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Detection and Molecular Identification of Microsporidia in Urine Samples from School Children in South Sinai, Egypt.

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ABSTRACT

Microsporidia are obligate intracellular spore-forming protozoan parasites belonging to the phylum Microsporidia. They infect nearly all of the invertebrate phyla as well as all five classes of vertebrates. In this study, a total of 1720 human urine samples from school children in South Sinai, Egypt were separately collected, centrifuged and the obtained sediments were stained with modified trichrome (MT) stain to detect microsporidian spores. The microscopically positive urine samples for microsporidia were processed by PCR using generic and species specific primers. Microscopic examination revealed microsporidian spores in 7.4% of urine samples. In general, children living in Saint-Katherine city recorded the highest rate of infection with urinary microsporidiosis (19.9%), followed by those living in Abu-Rdees (12.4%), Al-Tour (5.4%), Ras-Sedr (4.7%) and Abu-Zneema (2.2%). On the contrary, no infection with urinary microsporidiosis was recorded among school children in Nuweeba city. Children of the primary schools showed the highest rate of infection with urinary microsporidiosis (8.5%), followed by those in the preparatory schools (7.3%) and lastly those of the secondary schools (3.3%). Molecular identification of the detected microsporidian spores at the species level revealed the presence of *Encephalitozoon intestinalis* and *Enterocytozoon bieneusi* in 38.7 and 97.3 % of PCR-positive samples, respectively.

Keywords: Microsporidia, Modified trichrome stain, PCR, South Sinai, School children.

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INTRODUCTION

Microsporidia are obligate, intracellular spore-forming organisms, initially classified as protozoa, while recent molecular phylogenetic studies have led to their reclassification as fungi [1, 2]. First recognized by Nageli in 1857 as the cause of disease in silkworms, microsporidia were not suspected as being a cause of human disease until 1959, when they were found in a child with encephalitis. Conclusive evidence of microsporidia as pathogens dates to 1973, when a 4-monthold athymic boy died from severe diarrhea and malabsorption caused by *Nosema* species (now *Anncalii aconnori*). Interest and knowledge of microsporidia as parasites of man have expanded dramatically since they were first described as a cause of persistent diarrhea and systemic disease in persons with AIDS [3]. Globally, few studies concerning the presence of microsporidia in urine were published. The detected species of microsporidia in urine samples were *Enterocytozoon bieneusi*, *Encephalitozoon intestinalis* and *Encephalitozoon cuniculi* [4, 5].

The most common symptomatic microsporidian infection is diarrhea [6, 7]. *E. bieneusi* or *E. intestinalis* are most commonly associated with diarrheal illness [8]. In immunocompetent patients, diarrhea is usually self limited [9]. Microsporidia also cause ocular infections (keratoconjunctivitis). *Trachipleistophora*, *Nosema ocularum*, *Microsporidium africanus*, *Vittaforma corneae*, *Annicaliia algerae*, *Microsporidium ceylonesis*, and some *Encephalitozoon* species have been reported to cause ocular microsporidiosis [10-12]. Other disorders have been associated with individual microsporidia species, including myositis, cholangitis, bronchiolitis, pneumonitis, sinusitis, cystitis, nephritis, hepatitis, peritonitis, prostatitis, and nodular cutaneous lesions [13, 14]. Microsporidian spores can be detected in urine by simple staining methods such as Giemsa stain [15] and Gram stain [16]. Other staining methods such as Calcflour white 2MR, Modified trichrome blue and indirect fluorescent antibody stains [17] were also used. Transmission electron microscopy was extensively used to illustrate the diagnostic ultrastructural morphology of microsporidian spores [18], while PCR-based methods were accomplished for genotyping of microsporidian spores [19, 20]. To our knowledge little information is available concerning the occurrence of urinary microsporidiosis in Egypt. The present work aimed to detect microsporidian spores in urine samples by light microscopy, to identify species by PCR and estimate prevalence of infections among school children from six cities in Egypt.

