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# Symptomatic respiratory *Encephalitozoon cuniculi* infection in renal transplant recipients

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ABSTRACT

*Objectives:* *Encephalitozoon* spp. and *Enterocytozoon bieneusi* are intracellular parasitic fungi from the phylum Microsporidia, which initially localize to the intestine. As opportunistic pathogens, *Encephalitozoon* spp. in particular can disseminate to the respiratory tract, among other locations. Patients on life-long immunosuppression are at higher risk of such infections, mostly symptomatic.
*Methods:* Sputum samples and bronchial washings from 72 renal transplant recipients and 105 patients with various respiratory diseases were screened for *Encephalitozoon* spp. and *E. bieneusi* by microscopic examination and genus-specific nested PCR followed by genotyping.
*Results:* A total of 8.3% (6/72) of immunosuppressed renal transplant recipients and 1.9% (2/105) of patients with various respiratory diseases, both immunocompetent and immunosuppressed, were positive for respiratory microsporidial infection. All six transplant recipients were *Encephalitozoon cuniculi*-positive by PCR/sequencing and five of them suffered from respiratory symptoms. The presence of microsporidial spores was also confirmed microscopically in three of the transplant recipients. Of the two immunocompetent patients with various respiratory diseases, one had an *E. cuniculi* infection, while the second had an *E. bieneusi* infection.
*Conclusions:* Life-long immunosuppression in renal transplant recipients increases the risk of respiratory infection by *E. cuniculi*. Microsporidia should be screened in respiratory samples of these patients, particularly when they have respiratory symptoms.
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## Introduction

Intracellular fungi of the genus *Encephalitozoon* (*E. cuniculi*, *E. intestinalis*, and *E. hellem*) and *Enterocytozoon bieneusi* are the most common microsporidial species infecting humans. Since they are able to infect a wide range of animal species, microsporidial spores are widely distributed in the environment. Spores are excreted in the faeces, urine, or sputum; therefore people become infected by ingestion of food or water contaminated with spores or, less frequently, by inhalation of spores.

After ingestion, microsporidial spores initially develop in the epithelium of the small intestine. *E. bieneusi* and *E. intestinalis* infections are mainly confined to the intestine, resulting in diarrhoea, and rarely disseminate, while infection with *E. cuniculi* and *E. hellem* often also involves the kidneys (Wasson and Peper, 2000; Matos et al., 2012). When inhaled with air and dust, spores of microsporidia may invade the sinus or the lung epithelium, leading to respiratory infection, as has been observed particularly for *E. cuniculi*, *E. hellem*, and *E. bieneusi* (Wasson and Peper, 2000).

As opportunistic pathogens, microsporidia cause symptomatic infections mainly in patients with impaired immune function. Moreover, microsporidial dissemination to the central nervous system, eyes, and respiratory and urinary tracts has been documented in these patients, especially in the case of *E. cuniculi* and *E. hellem*, causing fever, pneumonia, hepatitis, cholangitis,

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peritonitis, or nephritis (Mertens et al., 1997; Wasson and Peper, 2000; Didier and Weiss, 2006; Nagpal et al., 2013). Iatrogenically immunosuppressed patients, especially transplant recipients, are the group at highest risk of such opportunistic infections, and their dissemination is due to life-long immunosuppressive treatment. Nevertheless, routine differential diagnosis of these pathogens is not performed and little is known about the prevalence of microsporidia in this group of patients.

The aim of this prevalence study was to determine whether renal transplant recipients are at risk of respiratory microsporidial infection and to compare this group with a group of patients with various respiratory diseases.

## Materials and methods

### *Patients*

Clinical specimens were obtained from 72 HIV-negative renal transplant recipients (RTRs) who had been under the care of the Department of Nephrology and Transplantation Medicine of Wroclaw Medical University (Wroclaw, Poland), and from 105 patients with various respiratory diseases (VRD) under the care of the Department of Pulmonology and Lung Cancer of Wroclaw Medical University from 2015 to 2018. Bronchial washings (BW) were collected from all VRD patients during routine clinical bronchoscopy examinations, while sputum samples were obtained from the RTR patients. Additionally, BW samples were collected from two RTR patients and a pleural fluid (PF) sample from one RTR patient.

