



MORPHOLOGICAL CHARACTERIZATION OF MICROSPORIDIAN SPORES IN GROUNDWATER IN CENTRAL REGION (CAMEROON): SIZE-SHAPE RELATIONSHIP AND SPECIES DIVERSITY

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ABSTRACT

In order to describe the shapes and sizes of waterborne microsporidian spores in tropical areas, samples were taken from groundwater (wells and springs) in the sub-urban areas of the Central Region of Cameroon. This descriptive and analytical study was conducted from August 2018 to March 2019 in the council areas of Okola, Mbankomo, Mbalmayo and Soa municipality. Forms of dissemination of Microsporidia protozoans were observed using the Olympus CK2 inverted microscopy with immersion oil at the objective 100X after the calorimetric technique based on the use of Trichrome of Weber stain. The observations have revealed several shapes of spores (ellipsoid, oval, ovoid, round, pyriform and fusiform) in the environment. These sizes of $([1 - 1.6] \times [0.7 - 1.2]) \mu\text{m}$ (E1), $([1.8 - 2.4 \times [1.2-2.0]) \mu\text{m}$ (E2), $([2 - 2.5] \times [1.6 - 2]) \mu\text{m}$ (E3), $([2.8 - 3.2] \times [1.6 - 2.4]) \mu\text{m}$ (E4), $([3.2-3.6] \times [2-2.4]) \mu\text{m}$ (E5), $([3.2-4] \times [2-2.4]) \mu\text{m}$ (E8) and $([3,6 - 4] \times [1,2-1,6])\mu\text{m}$ (E6) morphologically may correspond to *Enterocytozoon bieneusi*, *Encephalitozoon intestinalis*, *Encephalitozoon hellem*, *Encephalitozoon cuniculi*, *Nosema* spp., *Pleistophora* spp. and *Vittaforma corneae* respectively. These results show that spores of Microsporidia are waterborne and that consumption of this water would be a health risk for the population.

KEY WORDS: morphological characterization, spores, Microsporidia, Weber staining, groundwater.

INTRODUCTION

Microsporidia are eukaryotic organisms belonging to the phylum microsporidia, the taxonomy of which has undergone several modifications before being classified into the kingdom Protista by microsporidiologists (Cali *et al.*, 2016). Currently, more than 200 genera are identified worldwide. The most common of which are generally 1-4 μm in length as reported in humans are *Enterocytozoon*, *Encephalitozoon*, *Nosema*, *Brachiola*, *Vittaforma*, *Anncaliia*, *Pleistophora*, *Trachipleistophora*, and *Microsporidium* (Webber *et al.*, 1994). They are usually gastrointestinal pathogens; other studies have shown their presence in other tissues and organs including respiratory, excretory, nervous and muscular systems. These spores are common and ubiquitous in the environment (Cali and Takvorian, 2003). They are very resistant to pollution and survive for more than four months in the environment (Omalu *et al.*, 2006) and more than one year in aquatic system (Cali *et al.*, 2016). Microsporidia are obligate intracellular organisms that infect a wide variety of Invertebrates and Vertebrates, including Fish, Birds, Mammals, and Humans (Cali and Takvorian, 2004). They have been recognized as opportunistic agents emerging since the beginning of the AIDS epidemic, but were previously known in some animals. Among patients with HIV/AIDS, microsporidiosis is recognized as the third important opportunistic disease responsible for gastrointestinal disorders after *Cytomegalo* virus and *Cryptosporidium* (Sokolova *et al.*, 2011; Ajeegah, 2014).

Microsporidiosis is not only the prerogative in immune-compromised patients; earlier studies have shown their presence in immunocompetent individuals. They exert an intense intracellular parasitism which can lead to a significant pathogenic activity leading to the formation of spores which contain the infective form or sporoplasm and a polar tube. The main aim of this research is to characterize the various sizes and shapes of microsporidian spores from groundwater and the diversity of species.

Morphology of microsporidian spores

Microsporidia are generally oval or pyriform and relatively small, and their spores vary in length from 1 μm to 10 μm and generally from 1 to 4 μm for those that are pathogenic to humans (Webber *et al.*, 1994). The morphological character defining them is the presence of a polar filament or tubule. Spores have common general characteristics, although their sizes and ultrastructures vary according to the genus: they have a wall, a sporoplasm, an anterior vacuole and a posterior vacuole, as well as the polar filament and its anchoring disc. The spore is surrounded by a classical plasma membrane as well as two rigid extracellular walls: exospore and endospore. The exospore is made of a dense glycoprotein and fibrous matrix. The endospore is composed of alpha chitin and other proteins. Its thickness is fairly uniform except at the apex where this wall is thinner.

Inside the membrane is the sporoplasm (cytoplasm of the spore) which is the infectious material. It usually contains

one nucleus. The polaroplast is a large membrane organization occupying the anterior vacuole of the spore. The anterior portion of the polaroplast is highly organized in the form of stacked membranes called lamellar polaroplast, while the posterior portion is less organized and is called a vesicular polaroplast. The organelle playing the most obvious role in infection is the polar filament (or polar tubule). In sporoplasm, it is composed

of glycoprotein layer; 0.1 to 0.2µm in diameter and 50 to 500 µm long. It is attached to the apex via an umbrella structure called an anchoring disk. On 1/3 of the spore in this filament is stiff and helicoidal (the number of coils and their angles are preserved and allow to identify certain species). This filament ends at the level of the posterior vacuole. It seems that there is physical contact between these two structures.

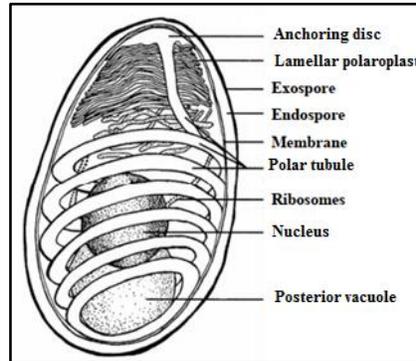


FIGURE 1: Diagram of the structure of a microsporidian spore (Franzen and Müller, 1999).

Life cycle and spore structure of Microsporidia

Typically, the live cycle of Microsporidia consists of three phases, in particular the infective or environmental phase, which represents the maturation of spores and infection of the host; the proliferative phase which represents the division of spores and the sporogonic phase which represents the formation of spores (Cali and Takvorian 1999). The infective spore contaminates the host orally. The spore extrudes its polar tubule into the membrane of

the target cell, usually an enterocyte, and injects the sporoplasm. This one becomes meronte (or schizonte) and initiates a process of division, binary or multiple. At some point, some cells differentiate into sporonts. These will develop a division-maturation process that leads to the formation of microsporidian spores. Man is contaminated by ingestion of spores contained in water and food contaminated with spores; or by inhalation and in direct contact.

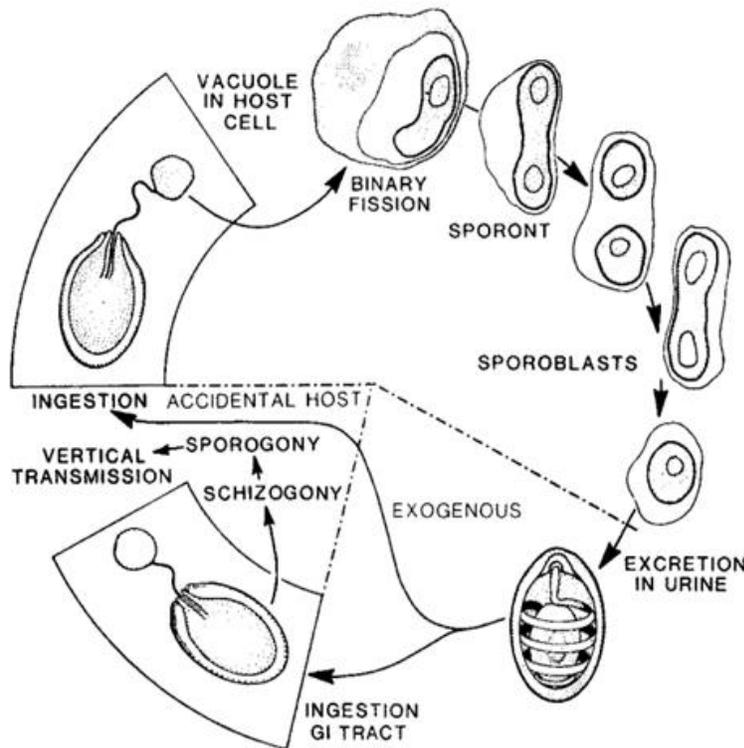


FIGURE 2: Live cycle of Microsporidia (Gardiner *et al.*, 1988)

