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Research Article

Molecular Detection of *Blastocystis* spp. in Cuban Pregnant Women

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Abstract

Introduction: *Blastocystis* spp. is a parasite of the chromistic kingdom capable of colonizing humans at the gastrointestinal level. During the last decades, several studies with different designs found an association between blastocystosis and iron deficiency anemia. Consequently, the involvement of *Blastocystis* spp. infection in the development of iron deficiency anemia in Cuban pregnant women should be carefully studied and, according to the results, adequately controlled.

Methods: Using the native Lugol method and concentration techniques, three stool samples collected from Cuban pregnant women were examined microscopically to demonstrate intestinal parasites. In addition, parasite molecular detection was performed on all samples. It was achieved by a conventional polymerase chain reaction method to specifically amplify a ~ 600-bp fragment of the *SSU rRNA* gene of the parasite. Blood specimens for hematologic parameters were collected.

Results: The presence of intestinal parasites was detected in 31.9% (43 out of 135) of the pregnant women screened. Protozoa were the only organisms found. Of all the pregnant women, *Blastocystis* spp. was the species more frequently present (in 28.9%, 39 out of 135). In 41 of the participants (30.4%, 41 out 135) was demonstrated anemia, and in 35 (25.9%, 35 out of 135), anemia was classified as iron deficiency anemia. The proportion of pregnant women parasitized by Blastocystis spp. who suffered from this type of anemia was significantly higher ($p = 0.02$).

Conclusion: This study microscopically revealed a high prevalence of *Blastocystis* spp. infection in Cuban pregnant women. The use of advanced molecular techniques permitted us to confirm that finding. At the same time, we demonstrated that gravid women infected with *Blastocystis* spp. are at high risk of suffering from iron deficiency anemia.

Keywords: *Blastocystis* spp. Infection; Molecular Detection; Pregnancy; Anemia; Iron Deficiency Anemia

Abbreviations

IDA: Iron Deficiency Anemia; PCR: Polymerase Chain Reaction; ST: Subtype; OR: Odds Ratios; ARN: Ribonucleic Acid; DNA: Deoxyribonucleic Acid

Introduction

Blastocystis spp. is a eukaryotic, anaerobic protist belonging to the phylum Stramenopiles [1,2]. It is composed of a heterogeneous set of subtypes (ST), and presents a wide pleomorphism with different replication strategies [3]. *Blastocystis* spp. is one of the parasites of the chromistic kingdom capable of colonizing humans at the gastrointestinal level; being the causal agent of blastocystosis or Ziert-Garavelli disease [2,4]. It is cosmopolitan, not restricted by climatic conditions or geographical area [5]. The infection is considered a zoonosis since, besides humans, *Blastocystis* spp. has been detected in mammals, birds, reptiles, amphibians and even insects [6,7].

To date, based on sequence analysis of the *Blastocystis* small subunit ribosomal of ribonucleic acid gene (SSU rRNA), at least 26 STs have been identified in humans and animals worldwide [8,9]. Subtypes 1-9 and ST12 have been found in humans [10,11]; some of which have also been observed in animals, such as ST3 in non-human primates, ST5 in cattle and pigs, ST7 in birds, and ST8 in non-human primates and birds [12,13]. On the contrary, some subtypes like ST10 and ST14 circulate predominantly in specific animals and have never been described in human infections [14], suggesting host specificity. Simultaneous colonization with different subtypes is not uncommon [15-17]. Subtypes 18 to 26 have recently been numbered [18], however, this may not be entirely true, as some researchers believe that some of these subtypes are molecular chimeras [19,20].

Regularly, the diagnosis of this protozoan consists of microscopic visualization in stool samples directly, or by the concentration technique, using stains such as Lugol, Giemsa, or trichrome [21,22]. Nevertheless, molecular detection using polymerase chain reaction (PCR) method is more sensitive than microscopy, and enables classification into STs [23-26].

During the last three decades, several studies with different designs found an association between blastocystosis and iron deficiency anemia (IDA) [27-29]. In 2012, El Deeb and Khodeer found that infection with *Blastocystis* spp. is a contributing risk factor for the development of IDA in pregnant Egyptian women [30]. More recently, to know the prevalence of blastocystosis and its possible association with IDA in Cuban pregnant women, we studied several parasitological and hematological variables in pregnant women from the city of Havana [29,31]. We observed that *Blastocystis* spp. was the parasite most frequently found in those women. At the same time, we encountered that the proportion of pregnant women suffering from IDA was significantly higher in the group of gravid women parasitized by *Blastocystis* spp. than in those not infected by that protozoon.

Globally, few studies address the diagnosis of infection by *Blastocystis* spp. in pregnant women [27-29]. Molecular studies to determine the prevalence of blastocystosis in women of childbearing age, including pregnant women, have not been carried out in our country. Taken into account the adverse effects of anemia on the health of mothers and their progeny, the contribution of infection by *Blastocystis* spp. in the development of IDA in Cuban pregnant women should be carefully studied and, according to the results that are reached, adequately controlled.