PCR-based tests for *Pneumocystis jirovecii* (Maitte et al., 2013) and standard laboratory tests for *Mycobacterium tuberculosis* were performed for all patients with respiratory symptoms. Clinical and demographic data, including age, sex, fever, respiratory symptoms, type of immunosuppressive drugs, and, in the case of RTRs, time after kidney transplantation (KTx), were taken into account. Fever was defined as a body temperature above 38 °C at the time of examination. Among respiratory symptoms, dyspnoea, progressive shortness of breath, exudation of fluid into the pleural cavity, and persistent cough were investigated, and radiological findings in the lungs (bilateral peripheral interstitial infiltrates and/or ground glass opacities, inflammatory changes) and respiratory failure were also considered.

### *Sample collection*

During the collection of BW, the bronchoscope was introduced into the main bronchi and 30–60 ml of warmed saline (room temperature) was instilled through the working channel into the airways. The BW was recovered via a suction channel into a suitable receptacle. Fresh BW samples were centrifuged at 2236 *g* for 20 min, and the sediment was resuspended in the remnant supernatant. Fresh sputa were treated with 1 M dithiothreitol as a mucolytic agent, incubated for 10 min at 37 °C, and centrifuged with the same parameters. The sediment was resuspended in the remnant supernatant. All samples were fixed immediately for microscopy or stored, without preservative, at −20 °C for 1–2 weeks before molecular analyses.

### *Microscopic examination*

The standard Calcofluor M2R staining method was used to detect microsporidial spores (Didier et al., 1995).

### *DNA isolation*

Sputum and BW samples were homogenized by bead disruption with a Precellys24 Instrument (Bertin Technologies, France), followed by digestion with proteinase K at 56 °C for 1 h. Total DNA was extracted using the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) following the manufacturer's instruction. Extracted DNA was stored at −20 °C until PCR amplification.

### *Molecular examination*

A genus-specific nested PCR protocol was used to amplify the partial sequence of the 16S rRNA gene, the entire ITS (internal transcribed spacer) region, and a partial sequence of the 5.8S rRNA gene of *E. bieneusi* and *Encephalitozoon* spp., with primer pairs

**Table 1**
Comparison of the basic characteristics of *Encephalitozoon cuniculi*-positive and negative renal transplant recipients.

| Characteristics | *Encephalitozoon cuniculi* | | *p*-Value |
|---|---|---|---|
| | Positive ($n=6$) | Negative ($n=66$) | |
| Average age in years (range) | 59 (45–68) | 52 (21–76) | 0.112 |
| Sex, number of patients (%) | | | |
| Male | 3 (50) | 33 (50) | 1.000 |
| Female | 3 (50) | 33 (50) | 1.000 |
| Average time after kidney transplantation in months (range) | 30.8 (2.4–90.5) | 83.2 (0.16–225.6) | 0.175 |
| Symptoms, number of patients (%) | | | |
| Respiratory symptoms[a] | 4 (66.6)[b] | 16 (24.3) | 0.046 |
| Fever | 2 (33.3) | 11 (16.7) | 0.295 |
| Immunosuppressive regimen, number of patients (%) | | | |
| CI, PDN, MMF | 3 (50) | 36 (54.5) | 1.000 |
| CI, PDN, AZA | 0 | 4 (6.1) | 1.000 |
| PSI, PDN, MMF | 0 | 1 (1.5) | 1.000 |
| CI, PDN | 3 (50) | 18 (27.4) | 1.000 |
| CI, MMF | 0 | 3 (4.5) | 1.000 |
| PSI, PDN | 0 | 1 (1.5) | 1.000 |
| PDN | 0 | 3 (4.5) | 1.000 |

CI, calcineurin inhibitors; PDN, prednisone; MMF, mycophenolate mofetil; AZA, azathioprine; PSI, proliferation signal inhibitors.

[a] Presence of dyspnoea, progressive shortness of breath, exudation of fluid into the pleural cavity, persistent cough, inflammatory changes in the lungs, radiological findings (presence of bilateral peripheral interstitial infiltrates and/or ground glass opacities), and respiratory failure.