TABLE 1: Microsporidian spores sizes associated to human infection

species	(2) Dimensions in μm	(3) Dimensions 100x in μm	Number of coils	Sites of infection	Contamination
(1) <i>Anncaliia connori</i> (<i>Branchiolaria connori</i>) (<i>Nosema connori</i>)	4 - 4,5 x 2 - 2,5	10 – 11.25 x 5 – 6.5	11	Systematic	Direct contact, Inhalation, ingestion
(1) <i>Vattiforma corneae</i> (<i>Nosema corneum</i>)	3,7 x 1	9.25 x 2.5	5-6	Cornea Urinary Tract	Ingestion, Contact oculaire
(1) <i>Anncaliia vermicularum</i> (<i>Branchiolaria vermicularum</i>) (<i>Nosema vermicularum</i>)	2,5 - 2,9 x 1,9 - 2	6.25 – 7,25x 4,75 - 5	7-10	Muscle	Direct, Contact, Inhalation, ingestion
<i>Nosema oculorum</i> *	3 x 5	7.5 x 12.5	9 - 1 2	Pancreas, liver, surrenales Systematic, surrenales, pancreas	Inhalation, Contact direct
(1) <i>Anncaliia algerae</i> (<i>Branchiolaria algerae</i>) (<i>Nosema algerae</i>)	3-4x2	7,5-10 x 5	8-11	Cornea, Muscle, Intestine	Ingestion, Eye Contact
(1) <i>Encephalitozoon cuniculi</i> * (<i>Nosema cuniculi</i>)	2,5 - 3,2 x 1,2 - 1,6	6.25 - 8 x 3 - 4	4,5 - 6	Systematic, Cornea Intestine, brain, heart, liver, kidneys	Ingestion, inhalation, Eye Contact
<i>Encephalitozoon hellem</i> *	2 - 2,5 x 1 - 1,5	5 – 6.25 x 2.5 – 3.75	6 - 8	Systematic surrenales, pancreás, liver, Cornea, Urinary Tract	Inhalation, Eye Contact, Ingestion
<i>Enterocytozoon bienewisi</i> *	1 - 1,6 x 0,7 – 1	2.5 – 4 x 1.75 – 2.5	5 - 6	Systematic, Intestine, skin, liver	Ingestion, Inhalation
(1) <i>Encephalitozoon intestinalis</i> * (<i>Septata intestinalis</i>)	1,7 - 2,2 x 0,8 -1,2	4.25 – 5.5x 2-3	5 - 6	Systematic, skin Intestine, Cornea,	Ingestion
<i>Microsporidium ceylonensis</i>	3,5 x 1,5	8.75 x 3.75	0	Cornea	Ingestion, Eye Contact
<i>Microsporidium africanum</i>	4,5 - 5 x 2,5 - 3	11.25 -12.5 x 6.25- 7.5	1 1 - 1 3	Cornea	Ingestion, Eye Contact
<i>Pleistophora</i> sp.	3,2 - 3,4 x2,8 4,0 x 2,4	8- 8.5x 7 10x6	11 11	Systematic Muscle Systematique muscle,	Ingestion
<i>Trachipleistophora hominis</i> <i>Trachipleistophora anthropophthera</i>	3,7 x 2,0 2,2-2,5 x 1,8-2,0	9,25 x 5,0 5,5-6,25 x 4,5-5,0	9	liver, brain, Cornéa Skeletal muscles, brain, heart, liver, kidneys	Ingestion Ingestion

(1) The specie recent name, (2) real dimensions of spores, *present in AIDS patient (3) increasing of image 2, 5(2) = (3)

Sources of information: (Pilarska *et al.*, 2015; Matthew *et al.*, 2014; Weiss, 2014; Sokolova *et al.*, 2010; Franzen *et al.*, 2006; Omalu *et al.*, 2006; Cali *et al.*, 2005; Vávra *et al.*, 1997; Weber, *et al.*, 1994).**TABLE 2: Infection Symptoms of Human Microsporidian Species**

microsporidia species	symptoms																
	diarrhea	cholangitis	cholecystitis	hepatitis	peritonitis	encephalitis	keratoconjunctivitis	rinitis	sinusitis	branchitis	pneumonia	urine infection	urethritis	nephritis	prostatitis	muscle infection	disseminated infection
<i>Enterocytozoon bienewisi</i>	x	x	x					x	x	x	x						
<i>Encephalitozoon intestinalis</i>	x	x	x				x	x	x		x	x		x			x
<i>Encephalitozoon hellem</i> (1)							x	x	x	x	x	x	x	x	x		x
(1) <i>Encephalitozoon cuniculi</i>	x			x	x	x	x	x	x			x					x
<i>Trachipleistophora hominis</i> (1)							x	x	x							x	
<i>Trachipleistophora anthropophthera</i> (1)						x										x	x
<i>Pleistophora</i> spp.																x	
<i>Vattiforma corneae</i> (2)							x					x					
<i>Nosema oculorum</i> (2)							x										
<i>Branchiolaria connori</i> (2)																	x
<i>Branchiolaria vermicularum</i> (1)																x	
<i>Branchiolaria algerae</i>							x										
<i>Microsporidium africanum</i> (2)							x										
<i>Microsporidium ceylonensis</i> (2)							x										

Table 2. Human microsporidiosis and clinical manifestations (from Franzen and Muller, 2001). The species noted (1) were only found in patients with AIDS whereas those noted (2) were only identified in immunocompetent individuals.

MATERIAL AND METHODS

Presentation of the central region

The Central Region is one of the ten regions of Cameroon, located in the Center of the country. Its capital is the city of Yaounde, the political capital of Cameroon, located in the south of the Central region between 3° 30 'and 3° 58' north latitude and between 11° 20 'and 11° 40' east longitude (Suchel, 1972). It is located at almost 750 m altitude, and is characterized by a particular climate in four seasons called "climate Yaounde" (Suchel, 1972) including: a Long Dry Season (LDS) which extends from mid-November to mid-March, a Small Rainy Season (SRS) that runs from mid-March to the end of May, a Short Dry Season (SDS) from June to August, a Long Rainy Season (LRS) that runs from September to mid-November. The thermal regime is hot and varies very little. Thus, average monthly temperatures range from 22.4° C to 27.2°C. The average annual rainfall is 1576mm.

Sampling

Samples were taken from groundwater (wells and springs) in the sub-urban areas of the Central Region; this study was conducted from August 2018 to March 2019 in the council Okola, Mbankomo, Mbalmayo and Soa Municipality. The water was collected in 1000cc flasks and transported to the Hydrobiology and Environment Laboratory for analysis.

Observations of spores of Microsporidia: WEBER staining technique

Among the various Trichrome techniques (WEBER, RYAN, KOKOSKIN and DELUOL WEBER staining) (1992), Weber's technique appears to be the most able to specifically distinguish spores from microsporidia. It is characterized by a good specificity, allows a satisfactory parasitological screening both in terms of specificity, sensitivity, and reliability (Sparfel *et al.*, 1998).

