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Original Article

Encephalitozoon cuniculi infection among immunocompromised and immunocompetent humans in Egypt

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Abstract

Background: *Encephalitozoon cuniculi* infects a wide range of homoeothermic animals, including man. Complications due to this microsporidian have been reported only in immunocompromised patients. Reports on *E. cuniculi* in immunocompetent humans are lacking, most probably, because it is not linked to any clinical manifestations in such hosts. The present work was carried out with the aim of studying, for the first time in Egypt, the prevalence of *E. cuniculi* infection of urinary tract among non-HIV immunocompromised patients and immunocompetent individuals. It tested also the influence of some factors on the risk of infection.

Methods: Blood and urine samples were collected from 88 persons (44 non-HIV immunocompromised patients and 44 subjects as immunocompetent control group). IFAT serological assay and Weber's green modified trichrome stain (MTS) urine smears were carried out. Molecular study by PCR was also performed to detect DNA of *E. cuniculi* in urine samples. A full history sheet was fulfilled for each subject to test the suspected risk factors.

Results: The IFAT examination confirmed the presence of antibodies against *E. cuniculi* in 44.3% of the human subjects. The seroprevalence of *E. cuniculi* was significantly higher in the immunocompromised patients compared with the immunocompetent individuals (77.3% versus 11.4%). Compared with IFAT (the gold standard), the sensitivity and specificity of Weber's green MTS smears were 69.23% and 89.80%. By using PCR, no positive cases were detected among human subjects.

Conclusion: A high prevalence of *E. cuniculi* infection in the studied individuals was noted. Although infection was found in some immunocompetent individuals, the immune status of the host remains the corner stone for occurrence of the infection.

Introduction

Encephalitozoon cuniculi is an obligate intracellular microsporidian species, which infects a wide range of birds and mammals (1-3). There was some doubt whether *E. cuniculi* did in fact cause human infection (4). However, the microsporidian is increasingly recognized as a cause of severe disseminated infections in immunocompromised patients (5-9). Such disseminated infections including the ocular, respiratory, and urogenital organs. There are, however, few reports of enteric localization of *E. cuniculi* (10, 11).

Spores of *E. cuniculi* can survive in macrophages and spread throughout the host, either human or animal, where they may cause various lesions affecting the nervous system, respiratory system, digestive tract, liver, peritoneum, lung, bladder, and kidney (8, 12-14). Infectious spores are excreted in the urine, feces, or respiratory secretions of the infected host (14). Because *E. cuniculi* has a low host specificity and its spores are resistant to adverse environmental conditions, man can easily get infected with this microsporidian (15, 16). Most microsporidian infections are thought to result from fecal-oral transmission of spores (17). Although, waterborne transmission of microsporidiosis may pose the greatest threat, nonaquatic transmission is also of public health concern. Spores have been identified on fruits, sprouts, and green-leafy vegetables. Such contamination maybe as a result of microsporidial contamination of agricultural irrigation waters, or due to sewage-sludge end products used as fertilizers (18-20). Infection with microsporidia through inhalation of aerosolized spores is possible (3). Additionally, transplacental transmission has been demonstrated in *E. cuniculi* (21). Human-to-human transmission is also possible via transplantation of solid organs from an infected donor (22, 23).

Complications due to *E. cuniculi* infection have been reported in immunocompromised patients, but reports on *E. cuniculi* in immunocompetent humans are lacking, most probably because it is not linked to any clinical manifestations in healthy population (24).

In Egypt, a recent study was done to evaluate the prevalence of *E. cuniculi* infection in seven different animal hosts (cattle, buffaloes, sheep, goat, rabbit, dog, and rat) in different provinces. The results of serological examination confirmed the occurrence of antibodies against *E. cuniculi* in 38.9 % of the examined animals. The highest positivity was observed in goats followed by buffaloes, rabbits, dogs, rats, and cattle, while the least was recorded in sheep. This indicates the wide distribution of *E. cuniculi* in Egypt and confirms that these animals may be reservoirs of the spores and thus potential sources of infection for human and animals (25). However, to the best of our knowledge, the epidemiological situation and public health importance of *E. cuniculi* infection in Egypt is unknown.

The present work was carried out with the aim of studying the prevalence of *E. cuniculi* infection in non-HIV immunocompromised patients and immunocompetent individuals in Egypt. It tested as well the influence of some factors on the risk of infection.