MATERIAL AND METHODS

Study Area

Six cities in South Sinai were included in our study. Five of these cities are coastal lying on the Gulf of Seuez (RasSedr, Abu-Zneema, Abu-Redees and El-Tour cities) and the Gulf of Aqaba (Nuweeba). The sixth city (Saint Catherine) is in the mountainous desert area of South Sinai (Fig. 1).

Figure 1: A map showing cities of Sinai Peninsula: 1: Ras-Sedr, 2: Abu-Redees, 3: Abu-Zneema, 4: Al-Tour, 5: Saint-Katherine, 6: Nuweeba.

Urine Samples

A total of 1720 urine samples were collected randomly from school children in South Sinai. Samples were collected from 6 cities in South Sinai governorate, namely Al-Tour, Abu-Rdees, Abu-Zneema, Ras-Sedr, Saint-Katherine and Nuweeba. Urine samples were separately collected in plastic containers labeled with the sex and age of the person from whom the sample was collected. Collected samples were preserved with 10% formalin and carried out after 3-4 days to Parasitology laboratory, Water Pollution Research Department, National Research Centre, Giza, Egypt. In the laboratory, about half the volume of each urine sample was centrifuged at 5000 rpm for 10 min [21]. The supernatant was discarded while the last drop (containing sediment) was fixed on a glass slide using absolute methyl alcohol (Merck) and then stained by modified trichrome staining technique [22]. Stained slides were examined using 100-x objective lens. Positive slides contained oval, crescent and/or rod shape spores stained red with characteristic for microsporidia staining pattern [22]. The second part of each sample was kept at -20°C until used for DNA extraction.

DNA Extraction

The preserved part of each sample was washed with phosphate buffer saline (PBS) and centrifuged at 2500 g for 5 min. The supernatant was discarded and the pellet was washed again 2 times as mentioned before. The final pellet was resuspended in 200 µl of PBS. Two hundred microliters of sample suspension were extracted using the Qiagen QIAamp DNA Stool Mini Kit (Stool Kit) (Qiagen, Valencia, CA). The manufacture's protocol was modified as follows. (i) The temperature of the initial lysis step increased to 95°C; (ii) incubation with Proteinase K was carried out for 4 h in a water bath at 55°C; (iii) 100 µl of AE buffer were used for elution. To increase the amount of DNA, the elution step was repeated by reapplying the original 100 µl to the spin column. DNA eluate was stored at −20°C until PCR analysis.

PCR Amplification and Electrophoresis

PCR was performed using three different diagnostic primer pairs: i) generic microsporidia primer pair (PMP1 and PMP2) used to confirm the presence of microsporidia [23]; ii) species specific primer pair (EBIEF1/EBIER1) for amplification of microsporidian small subunit rRNA (SSU-rRNA) coding regions of *E. bieneusi*[24]; and iii) species specific primer pair (SINTF/SINTR) for *E. intestinalis*[25]. Amplifi-cation of DNA was performed using Maxima Hot Start Green PCR master mix (Thermo Scientific). A hot-start procedure for microsporidia and *E. bieneusi* was used with an initial denaturation at 95°C for 4 min, followed by 35 cycles of denaturation at 94°C for 30s, primer annealing at 60°C for 30s, and extension at 72°C for 30s. A final extension step was performed at 72°C for 10 min [23, 24]. The optimal PCR conditions for the SINTF/SINTR primers were found to be an initial denaturation at 95°C for 4 min, followed by 35 cycles of denaturation at 94°C for 30s, annealing at 55°C for 30s, and extension at 72°C for 90s. A final extension step was performed at 72°C for 10 min [25]. The PCR products were detected by agarose gel electrophoresis and ethidium bromide staining.

Statistical Analysis

The obtained data were analyzed by two-way ANOVA using Minitab statistical program (Minitab Inc., Pennsylvania, USA).

RESULTS

Microsporidian spores stained with MT stain, appeared pink against a green counterstained background and contained a central pink band and posterior vacuole. This staining pattern helped to differentiate microsporidia from yeasts also stained pink and that have similar size (Fig. 2).

