



## OCCURRENCE OF INTESTINAL *MICROSPORIDIUM SPP* IN PEDIATRIC PATIENTS

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### ABSTRACT

Microsporidia includes about 1,300 species of obligate intracellular organisms that affect a wide range of hosts, even human. 15 species are recognized as pathogenic for humans. The most frequent genres that infect human are *Enterocytozoon spp* and *Encephalitozoon spp*. *Microsporidium spp* is considered opportunistic, but the infections have increased recently in both immunocompetent and immunocompromised individuals becoming in a public health problem. The aim was to establish the species of *Microsporidium spp*. present in fecal samples of children with and without gastrointestinal symptomatology using Faust coproparasitoscopic method (CPS), staining and molecular techniques. 130 stool samples from ≤11 years old children were examined by microscopy examination/Lugol's iodine and Kinyoun staining and Polymerase Chain Reaction (PCR) to identify a fragment of 18s ribosomal gene of intestinal *Microsporidium*. 18/130 (13.84%) samples were positive to *Microsporidium* by CPS and 20/130 (15.38%) samples were positive by PCR. All fragment amplified were sequenced, aligned and compared with sequences of species of *Encephalitozoon* reported in the genebank. Eleven samples were consistent with *Encephalitozoon hellem* (11/20, 55%) y nine corresponded to *Encephalitozoon intestinalis* (9/20, 45%), without correlation between the presence of diarrhea and geographic origin of samples. The correct identification of the species is of clinical importance to provide an effective treatment and, because the species cannot be differentiated by optical microscopy, the molecular tools have been very useful to know more about the infection by *Microsporidium*.

**KEYWORDS:** Microsporidia, *Enterocytozoon*, *Encephalitozoon*, Emerging, Parasite, Protozoa.

### INTRODUCTION

Microsporidia are unicellular opportunistic pathogens that infect a wide range of vertebrate and invertebrate hosts. These obligate intracellular eukaryotes consist of more than 170 genera and 1300 species<sup>[1]</sup> and at least 15 species have been implicated in human pathology since 1994, including *Enterocytozoon bieneusi*, *Encephalitozoon intestinalis*, *Encephalitozoon hellem*, and *Encephalitozoon cuniculi*. The life cycle of Microsporidia species that infect all major animal groups is direct and simple. Infection is transmitted from host to host by highly resistant spores, which have a unique organization including an infective sporoplasm and a coiled polar tube fixed to the anterior end of the spore, and ejects the spore contents into the host cell to indicate the proliferation.<sup>[2]</sup>

Parasite infections are common during the critical developmental period in children, especially in orphanage, nurseries, and schools. In recent years a high number of cases have been reported as opportunistic infections in immunocompromised patients, for example,

those who have received an organ transplant, VIH infected and individuals under cancer chemotherapy. Clinical manifestations of microsporidiosis are diverse and ambiguous and include fever, weight loss, and chronic or self-limiting diarrhea.<sup>[3]</sup>

Intracellular location of *Microsporidia* and highly natural resistant of the wall spore are factors that complicate the treatment. The usual treatment against *Microsporidia* includes the use of albendazole and fumagillin and some derivative drugs; however, they do not fully eradicate the parasites.<sup>[4]</sup>

*Microsporidium spp*. has been considered in the last decade a medical importance problem in Mexico and the world, although initially it was considered that only affected immunocompromised individuals, recent studies have shown that it can affect a large number of immunocompetent individuals causing gastrointestinal problems. The aim of this work was to establish the main species of *Microsporidium spp*. in fecal samples of children with and without gastrointestinal

symptomatology using Faust coproparasitoscopic method, staining, and molecular techniques.

## MATERIAL AND METHODS

**Samples.** We analyzed 130 fecal samples from  $\leq 11$  years old children obtained from patients with different pathologies who attend to the hospital (HIM) and children from rural communities with no apparent symptomatology (the data are summarized in table 1). All procedures performed in this study involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. The ethical considerations were following in accordance with the Institutional committee of HIM that reviewed and approved the project. Informed consent was obtained from all individual participants included in the study with the signature of a parent or legal tutor. Clinical data were gathered by asking the patients about their age, sex, time of chemotherapy treatment (In case) and clinical symptoms, especially diarrhea.

**Microscopy examination.** Fecal samples were collected and examined by microscopy after staining with Lugol's iodine<sup>[5]</sup> in order to find spores of *Microsporidium spp.* A slide preparation was made from each sample and it was fixed with methanol during 5 minutes, then slides were stained with Kinyoun<sup>[6]</sup>; fixed preparation was rinsed with concentrated carbol-fuchsin for 20 minutes, and then it was washed with 7% sulfuric acid and counterstained with Malachite green. Slides were dried and observed by light microscopy (Olympus BH2). The sample was viewed under oil immersion lens.