Materials and Methods

Sample collection

Blood and urine samples were collected in the period from November 2012 to March 2014 from 88 persons. Forty-four subjects were non-HIV immunocompromised patients receiving chemotherapy for treatment of malignancy (group I). They were chosen from among patients attending the Clinical Oncology Department at the main Alexandria University Hospital, El-Shatby Paediatric Alexandria University Hospital, and Fever Hospital (Alexandria, Egypt). In addition, 44 subjects were included in the immunocompetent control group (group II). Informed consents from all subjects preceded sample collection. A full

history sheet was fulfilled for each subject including; age, sex, diagnosis, disease stage, treatments received as well as contact with animals. Complete blood count with differential leukocyte count was considered as a laboratory indicator of the immune status and was performed for all subjects.

Spores of *E. cuniculi*

Spores of *E. cuniculi* (rabbit isolate) kindly supplied by Prof. Dr. Peter Deplazes (Institute of Parasitology, University of Zurich, Switzerland) were used as an antigen in serological tests.

Reference sera

A negative reference serum was kindly supplied by Ms. Lisa Bowers (Division of Microbiology, Tulane National Primate Research Center, Covington, LA, USA). A positive reference serum was obtained by experimental infection of rabbits with *E. cuniculi* spores. Sera were collected after 3 weeks post infection (PI), stored at -20 °C, and used as the positive control.

Serological examination

Indirect fluorescent antibody test (IFAT): In this technique, unlabeled antibodies (in human serum) were added to *E. cuniculi* spores. In positive samples the antibodies combined with specific antigen, then the antigen-antibody complex was labeled with fluorescein-conjugated anti-immunoglobulin antibody, the resulting triple complex was then being detected by fluorescence microscopy (26, 27).

Blood samples were collected and centrifuged at 2000 rpm for 5 min and then serum was kept at -20 °C until used for serological examination. Sera were examined for antibodies of *E. cuniculi* using IFAT (26, 27). Briefly, slides were washed with absolute alcohol and then kept until air-dried. Using black nail varnish, eight small circles were made on each slide; each circle represents a well. Ten microliters of spore suspension (containing about 80,000 spores of *E. cuniculi*) were added to each well on each slide. Slides were incubated at 37 °C for 30 min. Ten microliters of methanol were added to each well for antigen fixation, and then slides were kept for another 10 min at 37 °C. Slides were then rinsed with distilled water and kept until air-dried. Serum was diluted 1:40 in PBS, and 20 µl of diluted serum were added to each well and then slides were incubated at 37 °C for 30 min. Slides were washed three times in PBS and then in distilled water, and the slides were kept until air dried. Ten microliters of diluted Fluorescein isothiocyanate (FITC) conjugated antibody (Sigma, St. Louis, MO, USA) were added to each well. Slides were then incubated at 37 °C for 30 min. Slides were again rinsed three times in PBS and distilled water, then kept until air-dried. Ten microliters of glycerin/PBS (1:9) were added to each well and kept until air-dried. Slides were covered with a cover slip and examined at ×40 with an Olympus immunofluorescent microscope. Positive and negative control sera were used as controls on each slide.

Based on previous studies, the IFAT was found to have a high reliability for diagnosis of *E. cuniculi* to the species level (25, 28-32). Thus it was used as the gold standard technique in some previous studies (25, 28), and in the current study.

Urine examination

Urine processing and preservation: Urine samples were collected in properly labeled screw capped plastic containers and taken directly to the laboratory of Medical Parasitology Department, Faculty of Medicine, Alexandria University, Egypt. Each sample was centrifuged at 15,000 rpm for 10 min and then supernatants were decanted (33). Each sample was divided into two portions. One part was freshly frozen at -20°C for DNA extraction and PCR. The rest of the sample was smeared for subsequent microscopic examination.

Because of the reported intermitted and scanty shedding of spores in urine of animals after experimental infections (34), none of the two

techniques used for examination of urine was considered as the gold standard for diagnosis.

Microscopic detection of *E. cuniculi* spores in urine: Smeared samples were left to dry then fixed with methanol and stained with Weber's green modified trichrome stain (Weber's green MTS). Stained smears were examined under the ordinary light microscope with oil immersion lens (35).

Molecular detection of *E. cuniculi* DNA in urine (PCR): DNA was extracted from urine samples according to the method described by Ghatak and colleagues in 2013 (36). The PCR was performed using *E. cuniculi*-specific primers that amplify a 549-bp fragment of the small subunit ribosomal RNA (ssrRNA) gene. The forward (ECUNF: 5'-ATGAGAAGTGA-TGTGTGTGCG-3') and reverse primers (ECUNR: 5'-TGCCATGCACTCACAGGCATC-3') were used (37). The PCR was performed using the following conditions: after initial denaturation of the DNA at 95 °C for 3 min,35 cycles were run 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 1 min with a 10-min 72 °C extension after the 35 cycles. Agarose gel electrophoresis was performed on PCR products, and the resulting bands were visualized using ethidium bromide and UV light. One positive and another negative control reaction without template DNA were also performed. The size of the PCR products was calculated using a 3000-bp ladder.