Material and Methods

Stool samples collection and microscopy

A descriptive and cross-sectional study was carried out in pregnant women attending at three polyclinics from La Lisa municipality, Havana, Cuba. From each pregnant woman, all enrolled in the study at the time of their recruitment, was obtaining the corresponding informed consent. Three serial stool samples (obtained spontaneously and on alternate days) were collected per each. All samples were examined microscopically for the presence of intestinal parasites, included *Blastocystis* spp., using native Lugol for the detection of protozoan (both cysts and trophozoites) and helminth ova, and concentration techniques (Willis and Kato-Katz methods) for helminth additional detection [29]. All fecal samples microscopically positive to *Blastocystis* spp. were preserved and stored at − 20 °C for further molecular analysis. Blood specimens for measuring hematologic parameters, including hemoglobin and hematocrit, were collected in tubes with EDTA and analyzed after 1-2 h. Blood specimens for serum iron parameters were collected in tubes without anticoagulant and analyzed after 4 h.

Deoxyribonucleic acid extraction and amplified of blastocystis isolates

The genomic deoxyribonucleic acid (DNA) was extracted from the stool samples using a QIAamp DNA Stool Mini Kit (QIAGEN Inc., Hilden, Germany) according to the manufacturer's instructions. The DNA concentrations were determined using a NanoDrop ND-1000 spectrophotometer (Thermo Scientific Inc., Wilmington, DE, USA). The SSU-rRNA ~ 600 bp fragment amplification was carried out by using primers RD5 (5'-ATCTGGTTGATCCTGCCAGT-3') and BhRDr (5'-GAGCTTTTTAACTGCAACAACG-3') to amplified *Blastocystis*-positive stool, using the PCR-conditions described in Scicluna., *et al*. 2006 [32]. PCR products were visualized under UV exposition in transilluminator equipment (Macrovue 2011, LKB, Sweden) after running 15 µL in 1.2% agarose gel staining with ethidium bromide.

Statistical analysis

Statistical analysis was performed using the Statistical Package Epidat 4.0 and EpiInfo 6.02. Pearson's Chi Square tests were used to examine the associations of *Blastocystis* prevalence with anemia and IDA. Odds ratios (OR) and 95% confidence intervals (95% CI) were computed, as well. All $p \leq 0.05$ were considered statistically significant.

Results

A total of 147 pregnant women from the municipality of La Lisa were recruited to participate in the study. Of them, 12 did not meet one of the inclusion criteria and were excluded from the research. Of the 135 pregnant women finally enrolled in the investigation, 31.9% (43 out of 135) were infected with one or more species of intestinal parasites. It could be observed that protozoan infections were the only ones detected. *Blastocystis* spp. (28.9%, 39 out of 135) was the most prevalent parasite. Two other protozoa were less frequently found: *Giardia lamblia* (3.7%, 5 out of 135) and *Entamoeba histolytica/dispar* (2.2%, 3 out of 135) (Table 1).

Table 1: Frequency of parasites detected in fecal samples of pregnant women enrolled in the study.

Parasite detected	No	%	[IC al 95%]
One or more parasites	43	31,9	[23,62-40,08]
Blastocystis spp.	39	28,9	[20,87-36,91]
Giardia lamblia	5	3,7	[1,21-8,43]
Entamoeba histolytica/dispar	3	2,2	[0,46-6,35]

In 41 of the pregnant women (30.4%, 41 out 135), it was demonstrated anemia, and in 35 (25.9%, 35 out of 135), anemia was classified as IDA. The proportion of pregnant women parasitized by *Blastocystis* spp. who suffered from anemia was higher than in those who were not infected with it (p = 0.01). The proportion of pregnant women parasitized by *Blastocystis* spp. who suffered from IDA was also significantly higher (p = 0.02) (Table 2).

Table 2: Relationship between anemia and IDA with the presence of *Blastocystis* spp infection in pregnant women enrolled in the study.

Clinical features	*Blastocystis* spp. Infection		Odd ratio, (95 %CI)	*P* values
	Positive (n = 39), No. (%)	Negative (n = 96), No. (%)		
Anemia				
Yes	19 (44,8)	22 (22,9)	3,19	0,01
No	20 (55,2)	74 (77,1)	(1,46-6,98)	
IDA				
Yes	16 (41,0)	19 (19,8)	2,82	0,02
No	23 (59,0)	77 (80,2)	(1,26 -6,30)	

With independence of microscopy results, the genomic DNA of each stool samples was analyzed by PCR targeting *Blastocystis* spp. (Figure 1). All *Blastocystis* spp. microscopic positive samples were positive by PCR and all negative ones were negative. This not only confirms and supports the positive microscopy results, but also, for the first time in Cuba, enables the implementation of molecular procedures for complementing microscopic diagnosis of blastocystosis. Barcoding PCR relies on the protocol originally described by Scicluna., *et al.* [32] and involves amplification of partial *SSU rRNA* genes. When used on genomic DNA from stool, amplification of non-*Blastocystis* DNA may occur, especially in the absence of *Blastocystis*-specific DNA in the sample [32]. This is also one of the reasons why this PCR should not be used alone for diagnosis. Hence, it is recommended that barcoding be used only for molecular characterization of already known positive samples; not for screening.