**Molecular assay.** DNA was extracted from all feces by use of the QIAamp™ DNA stool mini kit (Qiagen inc., Valencia, CA) according to the manufacturer's instructions, then PCR was performed with universal primers V1/PMP2 primers described by Chabchoub<sup>[7]</sup> to obtain a 300 bp DNA fragment. PCR reaction was performed in 25  $\mu$ L reaction mixture containing: 1x PCR amplification buffer, 1.2  $\mu$ M each dATP, dGTP, dCTP and dTTP, 0.5ng each primers and 1.5 U of Taq DNA polymerase (Roche; Mannheim, Germany), the amplification procedure included 5 min of initial denaturation at 95°C, followed by 35 cycles at 94°C for 30 sec, an annealing step at 55°C for 30 sec, an extension step at 72°C for 1 min and a final extension step at 72°C for 10 min, following the last cycle. The Amplified products were electrophoretically resolved on a 2% agarose gel (V3121, Promega, USA) and stained with ethidium bromide to visualize the amplified-PCR products under UV illumination.

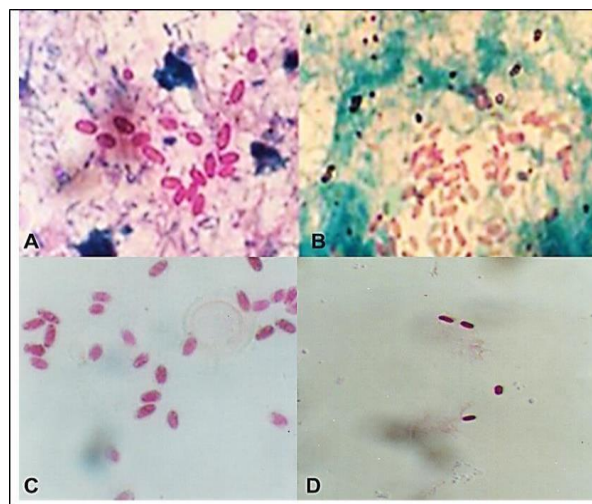
PCR products for *Microsporidium* species were sequenced on an ABI3730 automated sequencer (Applied Biosystems, ThermoFisher, UK). Consensus sequences for each strain were prepared using the Genius 8.0.3 software (Biomatters Ltd.). The sequences obtained

from the amplified fragments were compared with using BLAST and aligned by using Clustal Omega software to determine the species to which they belonged. Sequenced samples were compared with published sequences corresponded to 18s ribosomal gene available in the GeneBank of the following species: *E. hellem* (GenBank accession number **L39108**), *E.cuniculi* (GenBank accession number **L39107**) and *E. intestinalis* (GenBank accession number **EU436735.1**).

## RESULTS AND DISCUSSION

In the present study, we analyzed fecal matter isolates from 130 patients from symptomatic and asymptomatic children in order to identify the infective species of *Microsporidium spp.* After coproparasitoscopic analysis *Microsporidium spp.* positive samples and they were analyzed by molecular tools.

20/130 (15.38%) analyzed stool samples obtained from children were positive to *Microsporidium spp.* The microsporidial spores were identified on a fecal material spread on slide staining with Kinyoun technique; in Figure 1 the colored spores vary from pink to red on a green or blue background.



**Figure 1: Light microscopy view of slides stained with Kinyoun method. We can see the *Microsporidium spp* spores exhibiting morphological differences under immersion oil (A and B). C corresponds to *E. intestinalis* and the polar tube can be observed. D shows a slide of *E. hellem*. (Microscopy Olympus BH2, 100X).**

The CPS analysis shows different morphologies in the isolates when viewed under a microscope stained with Kinyoun method, the variations are observed in size, shape and way of conglomeration; the size varies from 1 to 2 microns and they are sometimes organized as single cells or in other cases in clusters like "bunch of grapes". The CPS analysis is a useful diagnostic tool, although it does not provide information about the species, since the mentioned variations are not sufficient parameters to determine the species to which they belong, provide an idea of the heterogeneity in the morphology of

*Microsporidium*, and could give an erroneous conclusion that these are different species, which is necessary to perform molecular tests.