Statistical analysis

Data were fed to the computer and analyzed using IBM SPSS software package version 20.0. Comparison between different groups regarding categorical variables was tested using Chi-square test. When more than 20% of the cells had expected count less than 5, correction for chi-square was conducted using Fisher's exact test. For normally distributed data, comparison between two independent populations was done using independent t-test. Agreement of the different predictive with the outcome was used and was expressed in sensitivity, specificity, positive predictive value, negative predictive value and accuracy. Significance of the obtained results was judged at the 5% level (38, 39).

Results

Seroprevalence of E. cuniculi in the studied groups

The results of serological examination (IFAT) confirmed the presence of antibodies against *E. cuniculi* in 39 out of 88 (44.3%) human subjects. The seroprevalence of *E. cuniculi* was significantly higher in group I (77.3%) compared with group II (11.4%) (Table 1).

Nearly half the number of the infected subjects, whether immunocompromised or immunocompetent, was above 50-year-old (Table 2). Comparing the seroprevalence by gender did not show any statistical significance in the studied subjects (Table 2). Difference as regards history of contact with animals was shown to be statistically significant only in immunocompetent individuals (Table 2).

Table 1: Diagnosis of *E. cuniculi* infection in the studied groups as detected by IFAT, Weber's green MTS smears and PCR

		Group I (n = 44)	Group II (n = 44)	*p*
Positive cases	IFAT	34 (77.3%)	5 (11.4%)	<0.001*
	Weber's green MTS	30 (68.2%)	2 (4.5%)	<0.001*
	PCR	0 (0.0%)	0 (0.0%)	-

Group I: Immunocompromised patients/Group II: Immunocompetent individuals/*: Student t-test statistically significant

Table 2: Relation between infection and demographic data in the studied groups

	Group I			Group II		
	Total (n = 44)	Positive (n = 34)	χ^2 (^{FE}p)	Total (n = 44)	Positive (n = 5)	χ^2 (^{FE}p)
Age						
≤50	21	16 (76.2%)	0.027	37	3 (8.1%)	2.447
>50	23	18 (78.3%)	(1.000)	7	2 (28.6%)	(0.173)
Sex						
Male	17	12 (70.6%)	0.705	26	4 (15.4%)	1.202
Female	27	22 (81.5%)	(0.473)	18	1 (5.6%)	(0.634)
Contact with animals						
No	18	14 (77.8%)	0.004	31	1 (7.7%)	6.899
Yes	26	20 (76.9%)	(1.000)	13	4 (30.8%)	(0.022)*

Group I: Immunocompromised patients/Group II: Immunocompetent individuals/χ^2: Chi square test/FE: Fisher exact test/*: Statistically significant

Microscopic examination of Weber's green MTS smears

Spores of *E. cuniculi* are colorless. Microscopic examination of stained smears showed spores of *E. cuniculi* as oval bodies with pink outline (about 1.5 µm wide and 2.5 µm long). Thirty cases in group I (68.2%) were positive for spores and this was significantly higher than in group II where two cases (4.5%) showed spores in their urine smears (Table 1). Compared with IFAT (the gold standard), the sensitivity and specificity of Weber's green MTS smears were 69.23% and 89.80%, respectively (Table 3).

Table 3: Agreement (sensitivity, specificity and accuracy) between IFAT, Weber's green MTS smears and PCR results

	IFAT		*p*	Sensitivity	Specificity	PPV€	NPV¥	Accuracy
	-ve	+ve						
Weber's green MTS smears								
-ve	44	12	<0.001*	69.23	89.80	84.38	78.57	80.68
+ve	5	27						
PCR								
-ve	49	39	-	0.0	100.0	-	55.68	55.68
+ve	0	0						

€: Positive predicted value/¥: Negative predicted value/*: Statistically significant

Molecular assays (PCR)

No positive cases were detected among human subjects in groups I and II (n = 88) (Tables 1 & 3).

Discussion

The present study applied different techniques to investigate the prevalence of *E. cuniculi* infection in immunocompromised patients and compared them with immunocompetent individuals. The results of the IFAT examination during the present study confirmed the presence of antibodies against *E. cuniculi* in

44.3% of the examined human subjects. Such high prevalence could be attributed to the enzooticity of *E. cuniculi* in domestic animals and the possible zoonotic transmission to man, a suspicion that was previously raised by Abu-Akkada and coworkers in 2015, when they reported a prevalence about 39% in animals in Alexandria and El Behira governorates (25). In addition, the present results revealed that individuals with history of contact with animals constituted 76.9% and 30.8% of the seropositive immunocompromised and immunocompetent subjects, respectively (Table 2).