5 mL of the pellet is taken and put into a test tube. To this, 1mL of 10% formalin was added to fix the organisms and 3mL of 33% zinc sulphate was added successively (Faust *et al.*, 1938). The resulting mixture will be centrifuged at 500 turns/min for 10 minutes using a centrifuge. With the

help of a syringe, 4mL of the supernatant is removed and spread on a slide due to 1mL per slide. Let dry in air for 24 hours. The slides are stained and immersed in the Trichrome solution for 90 minutes at room temperature [Trichrome composition: chromotrope 2R: 6g; Flast green: 0.15 g; phosphotungstic acid: 0.7 g; 3 mL of glacial acetic acid; wait 30 minutes; gradually add 100 mL of distilled water in a 125 mL flask]. The slides are rinsed in acetic alcohol for 10 seconds to differentiate (5 mL of acetic acid + 995 mL of alcohol at 90 °), then quenched successively in ethanol at 95° for 30 seconds; Absolute ethanol for 10 minutes and in the xylene for 10 minutes to dehydrate. The reading is done first with the 40X objective of the light microscopy and then with immersion oil at the objective 100X in which is found microsporidian spores. The spores, generally ovoid and round shapes and appear pinkish- red color, and present a constant and characteristic colorless eccentric vacuole. The measurements were taken using a micrometer integrated in the lens and the photographs using an Xplovview brand camera connected to the computer.

RESULTS AND DISCUSSION

Presentation of the morphology (shapes and sizes) waterborne of microsporidian spores. A total of 192 samples collected have been analyzed and 10mL of each samples were observed in order to identify various forms of microsporidian spores at it various sizes.

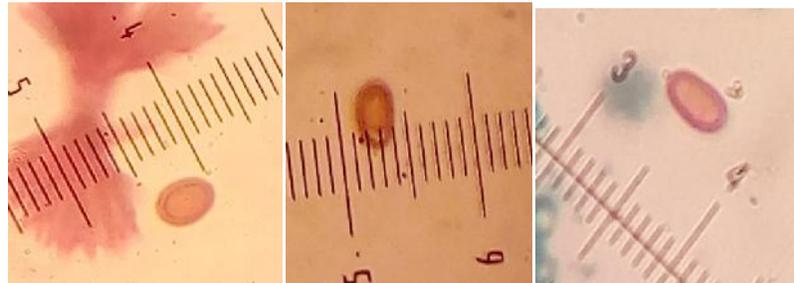
Ellipsoidal shapes

Shapes are generally oval with symmetrical poles. The spores have a straight, slightly curved shape with a rounded tip at both ends. These shapes are represented in the groundwater with various sizes.

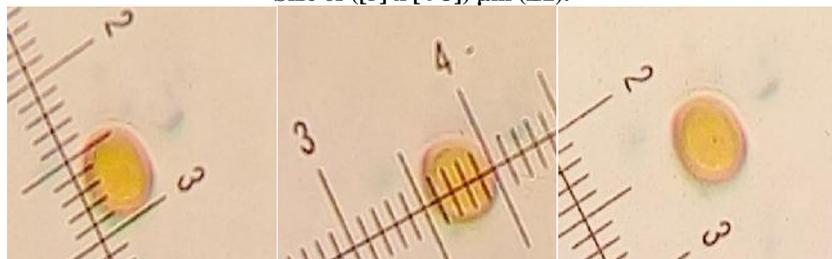
Size of ([2.5-4 x 2-3]) µm (E1)

Usually, they are the smallest intestinal microsporidian spores. They measure ([2.5-4] x [2-3]) µm at the objective 100x whose real size is given by the conversion factor [F (2/5)], corresponding to the size ([1 - 1, 6] x [0.8 - 1.2.] µm (E1) at the objective 40x which represents the real size (r) of the spore.

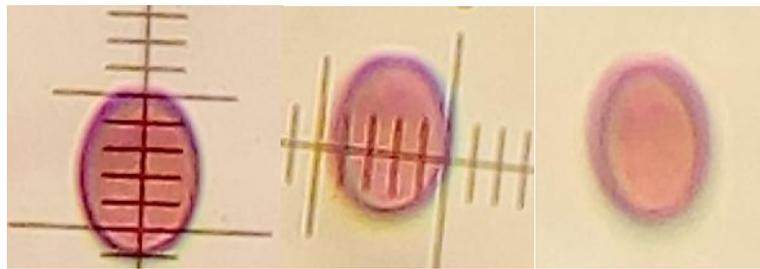
[F (2/5)] [2.5-4 x 2-3] (100x) = [1-6x0.8-1.2] (40x) = r



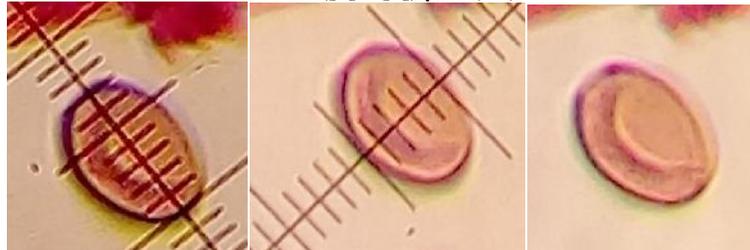
Size of ([5] x [4-3]) µm (E2).



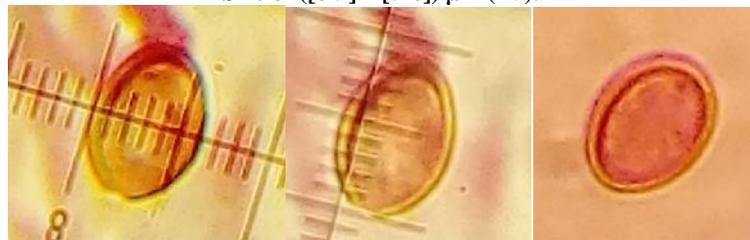
Size of ([6] x [5-4]) µm (E3).



Size of ([7] x [5]) μm. (E4)



Size of ([8-9] x [5-6]) μm (E5).

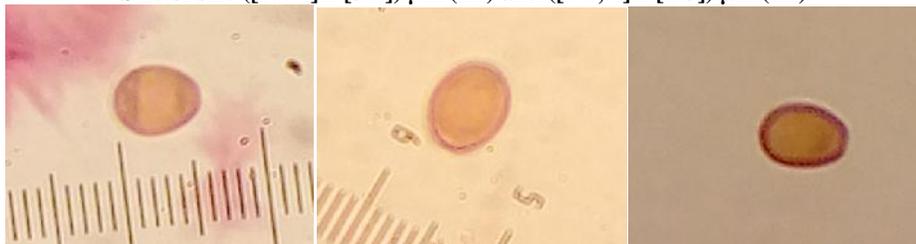


Oval shapes

This shapes presented unevenly symmetric forms at the poles of microsporidian spores. The spores have a straight to slightly curved shape with a rounded tip at both ends,

but one of the end is smaller than the other at the anterior pole where the Apex is. It presented various sizes ([2.5-4] x [3-4]) μm (E1) and ([5-4.4] x [4-5]) μm (E2) at objective 100X.

Size of size ([2.5-4] x [3-4]) μm (E1) and ([5-5, 4] x [4-3]) μm (E2)

