			
Increased	0	-	-
Decreased	1	1	0

Table S5.2 (*Continued*)

Biochemical analysis			
C- reactive protein			
Increased	11	8	3
Decreased	0	-	-
Total protein			
Increased	4	3	1
Decreased	0	-	-
Albumine			
Increased	3	3	0
Decreased	1	1	0
Globulin			
Increased	3	2	1
Decreased	0	-	-
Cholesterol			
Increased	2	1	1
Decreased	0	-	-
Triglycerides			
Increased	3	3	0
Decreased	0	-	-
Very low density lipoprotein			
Increased	1	1	0
Decreased	0	-	-
High density lipoprotein			
Increased	1	1	-
Decreased	7	7	-
Low density lipoprotein			
Increased	8	7	1
Decreased	0	-	-

[a]*RBC*, red blood cell

[b]*MCV*, mean corpuscular volume

[c]*MHC*, mean hemoglobin corpuscular

[d]*MCHC*, mean corpuscular hemoglobin concentration

Basophil, myelocyte and serum iron had their normal level

Chapter 6

General Discussion and Concluding Remarks

6.1. General Discussion

Brazil currently occupies a prominent position on the international scenario, but despite the economic growth, the reduction of poverty rate and inequality observed in recent decades, the population still lives with serious problems in health, education, water supply and sanitation. Unfortunately, intestinal parasitic infections continue to pose a public health problem (Aguiar *et al*., 2007; Castro *et al*., 2015), and studies reporting the prevalence of intestinal parasites still low and limited even in the big cities such as Rio de Janeiro.

The great social inequality is a common characteristic of Brazil, and Rio de Janeiro is an example of a city with economic and social contrasts. These disparities can be seen in the significant variation in the Human Development Index (HDI) (IBGE, 2010). According to Brazilian Institute of Statistics and Geography (2010) neighbourhoods such as Gávea, Leblon and Ipanema have a high HDI values (0.970, 0.967, 0.962, respectively), corresponding to HDI values of developed countries such as Norway, Australia and Switzerland (0.944, 0.935, 0.930, respectively), while, in others neighborhoods, like Rocinha, Acari and Complexo do Alemão the HDI values are below the municipal average (0.732, 0.720, 0.700, respectively). An urban feature of the city is the proximity of the slums and the poorer neighbourhoods to the most valued districts.

Our work demonstrates a high prevalence of intestinal parasites (17.5%) in Rio de Janeiro State, with an elevated prevalence of protozoan parasites (87%). The most predominant protozoa were *Endolimax nana* (28.8%), *Entamoeba coli* (14.8%), Complex *Entamoeba histolytica/Entamoeba dispar* (13.5%), *Blastocystis hominis* (12.7%), *Giardia lamblia* (8.1%), and *S. stercoralis* (4.3%) and *S. mansoni* (3.3%) were the most frequent helminths. Commonly helminths are the predominant enteric parasites (Santos *et al*., 2014; LaBeaud *et al*., 2015), but in this study they only appear in seventh position. Probably these results should be due to the regular deworming with the drugs albendazole and mebendazole, which is one of the intervention strategies to reduce the incidence of helminths in Brazil (MS, 2012).

It is well documented that intestinal parasitic infections are considered indicators of health and socio-environmental vulnerability, and that the poor sanitary conditions to which the population is exposed favours the acquisition of various pathogens. Therefore the high prevalence of intestinal parasites and co-infections (24.8%) observed in our survey is a reflection of infrastructural and socioeconomic conditions of Rio de Janeiro. Through the geospatial analysis was possible to observe that the distribution of the parasites was not

random or homogeneous, but was influenced by the material deprivation index (MDI). In areas with better socioeconomic, educational and sanitary conditions the number of infected patients was lower (8.3%); on the other hand, a larger number of infected patients were observed in areas with a high level of deprivation (26.6%).

One of the most common pathogenic species detected was *G. lamblia*, which is frequently found in Brazil (Mariano *et al*., 2015). The detection of *G. lamblia* cysts is particularly alarming since these are resistant to conventional routine disinfectants used in water treatment, and are frequently found in sewage effluent and surface water (Hatam-Nahavandi *et al*., 2015). *G. lamblia* is recognized as important waterborne disease pathogen that infects animals and humans worldwide, and due the vulnerability of drinking water supply systems of Rio de Janeiro, this parasite can be potentially spread by water.

In spite of its invariant morphology, genetic analysis have demonstrated that *G. lamblia* is a species complex composed of at least eight distinct assemblages (A to H), but only assemblages A and B have been detected in humans (Monis *et al*., 2003; Thompson, 2004; Lasek-Nesselquist *et al.*, 2010). Due to the lack of variation in morphology, the molecular characterization of cysts and/or trophozoites is of great importance for understanding the biology of the parasite, host preference, epidemiology and pathogenicity. Additionally, the distribution of assemblages A and B of *G. lamblia* varies greatly from one country to another and sometimes within the same country.

The distribution of *G. lamblia* assemblages and sub-assemblages in patients from Rio de Janeiro, determined by PCR-RFLP and qPCR using four different molecular markers (*bg*, *gdh*, *tpi* and *orf*C4), indicated that 52.7% of the patients were infected with isolates genotyped as assemblage A, 47.3% as assemblage B, and no mixed infections of assemblages A and B were detected.