The present results showed that the seroprevalence of *E. cuniculi* antibodies was significantly higher in the immunocompromised patients compared with immunocompetent controls (Table 1). Microscopic examination of Weber's green MTS urine smears prepared from immunocompromised patients showed significantly higher number of positive subjects shedding spore compared with the immunocompetent controls (Table 1). This goes with the fact that microsporidiosis is an opportunistic human infection. Most microsporidial infections caused by *E. cuniculi* were recorded in immunocompromised patients like those infected with HIV, patients undergoing organ transplantation, or patients with idiopathic $CD4^+$ T lymphocytopenia (40). The role played by the innate immunity in suppression of the microsporidian spread was proved, and the dissemination of infection in immunocompromised patients is well documented (22, 41, 42). Chronic infections caused by *E. cuniculi* in immunocompetent individuals are generally asymptomatic, probably reflecting a balanced parasite-host relationship. *E. cuniculi* represents the vast majority of the microsporidial species found in the healthy population in the Czech Republic (43). A competent immune response is unable to eliminate fully the infection even if there are no clinical signs and the carrier can be a source of infection. Unfortunately, such latent infections may be reactivated during immunosuppression (44).

The results of the present study showed that about half the number of the infected subjects, whether immunocompromised or immunocompetent, was above 50-year-old (Table 2). This could be explained by the age-related reduction in the immunity. Likewise, a recent parasitological evaluation of stool samples from Czech Republic citizens (immunocompetent individuals and foreign students of varying age groups) had identified 34% to 56% prevalence rates of shedding *E. bieneusi* and *Encephalitozoon* species with the highest prevalence in the group of 50 years and above (43). Similar results were recorded in Malaysia (45). On the contrary, in Japan the seropositivity for microsporidiosis was highest among people aged 19 years or younger and the seropositivity rates decreased among the older subjects (32).

The results of the present study revealed that gender had no significant influence on infection in infected subjects (Table 2). This finding also agrees with several previous studies (24, 32, 45-49).

According to the present results, the effect of contact with animals on infection was statistically significant only in the immunocompetent subjects (Table 2). This suggests that other sources of infection, such as contaminated water and food, may have a role in transmission of the pathogen. Such suggestion is supported by the fact that microsporidian spores can survive for several months in the environment and are resistant to moisture and desiccation (40, 50).

During the present study, urine samples were examined for detection of *E. cuniculi* spores by Weber's green MTS smears. This technique is a well-established method for diagnosis of microsporidian spores in body fluids (51). The results of the present study revealed that by comparing this technique with IFAT a sensitivity of 69.23% and a specificity of 89.80% were obtained. However, the stained urine smears could help to identify microsporidians to the species level. In addition, microscopic detection of spores in urine

requires well-trained personnel; as the spores could be readily mistaken with different precipitates in the sample.

In addition, during the present study urine samples were examined for *E. cuniculi* DNA by using the PCR. The technique failed to detect any infection in the 34 seropositive cases. Our present results are in agreement with previous results on infected animals in Alexandria and El Behira governorates, which revealed very low sensitivity of the PCR technique (25). Furthermore, our finding agreed with those of Jeklova and colleagues who found that experimental oral inoculation of *E. cuniculi* (10^3 spores) in rabbits, which is most likely to represent a natural infection, no DNA was detected (34). False negative PCR results are not attributed to intermittent shedding of spores but due to the low number of spores being shed that are below the detection threshold of the PCR (52).

Another explanation for the PCR failure is the presence of possible inhibitors (53). For example, urea concentrations above 50 mM are inhibitory for PCR (54). Interestingly, molecular methods for detection of other species of microsporidia in stools were reported to have some limitations (55). Mainly DNA extraction from spores has complexities. Such spores have very small size, rigid double layer wall and have low intensity in stool samples. Thus, DNA extraction methods strongly influence the PCR results (56-58).

Conclusion

The seropositivity recorded in our study (77.3% in immunocompromised and 11.4% in immunocompetent individuals) indicated an unexpected wide distribution of *E. cuniculi* infection. The study of risk factors showed that the immune status of the host remains the corner stone for occurrence of the infection. Therefore, we highlight the need to carry out further epidemiological and experimental work. More data about the magnitude of the problem and biology of the pathogen will help in developing preventive strategies for its eradication.

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