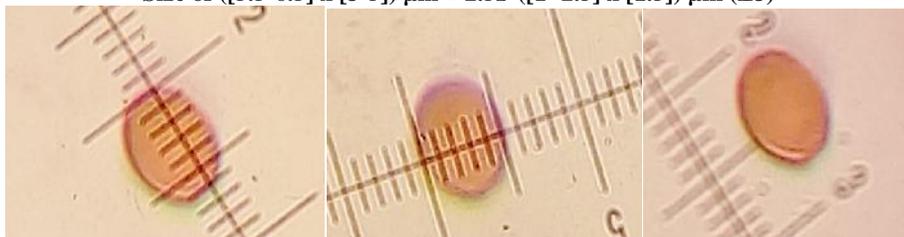


Size of ([5.5-8] x [3-6]) μm

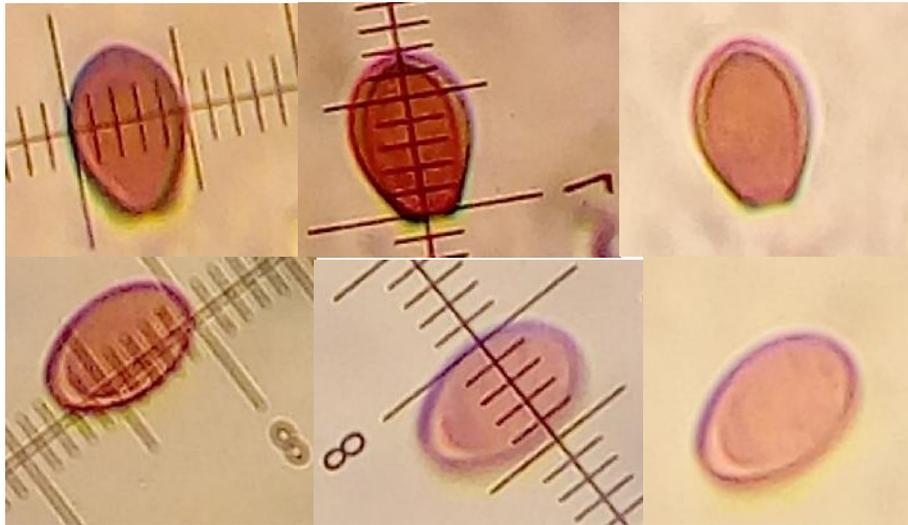
The spores have a straight to slightly curved shape with a rounded tip at both ends, but one of the ends is a little wider than the other; they are sometimes slightly narrower in the center or near the center; oval shapes may appear as asymmetrical. These spores represent morphologically

identical forms that are divided into three classes ([5.5-6.5] x [3-4]) μm. (E3); ([7-8] x [5-6]) (E4) μm; ([8 x 9] - [6]) (E 5) μm. At the objective100X these would represent respectively their real sizes ([2.5] x [1 - 1.5]) μm, ([2.5 - 3.2] x [1, 2- 1, 6]) μm and ([3, 2 - 3.6] x [2 - 2.4]) μm seen at the objective 40X.

Size of ([5.5-6.5] x [3-5]) μm = 2.5F ([1- 2.5] x [1.5]) μm (E3)



Size of ([7-8] x [5-6]) μm = 2.5F ([2.5-3.2] x [1.2-1.6]) (E4) μm

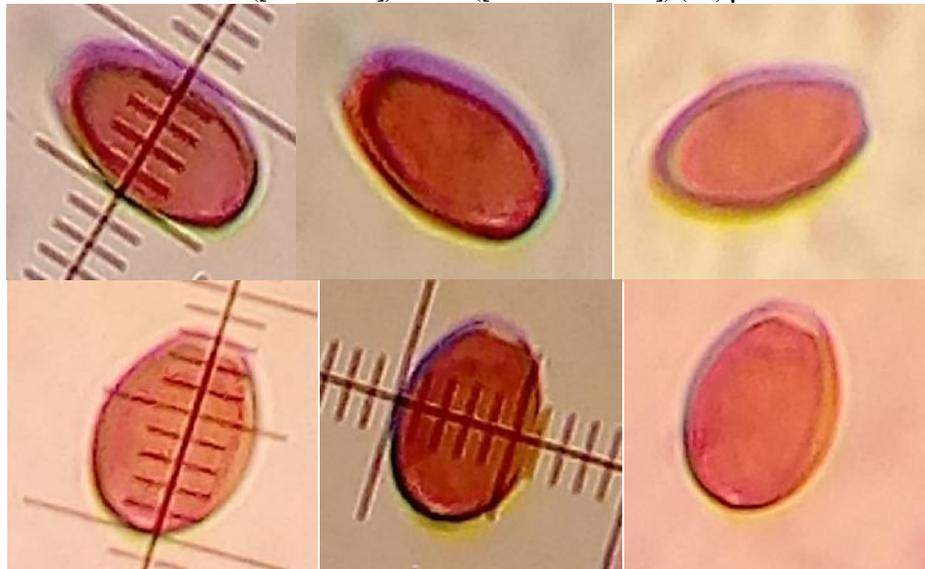


Size of ([7-8] x [5-6]) with the “apicule”

Oval shape, morphologically identical to size 7/5 μm (E4) but has a relatively smaller anterior pole and an “apicule” at the more rounded end of the posterior pole.



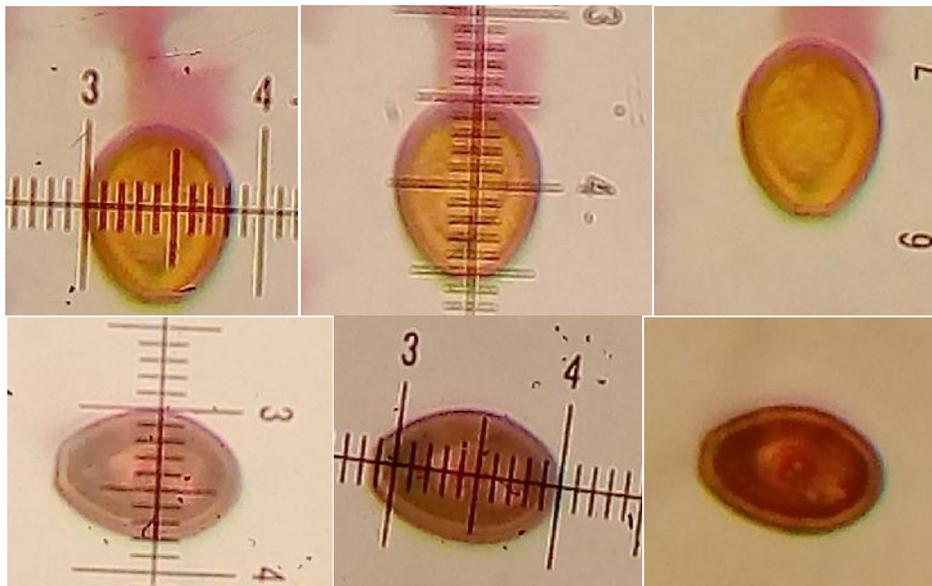
Size of ([8 - 9 x 5 - 7]) = 2.5F ([3.2 - 3.6 x 2 - 2.4]) (E5) μm



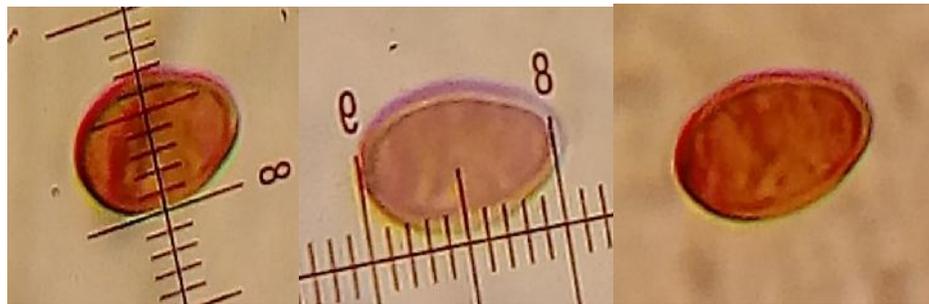
Oval at one of the accurate ends: Size ([10-11] x [8]) μm (E11)

The spores have a straight to slightly curved shape with a rounded tip at both ends, but one end is a little wider than the other; Oval forms measuring 10-11x8 μm with a mass of the internal apparatus or bulky sporoplasm of oval

shape and having approximately $\frac{3}{4}$ of the spore. The outer leaf or exospore is dark while the inner leaf or endospore is clear. The thickness of the layer is reduced to the anterior pole of the spore, zone where the polar tube is ejected.