**Table 1: Summary of analyzed samples.**

Sample	Code	Age (years)	Gender	Diagnosis	Diarrhea	Other parasites	Species of <i>Microsporidium</i>
1	16GRO	6	F	wd	-	<i>A. lumbricoides</i>	<i>E. h</i>
2	22CH	5	M	wd	-	-	<i>E. h</i>
3	33CH	6	M	wd	-	-	<i>E. h</i>
4	35CH	5	F	diarrhea	+	-	<i>E. h</i>
5	42CH	9	F	wd	-	<i>E. coli</i>	<i>E. h</i>
6	49CH	9	M	diarrhea	+	<i>E.coli</i>	<i>E. h</i>
7	59GRO	7	F	wd	-	<i>E. histolytica</i>	<i>E. h</i>
8	67GRO	3	F	diarrhea	+	-	<i>E. h</i>
9	GAPI*	7	M	ALL, with treatment	+	-	<i>E. h</i>
10	JAVI*	6	M	ALL, with treatment	+	-	<i>E. h</i>
11	MAVI*	8	M	ALL, with treatment	+	-	<i>E. h</i>
12	15HG	11	M	abdominal pain	+	-	<i>E. i</i>
13	16HG	9	F	chronic diarrhea	+	-	<i>E. i</i>
14	20HG	7	F	diarrhea	+	-	<i>E. i</i>
15	27HG	8	F	diarrhea	+	-	<i>E. i</i>
16	31CH	4	F	diarrhea	+	-	<i>E. i</i>
17	36CH	4	F	diarrhea	+	<i>E. histolytica</i>	<i>E. i</i>
18	49GRO	10	F	diarrhea	+	<i>E. histolytica</i>	<i>E. i</i>
19	5HG	9	M	diarrhea	+	<i>Cryptosporidium spp.</i>	<i>E. i</i>
20	7HG	10	F	diarrhea	+	-	<i>E. i</i>

Gender: F= female, M= Male. Presence (+) or absence (-) of diarrhea when the sample was taken. Wd: without a diagnosis of disease. ALL. Acute lymphoblastic leukemia. E.h: Encephalitozoon hellem, E.i: Encephalitozoon intestinalis. Samples labeled with GRO and CH correspond to children from rural communities of Mexico, GAPI, MAVI and JAVI belong to children diagnosed with ALL obtained at Children Hospital and the rest, (HG) were from children with gastric pathologies.

Table 1 shows a summary of the data obtained from twenty analyzed samples with presence of Microsporidia spores; the age ranging from 3-10 years old, with a media of  $6.77 \pm 2.25$ . 12 samples belonged to female patients and 8 to male patients, although no relation was observed with respect to the found species with genre or age. There was no important relationship between the presence or absence of diarrhea and other parasites with the determined species of *Microsporidium spp.* (table 1).

In this work, we analyzed three types of samples in the studied population (table 1), 11 samples of apparently healthy children without a clinical diagnosis, 6 of them with occasional diarrhea and 5 with no clinical symptoms. These children, belonging to a marginalized community, can present low levels of height and weight, affecting their physical and intellectual development, even when they do not show intestinal symptoms of infection. They can also be susceptible to parasitic infections due to the deficient sanitary conditions that exist in the region, in addition to the lack of drinking water and close cohabitation with domestic animals; the factors that determine the asymptomatic infection of *Microsporidium*. The presence of other parasites was also observed in these samples, such as *E. histolytica*, *A. lumbricoides*, and *E. coli*, which are often found co-parasitizing an organism and are indicative of poor hygiene in the water and food consumed.

3 samples from children with a diagnosis of ALL and under chemotherapy treatment phase, all three had chronic diarrhea that weakened their health, which had been attributed to chemotherapy treatment, however, in all three cases, spores of *Microsporidium* were found in fecal matter. In these cases, the infection by *Microsporidium* is even more serious, since diarrhea deteriorates the state of health, already vulnerable by the chemotherapy treatment, of the patient.

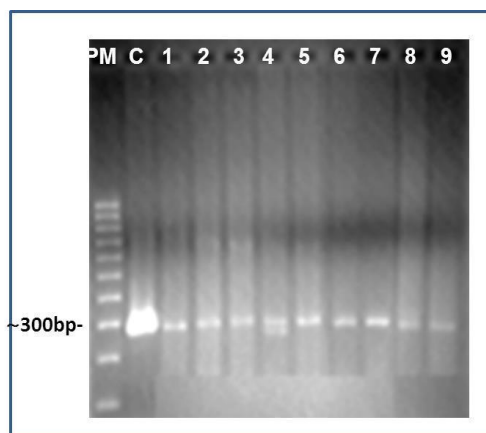
If a timely treatment is not administered, the infection can present complications that can even cause death. For this reasons, it is important to intentionally look for *Microsporidium spp* as a causal agent of diarrhea to stop the physical deterioration of the patient.