Discussion

Parasites infect pregnant women with relatively high frequency [33-35]. It is a consequence of two interacting physiological processes that occurs in the woman during pregnancy: the natural modulation of her immune responses and the occurrence of chang-

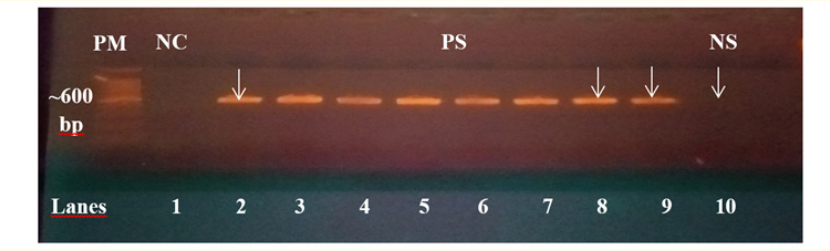

Figure 1: PCR gel electrophoresis of the isolated *Blastocystis* spp. revealed ~ 600 bp band size. Lane PM: 100 bp DNA ladder; Lane 1: negative control/adistilled water, Lane 2-8 amplified DNA of *Blastocystis* spp.: positive samples; Lane 9: positive control, Lane 10: negative sample.

es in her microbiota, both necessary for the healthy development of the fetus [33,36]. In addition, it is well documented that pregnancy, with its increased nutritional demands and altered immune defenses, is an especially vulnerable time to contract parasitic infections. Moreover, several of them can occur simultaneously, increasing the adverse consequences for mothers and their progeny [37,38].

The effects and severity of IP on pregnancy depend on different factors in relation with the mother and its scenario, including species involved, parasitic load, coexisting infections, intervals between pregnancies, nutritional health, immunity status, accessibility to safe drinking water, climate, and socioeconomic condition. IP during pregnancy is associated with serious adverse maternal and fetal outcomes [39]. Infections by intestinal parasites have a broad number of interrelated consequences for pregnant women and their offspring, including maternal anemia [33,40], low weight gain during pregnancy, poor fetal growth [41,42], low birth weight [42], and premature delivery [43].

Blastocystis spp. is a ubiquitous parasite with worldwide distribution [44,45]. Globally, the variability in the prevalence of this parasite may be due to the diversity of evolutionary forms that it presents, the different technological capacities used to detect it and, probably, the differences in the designs and in the characteristics of the sites where the investigations were conducted [29,34]. In the present study, *Blastocystis* spp. was the most common parasite among stool specimens, in which 28.9% of *Blastocystis* spp. positive samples were found by direct microscopic and confirmed by PCR.

Consensus recommendations define anemia as hemoglobin level (Hb) <10.5 g/dL during pregnancy and <10 g/dL during the postpartum period [46]. Anemia in pregnancy is a major public health problem affecting >56 million pregnant women worldwide. It is an important cause of maternal morbidity and mortality, preterm birth, intra uterine growth retardation, low birth weight and poor iron status in the infant [31,47,48]. *Blastocystis* spp. infection has been identified as a contributing factor to the pathogenesis of iron deficiency anemia in pregnant women [29,45]. In our study, a statistically significant association was found between the development of anemia, mostly IDA, and infection by *Blastocystis* spp. Cuba has reduced maternal and infant mortality rates to digits typical of high income countries. From now on, reducing these figures will require the prevention and control of less frequent and, in some cases, less known entities related with anemia. *Blastocystis* spp. infection may be one of them.

Several methods have been used for molecular characterization of *Blastocystis*. The two most commonly used are: PCR of unknown DNA targets using subtype-specific primers and PCR amplification of small subunit ribosomal RNA (SSU rRNA) genes followed by Sanger sequencing, one of which is the "barcoding" method [32,49]. Barcoding is currently the method of choice due to the sensitivity and specificity of this approach [14]. Finally, a modified version of the barcoding approach was recently introduced to facilitate better detection and differentiation of mixed subtype infections [15].

Recent literature shows that *Blastocystis* spp. is a complex of numerous subtypes associated with different types of clinical mani-

festations [50,51]. *Blastocystis* spp. infection has been identified as a contributing factor to the pathogenesis of IDA in pregnant women [29,45]. However, the causal mechanism underlying *Blastocystis* spp. infections and anemia, and the subtypes implicated remains to be demonstrated.

This study has some limitations. First, this is a cross- sectional study, and we are unable to draw causal conclusions. Second, a study that includes a greater number of samples and the aforementioned typing should be one of the recommendations for further research work.

Conclusion

This study microscopically revealed a high prevalence of *Blastocystis* spp. infection in Cuban pregnant women. The use of advanced molecular techniques permitted us to confirm that finding. At the same time, we demonstrated that gravid women infected with *Blastocystis* spp. are at high risk of suffering from IDA.

Conflict of Interest

Each author declares that he or she has no commercial associations (e.g. consultancies, stock ownership, equity interest, patent/licensing arrangement, etc.) that might pose a conflict of interest in connection with the submitted article.

Author Contributions

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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