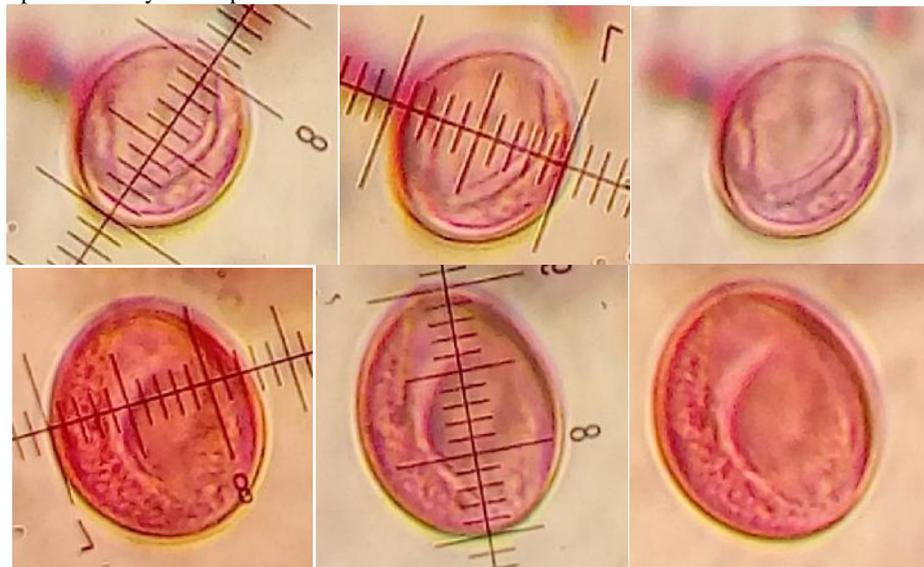


Oval shapes: Size([8-11]x [6-7]) μm(E12)



Oval shape to globular ([11-15] x [9-12]) μm (E13)

This oval shape tends to be globular: indeed, the length is close to the width. The cell presents an arc-shaped from one pole to the other pole that may be the polar tube.



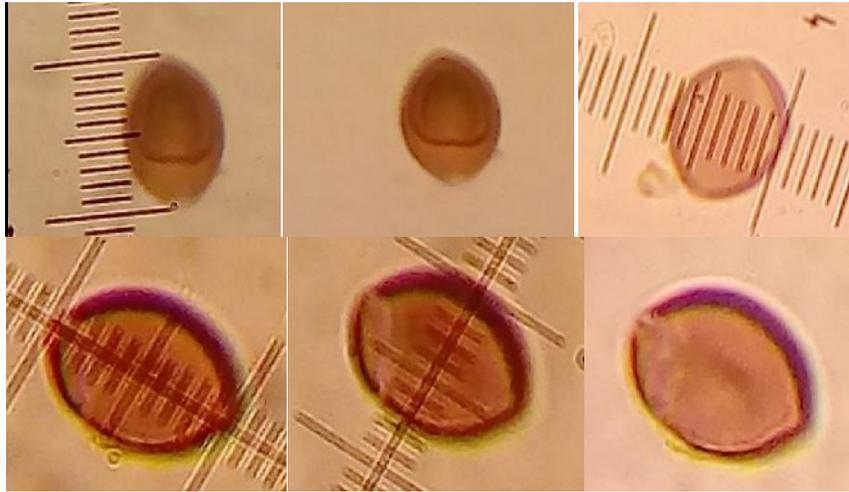
III.4. Ovoid shapes

Ovoid forms at both tapered ends: Size of ([9-10] x [7-8]) μm (E7)

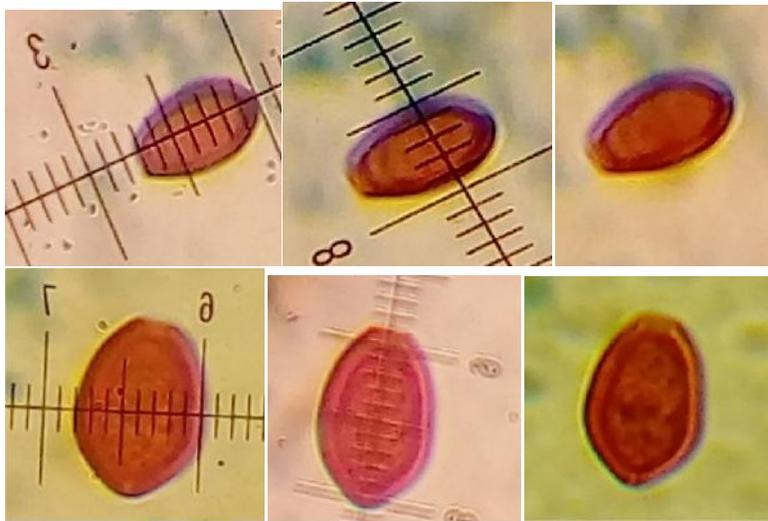
Acute rhomboid ovoid forms at both ends with a median bulge, having the shape of a kite or coconut. The

protective wall of the spore is clearly visible and thick. The sporoplasm may be visible more than 2/3 of the spore volume or measuring. This shape measures 10-9x8 μm at objective 100X.

Morphological characterization of microsporidian spores in groundwater

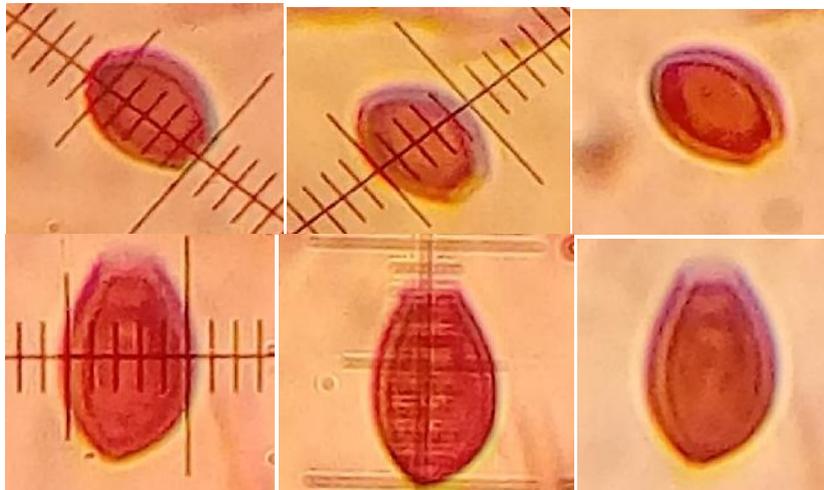


Ovoid forms of which one of the extremities presents an angular inclination with taper ending at the posterior pole and the other at the anterior pole is smaller with a flat ending at the objective 100X. It measures ([8-11]- [8x4]) μm (E10).



Size of ([7-11] x [8-5]) μm (E9)

Ovoid forms, one end of which has a convex inclination tapered at the posterior pole and the other arcuate at the anterior pole with a flat ending: Size of ([8-5] x [6-4]) μm (E3) and (E5).



Round shapes

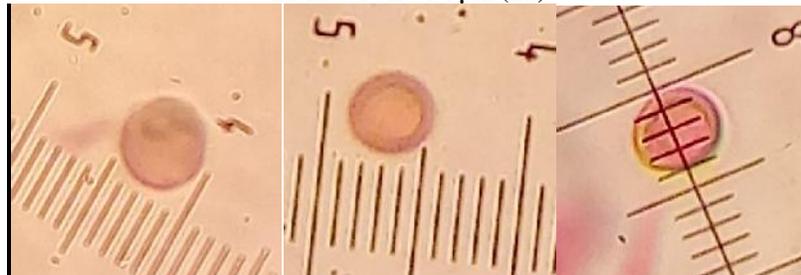
These shapes have a diameter ranging from 3 μ m to 7 μ m depending on the type of species observed. The sporoplasm may be visible with the vacuole or nucleus.