According to the literature, it is possible to observe that the first researches carried out in Brazil showed a predominance of assemblage A (Volotão *et al*., 2007; Souza *et al*., 2007), and in the following years this distribution began to be reversed, assemblage B became more prevalent (Kohli *et al*., 2008; Santos *et al*., 2012; Colli *et al*., 2015; Oliveira-Arbex *et al*., 2015) or had an equal distribution to the assemblage A (Durigan *et al*., 2014; David *et al*., 2015; Nunes *et al*., 2016). This change in the genetic profile over the years could also be observed in our work. Assemblage A was more prevalent until 2012, whereas from 2013-2015 assemblage B was the most frequent.

Regarding subtyping, we reported the presence of sub-assemblages AII (16 of 35), BIII (15 of 35), and BIV (12 of 35) in the clinical isolates. Among assemblage A, sub-

assemblage AII is identified more often in studies on human giardiasis in Brazil, which is similar with the data obtained in Europe and other parts of the world (Sprong *et al*., 2009). Conversely, among the assemblage B, our study observed a greater number of patients with sub-assemblage BIII, in contrast with previous results from Brazil, where sub-assemblage BIV was identified more often (Durigan *et al*., 2014; Colli *et al*., 2015; Oliveira-Arbex *et al*., 2015).

As the frequency of assemblages was very similar, the presence of mixed infections A and B was expected, however only the mixing of the sub-assemblages BIII and BIV within isolates was detected (8 of 35). Sometimes the failure to detect these mixtures is not due to the absence of the mixed infection but because one assemblage can be preferentially amplified over another at one locus (Ryan and Cacciò, 2013), and to prevent such situation we used qPCR assemblage-specific primers. This qPCR assay had provided a simultaneous detection of assemblages A and B, and improved the *G. lamblia* detection in relation to nested-PCR of *bg* and *gdh* genes. We verified that for the isolates typed at more than one locus, assignment to assemblages was always concordant, confirming the absence of mixed infections with assemblages A and B.

The investigation of the genetic variability of *G. lamblia* isolates via MLST using *bg*, *tpi* and *gdh* genes, allowed us identify inter- and intra-assemblage level of genetic variation, and describe two novel assemblage A multilocus MLGs. Sequence analysis showed that assemblage B isolates have a higher genetic polymorphism than assemblage A isolates, with most of the sequences (66.7%) presenting heterogeneous nucleotides, and displaying mainly novel polymorphisms. The presence of double peaks hindered the classification of sub-assemblages and subtypes and in most cases prevented the unambiguous identification of MLGs. We observed that the sequenced isolates displayed mainly novel polymorphisms and that assemblage B isolates exhibited many more subtypes than assemblage A, which is in agreement with several works from other research groups (Sprong *et al*., 2009; Feng and Xiao, 2011; Ryan and Cacciò, 2013; Minetti *et al*., 2015).

Typically sequencing profiles from assemblage A isolates rarely show heterogeneity, thereby allowing the grouping and classification of assemblage A sequences, as proposed by Cacciò *et al*. (2008). Sequencing and phylogenetic analysis of the concatenated *bg*, *tpi* and *gdh* genes showed that most of the assemblage A isolates clustered with other human-derived MLGs, all belonging to sub-assemblage AII (AII-1 and AII-4). Nevertheless, in seven assemblage A isolates the combination of the subtypes found at the MLST loci were different from those described before, so our group suggested following the numbering of MLGs

proposed by Cacciò *et al*. (2008), and named the novel combinations as AII-8 and AII-9. Moreover, a new assemblage A MLG was observed (INI 44), but could not be classified by previous proposed nomenclature.

The detection of a great number of sequences showing heterogeneous nucleotides, the description of new subtypes and multilocus (both in assemblage A and B) made us reflect on the genetic diversity of the parasite that is circulating in Rio de Janeiro, and how this variability could be related with symptoms in patients with and without HIV/AIDS virus, as well as its association with CD4 T cell counts. Generally, *G. lamblia* is one of the most common non-opportunistic parasites found in HIV infected patients (Boaitey *et al*., 2012; Kiros *et al*., 2015; Metha *et al*., 2013; Nkenfou *et al*., 2013).

The higher prevalence of assemblage B observed in HIV infected patients with CD4 T cell count less than 200 cell/mm^3, suggests that the low immunity can give more advantages for assemblage B replication than assemblage A. Besides, we observed a greater number of samples belonging to assemblage B in symptomatic cases (57.9%). Since most of the patients were asymptomatic (67.7%) they can act as unidentified carriers, spreading the infection, and thus contributing to the increase of epidemic rates.

6.2. Concluding Remarks

Overall, this work estimates the prevalence and gives a detailed description of the geographical distribution of intestinal parasites in Rio de Janeiro population, as well as identifies vulnerable areas, providing important additional information on the epidemiology of parasites in the country. Also reports the molecular epidemiological data of *G. lamblia* and evaluates its association with symptoms in patients with and without HIV virus.

It is the first time that MLG and MLST are applied in samples from Rio de Janeiro State, allowing the reliable genetic characterization of *G. lamblia* assemblages and sub-assemblages. We detect for the first time *G. lamblia* assemblage B in Rio de Janeiro, report a switch in genetic profile over the years, identify novel subtypes in both assemblages, and describe novel assemblage A MLGs. The results suggest that assemblage B has a good chance of being found in HIV infected patients and seem to be associated with symptomatology. Probably, the lower CD4 T cell count gives advantages for parasite replication.

This work provided a new insight into the genetic diversity of *G. lamblia* parasite and improved our understanding of the epidemiology of the disease by elucidating the dynamics of giardiasis in the population of Rio de Janeiro.

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