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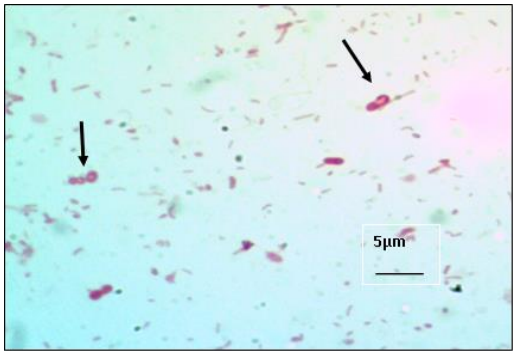

Figure 2: Trichrome stained specimen showing pink spores of microsporidia with colorless vacuole, against green background.

Microsporidian spores were detected in 127 (7.4%) out of 1720 children's urine samples stained with MT stain. The highest occurrence of microsporidia was recorded in SaintKatherine city (19.9%), followed by 12.4, 5.4, 4.7 and 2.2 % in Abu-Redees, Al-Tour, Ras-Sedr and Abu-Zneema cities, respectively, while no microsporidian spores were detected in urine samples from school children in Nuweeba. By PCR microsporidian spores were recorded in 111 (87.4%) out of the 127 microscopically positive samples (Table 1, Fig. 3).

Table 1: Prevalence of Urinary microsporidian spores in South Sinai.

City	Total examined samples	Positive samples by MT stain		Positive samples by PCR	
		No.	%	No.	%
Al-Tour	296	16	5.4	15	93.8
Ab-Redees	299	37	12.4	33	89.2
Abu-Zneema	139	3	2.2	3	100
Ras-Sedr	402	19	4.7	13	68.4
Saint-Katherine	262	52	19.9	47	90.4
Nuweeba	322	0	0	0	0
Total	1720	127	7.4	111	87.4

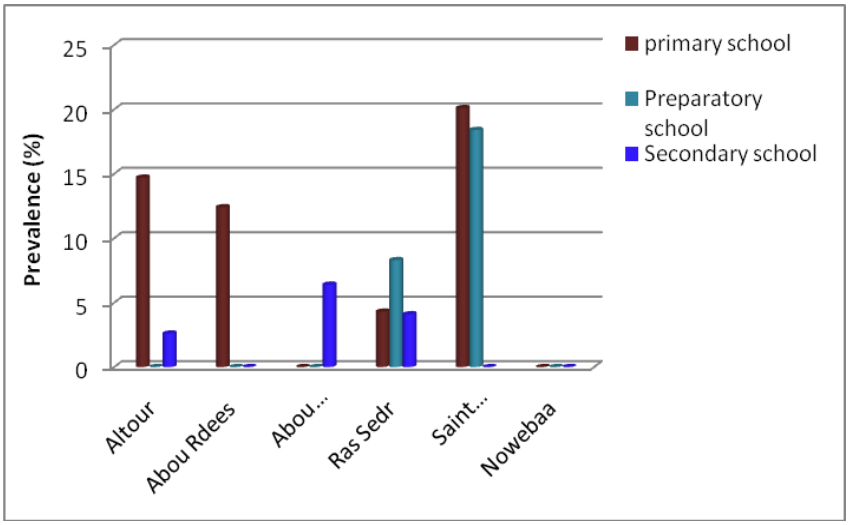

Figure 3: Prevalence of urinary microsporidia in school children of South Sinai

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It was found that in south Sinai cities, 2.7%, 61.3% and 36 % of PCR-positive urine samples had *Encephalitozoon intestinalis*, *E. bieneusi* and mixed infection (*E. intestinalis* + *E. bieneusi*), respectively. In South Sinai cities, *Encephalitozoon intestinalis* occurred only in primary school with the prevalence equaled 3.4%. The highest prevalence of mixed infection (*E. intestinalis* + *E. bieneusi*) reached 39.3% in primary school, followed by 36.4 and 9.1% in preparatory and secondary schools, respectively. The highest rate of infection with *Enterocytozoon bieneusi* reached 91% in secondary school, followed by 63.6 and 57.3% in preparatory and primary schools (Table, 2, Fig. 4).