Six more samples of children with gastric pathology that included pain, distention and mainly chronic or recurrent diarrhea, without knowing a definitive etiology. Two of these patients had received a kidney transplant in the last three years and two of them had minor non-gastric surgeries. The rest did not have important medical antecedents and the reason for attending the consultation for their analysis was done to identify the cause of the diarrhea and gastrointestinal symptoms that they presented at that moment.

Although a higher incidence of infection would be expected in immunosuppressed patients, it was observed that in immunocompetent cases there are also reported cases of infection. A previous study was conducted to determine the presence of *Microsporidium spp* in ALL patients at different stages of treatment and it was observed that 20% of the children were infected with *Microsporidium*.<sup>[8]</sup> Although the frequency of *Microsporidium* was initially considered mainly in immunosuppressed patients such as those infected with HIV, those diagnosed with leukemia and in treatment, transplanted individuals, among others, it is now observed that the frequency indices have increased in both immunocompromised and immunocompetent individuals.

The search for *Microsporidium* is not done in routine parasitoscopic studies, because there is little information about the pathology that can cause, however, it has been observed that the prevalence in immunocompromised patients is high and this could complicate the general state of the people who also have a disease that causes their low immunological efficiency. In immunocompetent people, infections by *Microsporidium* have also been reported and the problem is that they do not present clinical symptoms, and consequently there is no etiological diagnosis and these individuals act as carriers transmitting the infection to children and adults around them, these reasons enhance the importance of molecular techniques to be able to establish the etiology of the symptomatology that is mistakenly attributed to other pathologies.

The amplification of a ribosomal gene fragment yielded a band of approximately 300 bp for all samples, (figure 2), so the amplification was sequenced to classify them according to their species.



**Figure 2: 1.5% Agarose gel electrophoresis of PCR stained with ethidium bromide. Amplicons were run simultaneously with molecular weight (MW) 100bp ladder (1); A band of 300bp corresponding to *Microsporidium spp* control (C) and amplification of *Microsporidium* present in feces from patients (1-9).**

The different concentrations of the amplicon observed in the agarose electrophoresis are due to the quantity obtained from fecal matter is not exclusive to *Microsporidium*. It was observed that all the isolates amplified in the same way there was not a difference that allowed to classify them, so the sequencing of the amplified fragment and analyzed by multiple alignment.

11/20 samples were consistent with *E. hellem* and 9/20 with *E. intestinalis*, although *E. cuniculi* was included, and this served only as a reference, there was no consensus with any sample (figure 3). No relationship was observed between the species of *Microsporidium* and the age, sex or symptomatology of patients.

Although, there are many infectious species of *Microsporidium*, only a few of them are capable of infecting humans and this is important because it allows expanding the knowledge to understand the epidemiology of infection and to open new horizons to improve the care of infected patients improving prevention and the treatment to avoid complications that compromise the quality of life.

While immunocompetent and healthy individuals usually develop an unnoticed and self-limited infection, the infection in immunosuppressed patients, who can act as carriers maintaining the transmission and spread of the infection. Although the infection can pass asymptomatic when there is an imbalance in the immune defenses, the symptoms can be serious and compromise the health state of the infected patient. Infection in humans can present a wide range of symptoms and clinical manifestations can be found in different tissues, such as the intestine, kidney, liver, lung, and cornea. Diarrhea, intestinal malabsorption, and severe weight loss may be observed.

The genera of *Microsporidium* that have been reported more frequently in humans are *Enterocytozoon*, and *Encephalitozoon*. *E. bienewisi* has been limited to the intestinal tract while *E. intestinalis*, *E. cuniculi* and *E. hellem* are described as a cause of disseminated disease.<sup>[10]</sup> In this study, it was only observed in fecal matter, since the analysis of other biological samples was not performed, however it is probable that it could be found when it was done, since they usually infect enteric first and later spread to other organs, where it can cause serious damage, in immunocompromised patients.

In the world there are just a few studies about the prevalence of *Microsporidium* on immunocompromised patients, specifically those that are under anti-cancer chemotherapy. The data are variable because of the difficulties in the identification of the organism and because the parasitic search is focused on more common parasites.



of feces samples stained by using fluorescent or chromotrope-based stains. However, due to correct identification of species is of clinical importance to give an effective treatment and considering that the species cannot be differentiated from each other by light microscopy; several molecular techniques have been reported.

#### Declarations

All procedures performed in this study involving the use of human samples were in accordance with the ethical considerations were following in accordance with the Institutional committee of HIM that reviewed and approved the project (number HIM/2014/004. Informed consent was obtained from all individual participants included in the study with the signature of a parent or legal tutor.

All participants in this work have read and approved the manuscript, and declare that they have no conflict of interest.

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