ISHAM INTERNATIONAL SOCIETY FOR HUMAN AND ANIMAL MYCOLOGY

Medical Mycology, 2020, 58, 1191–1194
doi:10.1093/mmy/myaa048
Advance Access Publication Date: 4 June 2020
Brief Report

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Pneumocystis jirovecii and microsporidia: An unusual coinfection in HIV patients?

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Received 20 December 2019; Revised 3 April 2020; Accepted 19 May 2020; Editorial Decision 13 May 2020

Abstract

Pneumocystis jirovecii and microsporidia species are recognized as opportunistic infectious pathogens in AIDS patients. Coinfection of both in one patient has been rarely reported. The aim of the present study was to investigate the coinfection of *P. jirovecii* and microsporidia in different tissues from AIDS deceased patients. Post mortem histological finding of *P. jirovecii* and microsporidia was demonstrated by means of the Grocott's methenamine silver and Brown Brenn staining, respectively. Molecular technique was used for identification and characterization of both fungi. Out of the 514 autopsied cases *P. jirovecii* and microsporidia species were identified in 53 (10.3%) and 62 (12.1%) cases respectively. A total of five cases (0.97%) coinfected with *Pneumocystis* and microsporidia were recovered from all analyzed autopsies. Coinfection of *Pneumocystis* and microsporidia is very challenging and raises interesting issues about host-parasite relationship. The early diagnosis of both pathogens must be crucial to establish correct and early treatments, improve the patient's evolution, reducing the risk of death.

Key words: *Pneumocystis*, microsporidia, coinfection, AIDS, autopsy.

Introduction

Pneumocystis jirovecii and microsporidia species are recognized as opportunistic infectious pathogens in AIDS patients.[1,2] However, coinfection of both in one patient has rarely been reported. In Medline searches only one case report has been described.[3] Here we present the epidemiological data of five additional cases of these concomitant infections in different tissues, as well as the identification of microsporidia species and genetic characterization of *P. jirovecii* in 14 years of the Cuban AIDS epidemic.

Methods

Between January 1995 and May 2008, complete autopsies were performed on 514 AIDS deceased patients. In each case, a sample of approximately 1.0 g was taken from each tissue using sterile equipment, placed in sterile receptacles, and fixed in buffered formalin 10% for 24 hours. Only one sample was processed on a single day. Post mortem histological finding of *P. jirovecii* in lungs and microsporidia disseminated to lungs, kidneys, and ovaries was demonstrated by means of the Grocott's methenamine silver and Brown Brenn staining, respectively. Five cases of the

Table 1. Patients' characteristics, identification of microsporidia species and *Pneumocystis* genotype of five AIDS deceased patients with concomitant infection, IPK, Cuba 1995–2008.

Autopsy year/no.	CD4 cell count (cells/μl)	Year HIV diagnosis	Paraffin tissues for diagnosis micros/PcP	Anti-retroviral treatment (ART)	Microsporidia species PCR	*Pneumocystis* genotype mt LSU rRNA
97/18	31	1988	Kidney/lung	Yes	*E. cuniculi*	3 (85T/248C)
97/36	135	1989	Kidney/lung	Yes	*E. cuniculi*	3 (85T/248C)
99/02	39	1997	Kidney/lung	No	*E. cuniculi*	3 (85T/248C)
99/14	72	1987	Ovary/lung	No	*E. cuniculi*	3 (85T/248C)
05/25	83	2005	Lung/lung	No	*E. bieneusi*	3 (85T/248C)

deceased patients showed *P. jirovecii* pneumonia (PcP) and microsporidia infection simultaneously.

DNA extraction was performed using three 10-μm sections taken from each paraffin block of infected tissues. The pooled sections from each block were individually deparaffinized with xylene, then washed with ethanol, and digested with proteinase K (Roche, Basel, Switzerland).[4] For microsporidia cases, sections were treated with 0.4 U chitinase (Sigma-Aldrich, St. Louis, MO, USA) at 55°C for 2 hours with additional mechanical disruption of the spores by glass beads (425–600 μm, Sigma-Aldrich, USA).[5] DNA extraction was performed using QIAmp kit (Qiagene, Hilden, Germany) following manufacturer´s protocol.