Table 2: Identification of microsporidian species in South Sinai schools by PCR

School	Total PCR+	*Encephalitozoon intestinalis* only		*Enterocytozoon bieneusi* only		Mixed infection (*E. intestinalis* and *E. bieneusi*)	
		No.	%	No.	%	No.	%
Primary	**89**	**3**	**3.4**	**51**	**57.3**	**35**	**39.3**
Preparatory	**11**	**0**	**0**	**7**	**63.6**	**4**	**36.4**
Secondary	**11**	**0**	**0**	**10**	**91.0**	**1**	**9.1**
Total	**111**	**3**	**2.7**	**68**	**61.3**	**40**	**36**

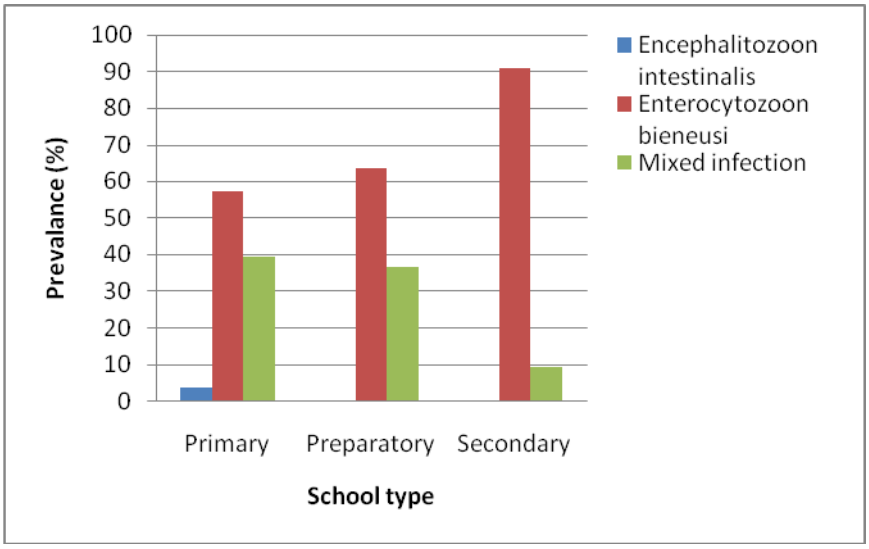

Figure 4: Distribution of microsporidian species among South Sinai schools.

p value = 0.1 (i.e. more than 0.05) so that the age had no significant effect on the prevalence of different microsporidian spp. Also there was no significant effect between the prevalence of *Enterocytozoon bieneusi* versus *Encephalitozoon intestinalis* in school children (p = 0.236) (Table 3).

Table 3: Two-way ANOVA for the effect of the children age against the prevalence of microsporidian spp

Sources	SS	MS	F	P
Age	4221.0	2110.5	8.96	0.100
Microsporidian spp.	661.5	661.5	2.81	0.236

SS: Sum of squares **MS:** Mean square **F:**F-distribution variable **P:** Significance

In South Sinai cities, *Encephalitozoon intestinalis* occurred only in Al-Tour city with the prevalence of 20 %. The highest rate of infection with *Enterocytozoon bieneusi* reached 100% in Abu-Redees and Abu-Zneema, followed by 61.5, 53.3 and 34.0% in Ras-Sedr, Al-Tour and SantKatherine, respectively. There was no infection with *Enterocytozoon bieneusi* in Nuweeba city. Mixed infection (*E. intestinalis* and *E. bieneusi*) was recorded only in SantKatherine, Ras Sedr and Al-Tour with the prevalence of 66, 38.5 and 26.7%, respectively (Table 4, Fig. 5).