[b] The patient with respiratory symptoms and *Pneumocystis jirovecii* co-infection has not been considered.

EBITS3/EBITS4 or ITS580F/ITS580R in primary reactions and EBITS1/EBITS2.4 or MSP3/MSP4A in secondary reactions (Katzwinkel-Wladarsch et al., 1996; Buckholt et al., 2002). PCR conditions were as described previously (Sak et al., 2011). A negative control (molecular grade water) and positive controls (DNA from *E. cuniculi* genotype III and *E. bieneusi* genotype CZ3) were included in each PCR amplification.

*Sequencing and phylogenetic analyses*

PCR products were sequenced in both directions using the Sanger sequencing method. Amplification and sequencing of each sample was repeated two times. Nucleotide sequences were edited using the program ChromasPro 2.1.5 and aligned with each other and with reference sequences from GenBank (http://www.ncbi.nlm.nih.gov/blast) using MAFFT version 7 (http://mafft.cbrc.jp/alignment/software/). Phylogenetic analyses were performed using MEGA6 software and trees were inferred by maximum likelihood method. Bootstrap support for branching was based on 1000 pseudo-replicates.

*Statistical analysis*

The Chi-square test or Fisher's exact test was used to compare categorical variables (sex, fever, respiratory symptoms, immunosuppressive regimen) between microsporidia-positive and negative patients, while continuous variables (age, time after KTx) were compared using the Student *t*-test. A *p*-value of $<0.05$ was considered significant.

## Results

The mean age of the RTRs ($n = 72$) was $52.5 \pm 13.9$ years (range 21–76 years), while that of the VRD patients ($n = 105$) was $62.0 \pm 12.7$ years (range 27–87 years). Overall, the male-to-female ratio was 1:1 (36 (50%) male, 36 (50%) female) in the RTR group and 2:1 (70 (66.7%) male, 35 (33.3%) female) in the VRD group. The mean time after KTx among renal transplant patients was 78.7 months, ranging from 5 days to 19 years. The immunosuppressive regimen in all RTRs included prednisone, calcineurin inhibitors (tacrolimus or cyclosporine), proliferation signal inhibitors (sirolimus), mycophenolate mofetil, or azathioprine (Table 1). Among the final respiratory diagnoses of the VRD patients, lung cancer (43/105, 41%), chronic obstructive pulmonary disease (12/105, 11.4%), and interstitial lung disease (11/105, 10.5%) were the most common. Only 11 of all VRD patients received immunosuppressive treatment (prednisone or methotrexate) and this was for cancers, chronic obstructive pulmonary disease, or autoimmune disorders.

Out of 72 RTRs and 105 VRD patients, 8.3% (6/72) and 1.9% (2/105), respectively, were PCR-positive for microsporidia. Additionally, the presence of microsporidial spores (two to three spores per slide) was confirmed in three RTRs by microscopy. Phylogenetic analysis revealed the presence of *E. cuniculi* genotype II in all RTRs and one VRD patient. One VRD patient had an *E. bieneusi* genotype D infection. Among RTRs from whom additional samples were examined, one was *E. cuniculi*-positive in BW and one in PF.

The results obtained indicated a significant association between *E. cuniculi* infection and respiratory symptoms in RTR patients ($p = 0.046$). Since a concomitant *P. jirovecii* infection was confirmed in the BW of one patient infected with *E. cuniculi* and Pneumocystis pneumonia (PcP) was diagnosed (Table 2), the respiratory symptoms of this patient were not considered in the statistical analysis as being associated with microsporidia. No *M. tuberculosis* infection was confirmed in any of the patients. Although the time after KTx in positive patients was more than 2.5 times shorter than in negative patients, no significant association was found for this parameter or for the other variables tested ($p > 0.05$; Table 1).