P. jirovecii DNA was detected using a single-round polymerase chain reaction (PCR) at mitochondrial (mt) large subunit (LSU) ribosomal RNA (rRNA) gene. DNA amplification was done using protocols described elsewhere.[4] Amplicons of positive PCR results were sequenced to detect polymorphisms at nucleotide positions 85 and 248.

For the identification of microsporidia species immunological and molecular methods were carried out. Initially, the immunohistochemistry technique (IHC) by indirect immunoperoxidase method was used. In brief, rabbit polyclonal antibodies anti-*Encephalitozoon intestinalis*, *Encephalitozoon cuniculi* (Ec), and *Encephalitozoon hellem*, at 1:1600 dilution and mouse monoclonal antibodies anti-*Enterocytozoon bieneusi* (Eb) and anti-*E. intestinalis* at 1:1000 dilution were used as primary antibodies.[6] The confirmation of microsporidia species was carried out by two PCR. First, differentiation of *E. bieneusi* from *Encephalitozoon* spp. was possible by the different size of the amplified DNA products (250 bp vs 270 to 279 bp) by restriction digestion analysis with *Pst*I (Sigma-Aldrich, USA) and *Hae*III (Sigma-Aldrich, USA).[7] In addition, species specific primers were used to amplify DNA regions encoding the small subunit of rRNA. The primer pairs ECUNF/ECUNR for Ec and EBIEF1/EBIER1 for Eb were used as described previously.[5]

All participants completed written informed consent and, according to our hospital's regulations, the procedure for requesting and authorizing research studies was completed.

Results

Out of the 514 autopsied cases, *P. jirovecii* and microsporidia species were identified histologically in tissues of 53 (10.3%) and 62 (12.1%) cases, respectively. All the deceased patients were severely inmunonosuppresed with T CD4 + lymphocytes count below 200 cells/μl (PcP cases 90.8 ± 12.9 [rank 10–198] vs Microsporidia cases 50.2 ± 15.7 [rank 8–150]). In parallel, the use of antiretroviral therapy (ART) in this series was 29.3% and 1.8% for PcP and microsporidia, respectively.

A total of five cases with the coinfection were recovered from the 514 autopsied cases (0.97%). These patients were aged 24–45 years, three were males and two patients received ART. Of the five coinfected patients, three had their kidneys affected. The IHC assay signed out the pathogen as Ec in all five cases, but molecular characterization demonstrated that only one out of five cases was due to Eb and the other four were Ec. Genotype 3 of *P. jirovecii* mt LSU rRNA was found in all PcP cases. Table 1 shows data for five AIDS deceased patients, such as year of death, T CD4 + lymphocytes count, year of human immunodeficiency virus (HIV) diagnosis, use of ART, paraffin tissues for diagnosis, and identification/characterization for Microsporidia/*Pneumocystis*.

Discussion

Pneumocystis and microsporidia were originally classified as protists, but are now accepted as fungi based on phylogenetic analysis. Both pathogens can cause severe disease in immunosuppressed patients, such as organ transplant recipients, under chemotherapy and patients with HIV infection.[1,2] Also, they are strict obligate parasites that infect humans and their genome sequence analyses show that they have lost most of the genes needed for making primary metabolites, such as amino acids and nucleotides. Finally, both fungi have progressively been found also in immunocompetent persons.[1,8] The above mentioned features for these species may justify that their concomitancy could interfere with each other life cycle. However, the intracellular nature of microsporidia and the extracellular interaction of *Pneumocystis* with the human lung epithelial cells should facilitate the coinfection in the same tissues.

Recently, a meta-analysis showed that the prevalence of microsporidia infection in people with HIV ranged between 0.7 and 81.3%, with an estimated prevalence of 11.8% worldwide and 5.6% in Latin America (only three articles were reviewed).[9] Previous report described that microsporidia infection in HIV/AIDS patients has a moderate incidence in Cuba. Once ART was supplied to Cuban AIDS patients in 2001, microsporidiosis has literally disappeared as cause of death. [6] However, based on data in the literature, the incidence of microsporidial infections is much higher than previously reported, and microsporidia may represent a neglected etiological agent of more common diseases.[9]

E. cuniculi was demonstrated as the most predominant microsporidia species in this study. A serological study among men who have sex with men (MSM) in Sweden showed that 33% of them carry antibodies to Ec suggesting that homosexual practices may contribute to horizontal transmission of microsporidiosis.[3] This aspect is very important for our study since 90% of the Cuban AIDS reported cases are MSM. The identification of microsporidia in the kidneys in 54 of 62 cases with disseminated microsporidiosis found among 514 post mortem studies in the study period corroborated this hypothesis.