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Table 4: Identification of microsporidian species in South Sinai cities by PCR

South Sinai cities	PCR+ samples	*Encephalitozoon*		*Enterocytozoon bieneusi*		Mixed infection	
		No	%	No	%	No	%
Al-Tour	15	3	20	8	53.3	4	26.7
Abu-Rdees	33	0	0	33	100	0	0
Abu-Zneema	3	0	0	3	100	0	0
Ras-Sedr	13	0	0	8	61.5	5	38.5
Sant-Katherine	47	0	0	16	34.0	31	66
Nuweeba	0	0	0	0	0	0	0
Total	111	3	2.7	68	61.3	40	36

Figure 5: Distribution of microsporidian species in South Sinai cities

P value (0.042) is less than 0.05 indicating that the cities had a significant effect against the prevalence of different microsporidian spp. On the contrary, there was no significant effect between the prevalence of *Enterocytozoon bieneusi* versus *Encephalitozoon intestinalis* in the cities (p = 0.081) (Table 5). *Encephalitozoon intestinalis* - and mixed infections were recorded only in males with the prevalence of 3.8 and 50.6%. On the other hand, the highest,100% infection with *Enterocytozoon bieneusi* was recorded in female urine samples (Table 6, Fig. 6).

Table 6: Distribution of species among genders

Gender	Total PCR + samples	*Encephalitozoon intestinalis* only		*Enterocytozoonbiene usi* only		*E. intestinalis* & *E. bieneusi*	
		No.	%	No.	%	No.	%
Male	79	3	3.8	36	45.6	40	50.6
Female	32	0	0	32	100	0	0
Total	111	3	2.7	68	61.3	40	36.0

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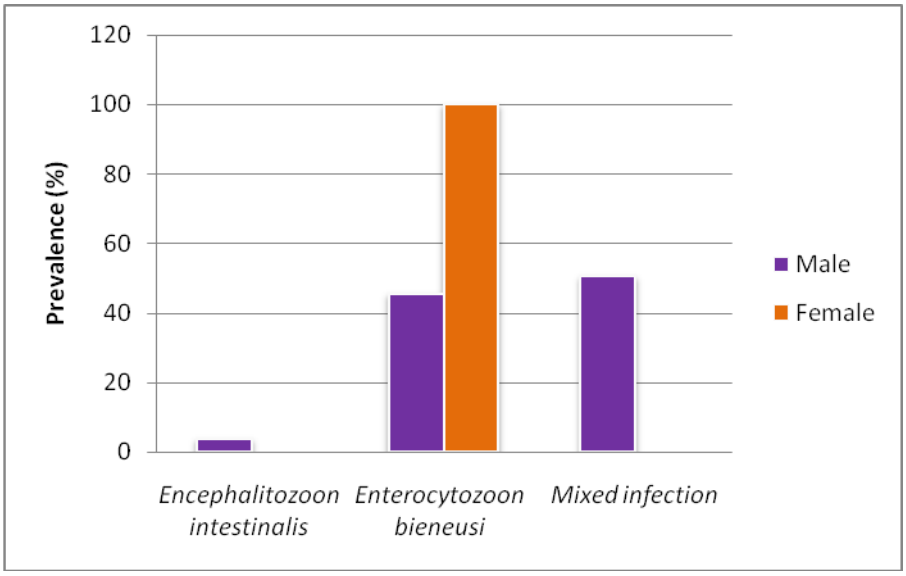

Figure 6: Distribution of microsporidian species among genders in South Sinai cities.

P value = 0.007 (i.e. less than 0.05) so that the children gender had a significant effect against the prevalence of different microsporidian spp. Also there was a significant effect on the prevalence of *Enterocytozoon bieneusi* versus *Encephalitozoon intestinalis* through children gender (p = 0.010) (Table 7).