Round with regular or spherical or globose shape: 3/3, 4/4, 5/5, 6/6, 7/7

Diameter size 3/3 μ m (E1)



Diameter size 4/4 μ m (E2)



Diameter size 5/5 μ m (E3)



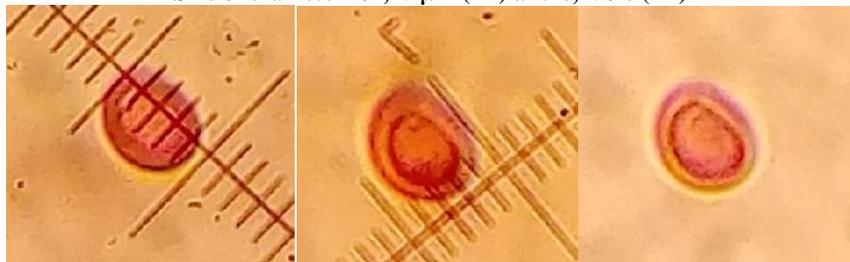
Diameter size 6/6 μ m (E3) and 7/7 (E7)



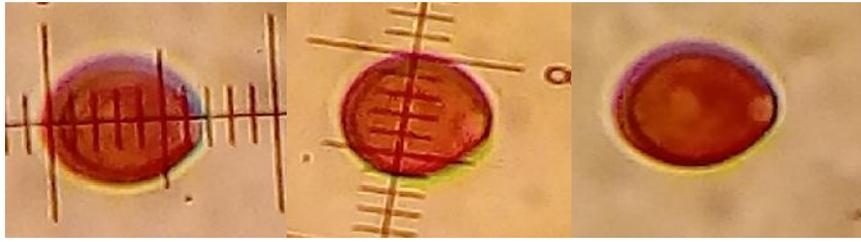
Roundish shapes with a distorted portion

The deformation spores can be presented by a semicircular form (E2). We can also note a globular shape with a bump, or the proximity of the ratio length / width (E3).

Size of diameter 4/4,25 μ m (E1) and 6,25 / 6 (E2)



Diameter size 5 / 5.25 and 6 / 6.25 μ m (E3)

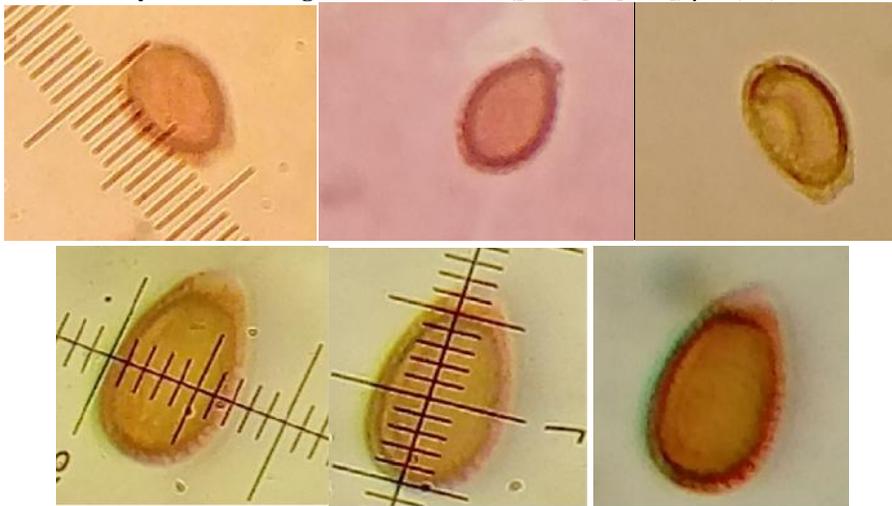


Pyriform shapes

The spores have a straight to slightly curved shape with one rounded end and the other is lightly curved at the tip or slightly truncated. This spore has a clearly visible double wall. There is a line that is sometimes visible from the posterior pole to the anterior pole and that may be polar filament. The anterior pole is less rounded than the posterior pole with a wall of the spore slightly thin at the

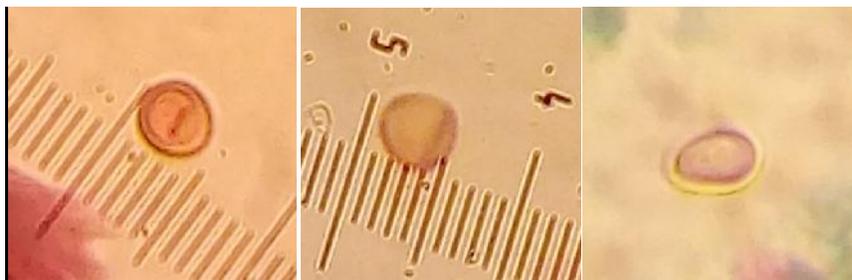
side of the apex through which the extrusion of sporoplasm occurs. This form measures (8-12) x (6-5) μm at the objective 100X. The wall flaps can be segmented with clear outer layer and a dark inner layer to the observation. For some pyriform shapes, the sheets are smooth whose sizes are ([4-7] x [3-5]) μm (E1, E2, E3 and E4).

Pyriform with segmented wall: Size ([8 - 12] x [6 - 7]) μm (E8)

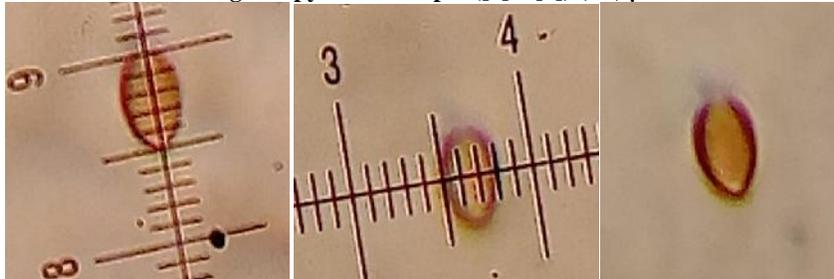


Pyriform shapes with smooth wall

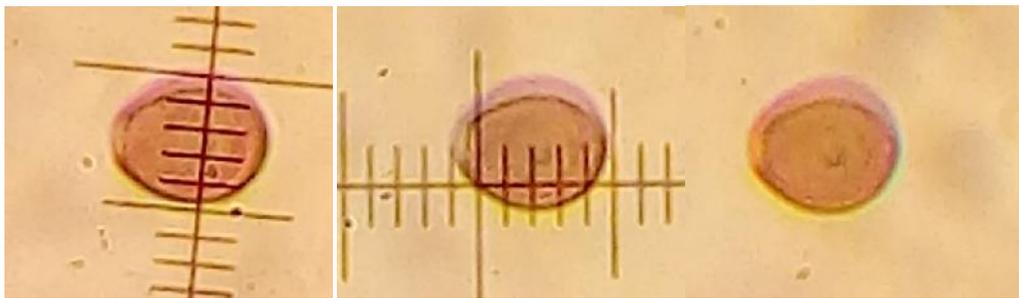
Shapes Tonsilotome one rounded end and the other acute ((E1)): ([4] x [3]) μm



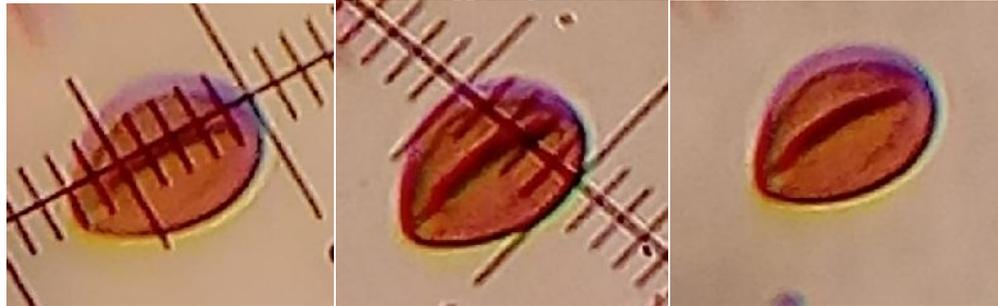
Elongated pyriform shape: ([5] x [3]) (E2) μm



Bulging pyriform shape: ([6] x [4,5]) μm (E3)



Pyriform with an egg- shape: ([7] x [5]) (E4) μm

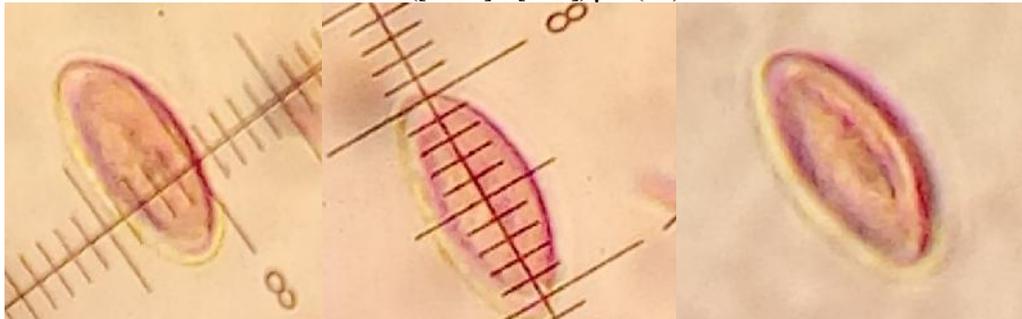


Fusiform shapes

This shape has a length usually more than twice the width. The spores have a straight to slightly curved shapes slightly rounded ends or slightly sizes. It can be irregularly

curved with two rounded ends; the length-width ratio is greater than one. It measures ([9-10] x [3-4]) μm and ([14] x [6]) μm.