## Discussion

Documented microsporidial infections with respiratory involvement are rare; to date, these have only been shown for immunosuppressed patients. Among transplant recipients, seven such cases have been documented (Kelkar et al., 1997; Mohindra et al., 2002; Teachey et al., 2004; Orenstein et al., 2005; George et al., 2012; Nagpal et al., 2013; Kicia et al., 2018). Four of these patients died as a result of cardiorespiratory failure, which suggests that microsporidia infection in the respiratory tract might be life-threatening. Moreover, the only data concerning the prevalence of microsporidia in respiratory samples showed 14.2% of iatrogenically immunosuppressed patients to be positive for

**Table 2**
Characteristics of microsporidia-positive patients.

| No. | Age, years | Sex | Time after KTx (months) | Immunosuppressive treatment | Pulmonary symptoms/radiological findings | Pulmonary co-infections | Sample | Microsporidia species/genotype |
|---|---|---|---|---|---|---|---|---|
| Renal transplant recipients (RTRs) | | | | | | | | |
| 1 | 63 | F | 90.5 | PDN, MMF, CI | Cough, yellow fluid in the pleural cavity | None | Sputum | *E. cuniculi* II |
| 2[a] | 60 | M | 4.8 | PDN, CI | Increased dyspnoea, Pneumocystis pneumonia/ground glass opacities | *P. jirovecii* | Sputum<br>BW[b] | –<br>*E. cuniculi* II |
| 3[c] | 58 | M | 7.0 | PDN, CI | Gradual deterioration of effort dyspnoea, cough with brown secretion, pneumonia/atelectatic-inflammatory densities, extensive inflammatory changes, congestive inflammatory changes, fluid in the pleura | None | Sputum<br>PF | –<br>*E. cuniculi* II |
| 4 | 45 | M | 28.2 | PDN, MMF, CI | Fever, cough/reticular shading in the right lung, interstitial infiltrates | None | Sputum[b] | *E. cuniculi* II |
| 5 | 62 | F | 2.4 | PDN, MMF, CI | Persistent fever, effort dyspnoea, symptoms of upper respiratory tract infection | None | Sputum[b] | *E. cuniculi* II |
| 6 | 68 | F | 51.7 | PDN, CI | None | None | Sputum | *E. cuniculi* II |
| Patients with various respiratory diseases (VRD patients) | | | | | | | | |
| 7 | 51 | M | – | None | N/A | None | BW | *E. cuniculi* II |
| 8 | 66 | M | – | None | Bronchiectasis, chronic bronchitis/post-inflammatory changes | None | BW | *E. bieneusi* D |

F, female; M, male; KTx, kidney transplantation; CI, calcineurin inhibitors; PDN, prednisone; MMF, mycophenolate mofetil; BW, bronchial washings, PF, pleural fluid; N/A, data not available at the time of examination; *E. cuniculi*, *Encephalitozoon cuniculi*; *E. bieneusi*, *Enterocytozoon bieneusi*.

[a] Sputum sample and bronchial washings tested.

[b] Microscopy positive.

[c] Sputum specimen and pleural fluid tested.

microsporidia in bronchoalveolar lavage, including two out of the six (33.3%) RTRs tested (Özkoç et al., 2016).

In the present study, 8.3% of the RTRs were found to be infected with microsporidia in respiratory samples. In contrast, no immunosuppressed VRD patients were infected, suggesting that the type and/or duration of immunosuppressive treatment may have an influence on the dissemination of infection or on higher susceptibility to localized respiratory infection. Indeed, all of the immunosuppressed VRD patients were treated with only one immunosuppressant, while more than 95% of all RTRs were treated with combinations of two or more immunosuppressive drugs (Tables 1 and 2). However, taking into account the high diversity of the VRD group in this study and the fact that only 11 patients from this group were receiving immunosuppressants, a more detailed investigation with a larger study group is needed to explain this phenomenon.

Respiratory microsporidial infection might ensue directly after the inhalation of spores or might develop as a result of dissemination after spore ingestion (Wasson and Peper, 2000). Since only respiratory specimens were tested in the study patients, it remains unclear whether the pathogens were acquired by inhalation or were haematogenously disseminated from another site of infection, and both transmission routes are probable. Considering that exposure to *Encephalitozoon* spp. is common among immunocompetent people (Sak et al., 2011) and that the lungs (besides the kidneys and cecum) are the place where *E. cuniculi* persist in chronic infections after dissemination from the intestine (Botterel et al., 2002; Wagnerová et al., 2013), reactivation of microsporidial infection in immunosuppressed RTRs in this study is possible, especially as the prevalence of urinary and intestinal microsporidial infections in RTRs is high (Kicia et al., 2016). Moreover, the donor–recipient transmission route with a graft cannot be excluded as well (Hocevar et al., 2014).