Table 7: Two-way ANOVA for the effect of the gender against the species type of microsporidia

Sources	SS	MS	F	P
Gender	1892.25	1892.25	7569.00	0.007
Microsporidian spp.	1056.25	1056.25	4225.00	0.010

SS: Sum of squares **MS:** Mean square **F:**F-distribution variable **P:** Significance

DISCUSSION

In this study the estimated prevalence of microsporidia infection was 7.4 %, which is less than it had been recorded by previous authors who examined AIDS patients (15%) [26]. It should be noticed that, first, the other authors studied immunocompromised patients, and, second, the numbers of urine and pulmonary specimens were not specified, making the comparison with our study inaccurate. The study of urine samples from immune patients in Tenerife (Spain) [27], stained with Weber's chromotrope stain, on the contrary, recorded a lower prevalence of microsporidia equaled to 2.5%. This difference can be explained by higher prevalence of microsporidiosis in children. Also, the authors examined only 40 samples, and their results might have not been representative. In the present work as many as 1610 urine samples were examined. Most of school children in Nuweiba were pure nomads living in a closed Bedouin community, while children in the other examined cities were mixed between nomads and those coming from other provinces of Northern Egypt. In another studies conducted in Giza governorate, Egypt, it was found that 13% of children aging 6 to 18 years old had microsporidian spores in their urine [5]. In other studies, microsporidian spores were detected in only one urine sample of AIDS man patient [28, 29]. Our result might be more accurate than those studies as we examined 878 random male urine samples for the microsporidian spores. Other workers [30] detected no microsporidian spores by using Weber's chromotrope based stain in the only examined one male AIDS patient. Didier et al. [31] also detected microsporidian spores in urine of a 37 years old AIDS patient. On the contrary, Del Aguila et al. [32] in Madrid (Spain) found no microsporidian spores in the examined 127 urine samples collected from 86 HIV positive children whose mean age was 6 years old. Concerning immunocompetent patients, other workers [33] examined urine of only one case aging 26 years old and detected microsporidian spores in it. Svedhem et al. [29] detected microsporidian spores in urine of a 39 years old patient. In our study, we randomly selected school children having only up to 18 years old without specification of their immune status. With respect to microsporidia in urine samples of children, a little data was published worldwide concerning microsporidia in urine samples of children. In the current investigation, microsporidial spores were

molecularly recorded in 111 (87.4%) out of the 127 microscopically positive samples. Most of children in South Sinai cities were found suffering from amorphous and triple phosphates (unpublished data) that might act as inhibitors for PCR. Other workers declared that inhibitor removal represented a major challenge to any DNA extraction process [34]. One tactic to avoid assay inhibitors is to filter the microsporidian spores away from the specimen. This approach has been successful for amplification of microsporidian DNA from as little as 100 to 500 spores/ml of sample but is labor-intensive [36]. While a number of PCR assays are available for microsporidia [21, 36-39], none of these assays has a ready mean by which the expected inhibitory effect caused by the specimen matrix could be overcome. Two different species of microsporidia were molecularly identified in the present work namely *Enterocytozoon bineusi* and *Encephalitozoon intestinalis*. Also, it was found that all the infected female children had only *Enterocytozoon bineusi*. Del Aguila et al. [39] detected microsporidian spores in the four examined urine samples by using PCR, the species were *Encephalitozoon cuniculi*, *Encephalitozoon hellem* and *Encephalitozoon intestinalis*. Didier et al. [32] detected *E. cuniculi*, *E. hellem* and *E. intestinalis* in the only one urine sample of an AIDS patient by using PCR. In other studies [34], only *Enterocytozoon cuniculi* was detected in all 12 urine samples of AIDS patients. In Spain [27], *Enterocytozoon bieneusi* was detected in 1/40 (2.5%) of immunocompetent patient urine samples. The presence of microsporidia in urine samples is rare [27, 35, 36]. To our knowledge this is the first large scale study concerning prevalence of microsporidia in urine samples from school children in Sinai Peninsula and there was no pervious published data in Egypt to compare with. In conclusion, we recommend using modified trichrome stain for the preliminary detection of microsporidian spores in the routine analysis of urine samples, as 87.4% of MT stained positive samples for microsporidia were also positive by PCR. Regarding PCR analysis, it is useful for confirmation and species identification.

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