Sizes ([9 - 10] x [3 - 4]) μm (E6)



Sizes ([14] x [6]) μm (E14)

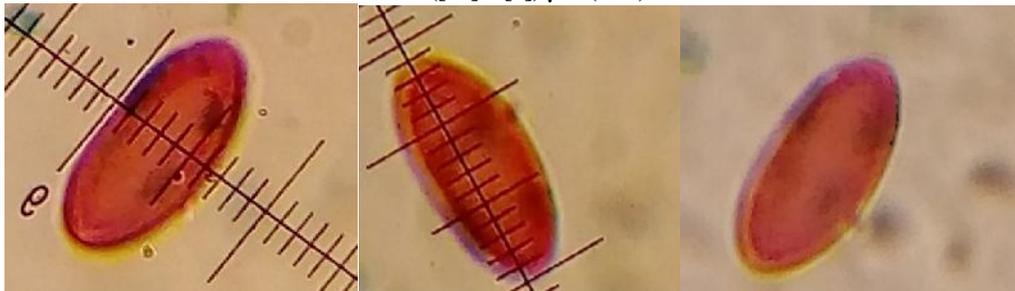


Table 3 presents the various sizes of microsporidian spores of waterborne in relationship with shapes observed at objectives 100X and their sizes corresponding to the objectives 40X give the real sizes (r) in other to better appreciate the organism in question. These sizes mark

their presences in relation to their shapes by the plus sign (+) and in case of no relationship, it is represented by the plus sign (-). The preference shapes is given by the double sign (++). The binary relationship illustrates the relationship between sizes and shapes of spore.

TABLE 3: Morphological identification of microsporidian spores

E	Size of 100x objectives in μm	b Size of 40x objectives in μm	Ellipsoidals	Ovals	Rounds	Ovoids	Pyriiforms	Fusifor ms	Real Dimensions in μm	Probability species
E1	[2.5 – 4] x [2 – 3]	[1 - 1,6] x [0,8-1,2]	++	+	+	-	+	-	1,08 - 1,64 x 0,7 – 0,98 Weber, <i>et al.</i> , 1994	<i>Enterocytozoon bieneusi</i>
E2	[4,5 – 6] x [3– 5]	[1,8 -2,4] x [1,2-2,0]	++	+	-	-	+	-	2,0-2,2x1,2 Omalu, <i>et al.</i> , 2006	<i>Encephalitozoon intestinalis</i>
E3	[5 – 6,25] x [4– 5]	[2 - 2,5] x [1,6 - 2]	+	++	+	+	+	-	2 - 2,5 x 1 - 1,5 Dei-CAS E., 1994	<i>Encephalitozoon hellem</i>
E4	[7-8] x [5– 6]	[2,8 - 3,2] x [1,6 - 2,4]	+	++	+	+	+	-	2,5-3,2 x 1,2-1,6 Omalu, <i>et al.</i> , 2006	<i>Encephalitozoon cuniculi</i>
E5	[8 – 10] x [5– 7]	[3,2 – 4] x [2 – 2,8]	+	++	-	-	-	-	3 - 5 x 2,0 – 2,5 Weber, <i>et al.</i> , 1994	<i>Nosema</i> spp.
E6	[9– 10] x [3– 4]	[3,6-4] x [1,2-1,6]	-	-	-	-	-	+	3,7-3,8x 1-1,2 Nicolas, 2003	<i>Vattiforma corneae</i>
E7	[9 -10] x [8]	[3,6-4] x [1,6]	-	-	-	+	-	-	3,5-1,6	<i>Microsporidium</i> spp
E8	[8 -12] x [6-7]	[3,2 – 4,8] x [2,4-2,8]	-	-	-	-	+	-	3,2 – 3,4 x 2,8 Nicolas, 2003	<i>Pleistophora</i> spp
E9	[7– 11] x [5– 8]	[2,8-4,4]x [2-3,2]	-	-	-	+	-	-	3,6-4x 2,4-2,8	<i>Microsporidium</i> spp
E10	[8– 11] x [4– 8]	[3,2-4,4]x [1,6-3,2]	-	-	-	+	-	-	3,5x1,6 Weber, <i>et al.</i> , 1994	<i>Microsporidium</i> spp
E11	[10- 11] x [8]	[3,6-4,4]x [2,8-3,2]	-	+	-	-	-	-	4,5x,2,5	<i>Microsporidium</i> spp
E12	[8-11]X [6-7]	[3,2-4,4]x [2,4-2,8]	-	+	-	-	-	-	4,4x2,5 Weber, <i>et al.</i> , 1994	<i>Microsporidium</i> spp
E13	[11-15] x [9-12]	[4,4-6]x [3,6-4,8]	-	+	-	-	-	-	4,4-6x 3,5-4,8	<i>Microsporidium</i> spp
E14	[14]X [6]	[5,6]x [2,4]	-	-	-	-	-	+	5,6x2,4	<i>Microsporidium</i> spp

(+): Presence of size-shapes relationship;
 (-): Absence of size-shapes relationship;
 (++) : Predominant shapes;
 (r): Real size according to previous research

(a): Probable species or genus
 (b): Corresponding size observe at objective 100x
 (E): variations of shapes of spores

The result of this study has shown variations of sizes and shapes of microsporidian spores corresponding to the diversity of species or genera in the groundwater environment. We observed several shapes belonging to the size ([1 -1,6] x [0,7 -1,2]) μm (E1) class that can be ellipsoidal, oval, pyriform and round. The morphological characters in size and mainly ellipsoidal shape show that these spores may belong to the specie *Enterocytozoon bieneusi* ([1 - 1, 6] x [0, 7 - 1] r) μm . According to Didier *et al.* (2004) and Weber *et al.*, (1994) the spores of *Enterocytozoon bieneusi* measure approximately 0.7-1.0 x 1.08-1.64 μm and are among the smallest of the

Microsporidia. These forms of spores are generally ellipsoidal or oval shape (Birkhead *et al.*, 2017). These spores are surrounded by a relatively thin chitinous endospore, possess a single nucleus and contain a polar filament that usually coils six times in a double-row alignment.

In addition, spore belonging to the size class of ([1.8 - 2.4 x [1.2-2.0]) μm (E2) can be ellipsoidal, oval, pyriform and round. The morphological characters on shape and size show whereas these spores are said to belong to the species *Encephalitozoon intestinalis* ([1, 7 - 2, 2] x [0, 8 - 1, 2] r) μm , generally of ellipsoidal shape. According to

Birkhead *et al.* (2017) and Weber *et al.* (1994), the mature spores of *Encephalitozoon intestinalis* measure approximately 1.2 x 2.0 µm rang in the class size of our observation with ellipsoidal shapes.