In this study, the confirmation of Ec species was carried out by two different molecular methods. The results differ from those of previous prevalence studies, since Ec was identified more frequently than Eb.[2,9] Contrary to Eb, Ec usually cause systemic disease, this clinical picture is similar to the analyzed patients in this series (Ec identified in kidney and ovary).[2] A high prevalence the Ec have been described in Slovakia (26.4%), the United States (84.6%), and Egypt (77.3%), respectively.[10–12] Ec infections in humans are considered primarily of zoonotic origin, more probably because the animals may be reservoirs of the spores and thus potential sources of infection for human in Cuba. Further molecular testing for the presence of Ec in Cuban HIV-infected patients may thus be warranted.

In our series, one case was mistakenly identified as Ec by IHC but later confirmed as Eb by means of two specific PCR. This discrepancy result is due to the use of polyclonal serum produced in rabbits that showed cross-reactions among the different species of microsporidia, indicating the presence of common antigens between species. In the same way, the studies by indirect immunofluorescence antibody test show different behavior depending on the monoclonal antibodies studied.[13]

Eb is known to infect the small intestine and could spread into the hepatobiliary tree in patients with AIDS. Pulmonary involvement by Eb is infrequent but may occur. Five cases are reported in the literature of severe immunosuppressed HIV-infected patients with intestinal and pulmonary symptoms until 2004. More recently, Kicia et al. described two additional cases of Eb infection with respiratory involvement (one in an HIV-negative transplant recipient and another case in 105 patients with various respiratory diseases).[14] The case described here reinforces the idea that Eb has the capability for pulmonary colonization.[2]

PcP remains as a major opportunistic infection in HIV-infected patients in both developed and developing countries and as an emerging problem in immunosuppressed patients without HIV infection worldwide.[1] A systematic review of hospitalizations in HIV-infected patients in the ART era reported that tuberculosis, bacterial community-acquired pneumonia, and PcP collectively accounted for 57% of inpatients death in adults globally.[15]

In this study, genotype 3 of *P. jirovecii* mt LSU rRNA was found in all cases. As previously reported, it occurred in 81.3% (13/16 samples) of autopsied AIDS patients in Cuba, suggesting that this genotype is very common in the country and its possible association with the virulence of *P. jirovecii* strains.[4]

Coinfection with *P. jirovecii* and microsporidia has not been evaluated previously due to the difficulty in the diagnosis of both pathogens. Some authors agree that the identification of intracellular stages compatible with microsporidia depends on careful observation and skillful interpretation of histopathology and cytological findings.[2] On the other hand, masking by tuberculosis, lack of awareness and of diagnostic facilities (e.g., bronchial lavage, high-resolution computed tomography, immunofluorescence staining, and molecular technique) could be responsible for the underreporting and late diagnosis of PcP.[1] This gets worse since some physicians think that both infections are only restricted to severely immunosuppressed AIDS patients.

Although relatively low number of cases with PcP and microsporidia coinfection were found in our series, the autopsies are performed in our institution in 75% of all deceased patients. Cuba is a country with a low prevalence of HIV cases (0.4% between 15 and 49 years with 2200 new cases per year), so this coinfection should not be considered relevant (22 cases per year), in contrast to countries with high HIV cases, where this phenomenon may be a more common medical problem than expected.

The study of coinfection, when human beings harbor two or more pathogenic parasites, requires in-depth investigation to have a comprehensive understanding of this multi-infectious process, in relation to its dynamics and consequences.[16]

In summary, coinfection of *Pneumocystis* and microsporidia is very challenging and raises interesting issues about host-parasite relationship. It needs to be taken into account that these results are findings to be confirmed by future studies. On the other hand, molecular techniques for the correct and fast identification of both infections are becoming an essential diagnostic tool for both fungi. The early diagnosis of both pathogens must be crucial to establish correct and early treatments, improve the patient's evolution, reducing the risk of death.

Funding

This work was supported by the 'Red Iberoamericana sobre Pneumocystosis' (212RT0450) in the framework of The Ibero-American Programme for Science, Technology and Development (CYTED).

Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and the writing of the paper.

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