The molecular investigation revealed that all of the positive RTRs and one of the VRD patients in this study were infected with *E. cuniculi*. It is noteworthy that in the majority of cases of known respiratory microsporidial infections in transplant recipients, Encephalitozoon was confirmed as a causative agent, while *E. bieneusi* respiratory tract infections have been shown mainly for HIV-infected patients (Sodqi et al., 2004). To date, the only case of *E. bieneusi* infection with respiratory involvement in an HIV-negative transplant recipient was observed in a hematopoietic stem cell transplant recipient (Kicia et al., 2018). The reason for the observed differences remains unknown and requires detailed investigation.

The clinical diagnosis of respiratory microsporidiosis is challenging. Even though in this study respiratory symptoms were more often observed in microsporidia-positive transplant recipients (the most commonly observed were persistent cough, dyspnoea, progressive shortness of breath, interstitial pneumonitis, acute respiratory distress syndrome, and interstitial opacities, as well as infiltrates on chest radiographs), they are non-specific and no consensus facilitating the recognition of microsporidial infection has been proposed. Moreover, respiratory localized microsporidia might coexist with other opportunistic pulmonary pathogens, such as *P. jirovecii*, as was seen in one of the study patients, making it more difficult to diagnose.

However, the importance of proper diagnosis is highlighted by the fact that the treatment of microsporidia is limited and genus-specific. In the treatment of human microsporidiosis, albendazole and fumagillin are known to have the highest clinical efficacy (Costa and Weiss, 2000; Champion et al., 2010). Albendazole is the drug of choice for the treatment of intestinal, respiratory, and disseminated microsporidiosis caused by *Encephalitozoon* spp., but has limited efficacy against *E. bieneusi* (Costa and Weiss, 2000). Fumagillin is highly effective against *E. bieneusi*, but shows toxicity and might cause thrombocytopenia, neutropenia, and hyperlipidemia when administered systemically in humans (Molina et al., 2002). Also aseptic meningoencephalitis has been reported as a result of fumagillin treatment in an RTR infected with *E. bieneusi*, in whom albendazole treatment failed (Audemard et al., 2012). Moreover, both albendazole and fumagillin may not be fully effective and do not eradicate pathogens, especially in severely immunocompromised patients, which results in pathogen persistence and may lead to relapse of symptoms after the completion of treatment (Molina et al., 1998; Costa and Weiss, 2000; Kotkova et al., 2013).

Since symptomatic microsporidial infection is related to the immune status of the host, restoration of the immune system may result in the resolution of microsporidiosis symptoms and elimination of the pathogen without the need for specific treatment. Such immune renewal might be achieved as a result of using highly active antiretroviral therapy (HAART) in AIDS patients (van Hal et al., 2007) or dose reduction or temporary withdrawal of the immunosuppressant in the case of pharmacologically immunosuppressed patients (Galván et al., 2011; Kicia et al., 2014).

The biological material in which microsporidia have been identified so far are nasal secretions, sputum, tracheobronchial aspirate, bronchoalveolar lavage, and lung biopsy (Teachey et al., 2004; Orenstein et al., 2005; Nagpal et al., 2013; Özkoç et al., 2016). The results of the present study indicate that sputum samples might be used in the diagnosis of microsporidial infection in transplant recipients suffering from respiratory symptoms and/or having radiological changes on chest radiographs, when bronchoscopy is unavailable. On the other hand, the finding of two patients infected in BW and PF while sputum remained negative suggests that the examination of sputum samples might result in misdiagnosis, and the prevalence of infection among the RTRs in this study might be underestimated.

In conclusion, the higher prevalence of *E. cuniculi* observed among patients after kidney transplantation testifies to the fact that life-long immunosuppression increases the risk of respiratory microsporidial infection in these patients. It is necessary to constantly monitor them, since symptoms of respiratory microsporidiosis are generally undistinguishable from those caused by more common infective agents. Moreover, when infection is untreated, it may lead to acute respiratory failure, which is life-threatening for the patient.

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## Conflict of interest

None.

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