However, we also observed several sizes of oval spores present in classes ([2 - 2.5] x [1.6 -2]) µm (E3), ([2.8 - 3.2] x 2, 4]) (E4), ([3,2-3,6] x [2-2, 4]) µm (E5) with some having ellipsoidal shapes. Some spores rang in these classes are morphologically identical which may respectively represent *Encephalitozoon hellem* ([2 - 2, 5] x [1 - 1.5]r) µm *Encephalitozoon cuniculi* ([2.5 - 3.2] x [1.2 -1.6] r) and *Nosema* spp. ([2.5-5] x [1, 9-3] r)µm. For this purpose, the work of Delage *et al.* (1995) and Cali *et al.* (2011) show that these three species generally, present shapes that are morphologically identical and differ only in size or genetic characteristics (immunological techniques).

According to these some authors (Adam *et al.*, 1971; Shadduckl and Greeley,1980; Levine, 1985 ; Weber *et al.*, 1994; Omalu *et al.*, 2006; Birkhead *et al.*, 2017) *Encephalitozoon cuniculi* spores are ellipsoid, round or oval and measure approximately 2.5-3,2 by 1,2-1.6µm and the internal structure presents one nucleus measuring 1/3 of the parasite with round or oval forms . While those of *Encephalitozoon hellem* which are more rounded, oval or ellipsoidal, measuring approximately 2.0-2.5 x 1.0-1.5 µm and *Nosema* spp. with the oval form, measure approximately 2-3 by 4-5µm. (2.0-2.5 x 4.0-4.5 for *Nosema connori* and 3.0 x 5.0 for *Nosema ocularum*.). As similar to *Encephalitozoon hellem*, the spores of *Encephalitozoon cuniculi* can also have the round form, the nucleus is compact round or oval measuring ¼ à 1/3 of the parasite and it is not place on the central of the spore (Levine *et al.*, 1985).

Pyriform spores size of ([3,2 - 4] x [2-2,4]) µm (E12) class may belong to the genus *Pleistophora* ([3,2 - 3,4] x [2,8] r) µm and the fusiform shape of the size ([3,6 - 4] x [1,2-1,6]) µm (E8) may be *Vittaforma corneae* ([3,7] x [1] r) µm. The other shapes of unclassified spores are attributed to the genus *Microsporidium* without distinction of their shapes (oval, ovoid).

The spores of *Pleistophora* were oval, approximately 2.8 by 3.2-3.4 µm while those of *Vittaforma corneae* measure 1.0 by 3.7- (Birkhead *et al.*, 2017; Weber *et al.*, 1994).

According to reviews, the collective name of organisms that cannot be classified according to taxonomy because the appropriate information is not valid, specifically details of the parasite cycle that are unknown belong to the genus *Microsporidium* (Canning *et al.*, 1986; Weber, *et al.*, 1994).

Microsporidium ceylonensis was identified in a corneal ulcer of a boy from Sri Lanka. Spores measuring 1.5 to 3.5 µm were detected free in the corneal stroma. Meronts and sporonts were not seen, and nucleation was not observed.

Microsporidium africanum was detected in the corneal stroma of a woman in Botswana suffering from a perforated corneal ulcer. The developmental stage of the parasite was not seen. The spores measure 2.5 to 4.5µm containing 11 to 13 coils in the polar tube.

Overall, the measurements of microsporidian spores observed varied from [3-15] x [2-12]) µm to objective

100X regardless of the shapes corresponding to the real dimensions ([1-6] x [0, 8 to 4.8]) µm. This allowed us to group the spores into three groups: microspores 1 to 2.5 microns length, mesospores 2.6 to 3.6 microns length and macrospores greater than 3.6 microns length according to our observations. On this established basis, spores of the same shapes can belong to one, two or all three groups. The variations of the shapes with the same size of class and the deformation of the shapes from oval to round and vice versa could be related not only to the extrinsic factors or environmental but also to the intrinsic factors or specific to the cell, as well as the proximity of the length and the width of some shapes (E12) may explain the polymorphism of microsporidian spores. For this purpose, the work carried out by Delage *et al.* (1995) states that polymorphism is related to the variation in the size and shape of spores. In addition, according to Cali *et al.* (2016) the spore stage is variable in its resistance and may survive years in the environment. This polymorphism could be related to a spore adaptation mechanism or evolution of the life cycle. Table 3 shows that smaller sizes of Microsporidia (microspores and mesospores) are more susceptible to variation of shapes than bigger sizes of spores (macrospores). Variation of spores in sizes by length, width, or diameter may be allowed to take many shapes. For this purpose, Vavra Yachnis *et al.*, 1997 specifies the first species (*Trachipleistophora antropoptera*) dimorphic described in humans. However, some species include *Encephalitozoon hellem*, *Encephalitozoon cuiniculi* and *Nosema* spp. May be morphologically identical in light microscopy. Although the morphological criteria by observation with light and electronic microscopy make it possible to identify the spores of Microsporidia base on their various sizes. The biochemical and antigenic analysis makes it possible to better characterize them.

The presence of spores in the wells and springs shows that man-made contamination would be waterborne. For this purpose, Dowd *et al.*, In 1998, undertook a study of different water. The Dowd and al. study was carried out on 14 samples of water analyzed by PCR: surface water, groundwater and tertiary wastewater effluents. Seven (7) samples contained Microsporidia (*Encephalitozoon intestinalis*, *Enterocytozoon bienewsi* and *Vittaforma corneae*). Specifically, tertiary wastewater effluents were isolated: *Encephalitozoon intestinalis* and *Vittaforma corneae* and in surface water: *Enterocytozoon bienewsi*. This study represents the first confirmation of the presence of pathogenic Microsporidia for humans in the water environment, indicating that these opportunistic and emerging pathogenic Microsporidia are waterborne. An epidemiological study has shown the direct correlation between the use of groundwater, well water and *Encephalitozoon intestinalis* infections (Enriquez *et al.*, 1997). In addition, this result corroborates with the research of Ajeegah *et al.* (2016) on the contamination of groundwater with other forms of resistance and dissemination protozoans. The diversity and abundance of parasites in aquatic environment show the possibility of cohabitation of waterborne diseases and the risk of co-infection. Ingestion of these spores is thought to be responsible for intestinal disorders accompanied by clinical manifestations, the most common intestinal

microsporidiosis in immune-compromise patients being non-mucous and non-bloody fluid diarrhea (Datry *et al.*, 1996, Kolter and Orenstein, 1998). Infection, which evolves chronically for months, causes the emission of 3 to 12 stools per day. It is associated with malabsorption, loss of appetite and a gradual loss in weight aggravated in severe forms by dehydration gradually leading to cachexia (Didier, 1998). Dissemination of infection to other organs is also possible from an intestinal focus. *Encephalitozoon intestinalis* causes nephritis and sinusitis, whereas *Enterocytozoon bieneusi* was found in the trachea-bronchial tree and liver cells (Kolter and Orenstein 1997, Dore *et al.* 1995). Both species may be the cause of cholangitis and cholecystitis (Sarfati *et al.*, 2001), whereas they are originally located in the gastrointestinal tract. According to studies, 15 to 50% of chronic diarrhea in AIDS patients is due to microsporidia (Sarfati *et al.*, 2001). These spores of infectious power would be the cause of several cases of undetected diarrhea in patients.

CONCLUSION

At the end of this research which present the morphological characterization of spores of Microsporidia, these spores are characterized by a variation of sizes and shapes light microscopy observations of objective 100X under immersion oil allow us to highlight several genera that may be *Enterocytozoon*, *Encephalitozoon*, *Nosema*, *Vittaforma*, *Pleistophora*, and *Microsporidium* from the morphological characteristics. However, precision on these groups of organisms requires antigenic, biochemical analysis to better differentiate them. The groundwater (wells and springs) is contaminated with microsporidian spores, showing that Microsporidia contamination is waterborne. Finally, the detection of Microsporidia in groundwater samples indicates that there may be the potential for subsurface transport of these protozoan parasites. As a result, the consumption of wells and springs water ignoring the origin and the quality would be a health risk for the surrounding population. The distribution of microsporidian spores in the water environment would allow better understanding of the origin, contamination and environmental phase of these spores to limit health risks.

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