

Pneumocystis jirovecii genotyping: experience in a tertiary-care hospital in Northern Italy

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SUMMARY

Respiratory samples from *Pneumocystis jirovecii* pneumonia (PJP) cases collected at a tertiary-care university hospital in Modena were analyzed for the presence of specific polymorphisms in the mitochondrial large subunit ribosomal RNA (mtLSU-rRNA). Retrospectively, 57 cases were selected in a six-year period and 34 out of the 57 processed BAL samples returned PCR positive results, thus allowing further molecular analysis. The following *P.jirovecii* genotype distribution was observed: genotype 3 (50%), genotype 2 (23%), genotype 1 (18%), genotypes 1 or 4 (9%). These data add novel insights on *P.jirovecii* epidemiology, investigating a previously unstudied area of Northern Italy. A peculiar local distribution is highlighted with respect to other areas within the national panorama, thus encouraging further in-depth studies in an attempt to better understand the overall situation concerning *P.jirovecii* genotype circulation.

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INTRODUCTION

Pneumocystis jirovecii is an ascomycetous fungus that causes opportunistic infections in humans: In particular, interstitial *P.jirovecii* pneumonia (PJP) is the most common clinical picture occurring in immunocompromised patients (Thomas and Limper, 2004). In the past, PJP was one of the prevalent opportunistic mycoses in acquired immunodeficiency syndrome (AIDS) patients. Nowadays, thanks to the introduction of highly active antiretroviral therapy (HAART) and the availability of different drugs for antifungal therapy and prophylaxis, the rate of PJP in HIV patients has been drastically reduced in industrialized countries (Mocroft *et al.*, 2003; Huang *et al.*, 2011), but remains a major concern in developing areas (Lanaspa *et al.*, 2015; Medina *et al.*, 2017). Besides HIV patients, PJP also afflicts individuals with chronic pulmonary disease, onco-hematologic patients, rheumatologic and dermatologic patients and individuals with inflammatory bowel disease (Norris and Morris, 2011; Cordonnier *et al.*, 2016; Mecoli *et al.*, 2017; Cotter *et al.*, 2016; Gonzalez Santiago *et al.*, 2016).

PJP may develop either by reactivation of resident fungal cells or by *de novo* infections (Morris and Norris, 2012; Kim *et al.*, 2015). It has been proposed that, following initial infection, the fungus is not cleared but rather persists as part of the commensal/resident microbiota for extend-

ed periods, eventually reactivating when the host becomes immunocompromised. If this is the case, the primary infection is acquired during childhood, according to the knowledge that most humans become seropositive for *P.jirovecii* early in life (Vargas *et al.*, 2001). Conversely, *de novo* infections may also occur as transient events during lifetime and likely related to active transmission (Kim *et al.*, 2015). The acquisition of *de novo* nosocomial *P.jirovecii* infections has been reported in numerous studies and reviews over the years, including both immunosuppressed and HIV patients (Chave *et al.*, 1991, 1991; Cheung *et al.*, 1994; de Boer *et al.*, 2007; Gianella *et al.*, 2010; Mori *et al.*, 2010; de Boer *et al.*, 2011; Sassi *et al.*, 2012; Yiannakis and Boswell, 2016). Moreover, detection of the same *P.jirovecii* genotype, both in patients and in air samples collected from their hospital rooms, strongly supports the possibility of nosocomial airborne transmission (Fréalte *et al.*, 2016; Choukri *et al.*, 2010; Damiani *et al.*, 2012; Le Gal *et al.*, 2015).

Multilocus genotyping of *P.jirovecii* will disclose the genetic diversity of the fungus, showing distinct epidemiological profiles with respect to geographic distribution, drug resistance and route of transmission (Matos *et al.*, 2010). Moreover, genotyping using multilocus sequence markers can be used to investigate suspected outbreaks, as proposed by the guidelines of The Fifth European Conference on Infections in Leukaemia, where such an approach has been rated with an A-II recommendation (Alaino *et al.*, 2016). Initial genetic and epidemiological data on *P.jirovecii* infections in Italy have focused on defined geographical regions, investigating both HIV-positive and HIV-negative individuals (Dimonte *et al.*, 2013).

The aim of this study was to expand knowledge on *P.jirovecii* diversity, genotyping samples from PJP cases collected at a tertiary-care hospital in Modena. In particular,

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the presence of specific polymorphisms in the mitochondrial large subunit ribosomal RNA (mtLSU-rRNA) of *P. jirovecii* was investigated.

MATERIALS AND METHODS

From 2011 to 2016, all broncho-alveolar lavage (BAL) specimens diagnosed as positive for *P. jirovecii* during routine activity [at the Laboratory of Microbiology and Virology of the University Hospital Trust in Modena] and frozen as sufficient leftover amounts were enrolled in the present study. The routine search for *P. jirovecii* in BAL samples had been performed by immunofluorescence (IFA) or PCR assays, namely, the MeriFluor Pneumocystis (Meridian Bioscience, Cincinnati, OH, USA) or the *P. jirovecii* Alert kit (Nanogen Advanced Diagnostic, Torino, Italy), respectively. Overall, 57 BAL samples from 57 patients were processed in the study. In 8 cases, DNA from BAL samples was manually extracted at the Hospital in Modena by MycXtra Fungal DNA Extraction Kit (Myconostica, Cambridge, UK). In the other 49 cases, BAL samples were sent to the Tor Vergata Hospital in Rome, where they were processed for DNA extraction by the automated EZ 1 Advanced XL platform using the QIAamp DNA kit (QIAGEN, Hilden, Germany). Subsequently, the extracted DNA samples were amplified by an in-house nested PCR of mtLSU-rRNA (260-bp fragment length) and sequenced to obtain the fungal genotype, as previously described (Dimonte *et al.*, 2013). As reported

in several studies, nested PCR targeting mtLSUrRNA was the most sensitive among different diagnostic methods, with a sensitivity of 98.3% (Robberts *et al.*, 2007; Fan *et al.*, 2013). Each PCR run included internal positive and negative controls.

The study was performed in agreement with the WMA Helsinki Declaration (Edinburgh 2000 and its subsequent modification) and with Italian National Law n. 675/1996 on the protection of personal data. The local Ethical Committee (n. 53/11) approved the present study and informed consent was obtained according to the approved protocol.

RESULTS

BAL samples recorded as *P. jirovecii* positive according to the Clinical Microbiology Laboratory routine analysis over a six-year period and frozen as adequate leftover amounts were included in the present study. PCR and sequencing analysis were performed to assess the presence of polymorphisms in the fungal mtLSU-rRNA DNA. A total of 57 BAL samples were selected and investigated. *Table 1* summarizes the patients' demographic and clinical characteristics. Two thirds of the patients were male, the mean age being 55±17 years; AIDS was the prevalent base disease (about 30%), followed by COPD (16%) and lymphoma (12,3%). Out of the 57 BAL samples, 34 returned PCR positive results (60%). By further aggregating the data depicted in the Table (data not shown), the rate of fungal DNA

Table 1 - Genotyping of *P. jirovecii* in BAL samples from patients in different clinical settings.

	All samples n=57 (100%)	Positive samples n= 34 (59,6%)	Genotype 3 n=17	Genotype 2 n=8	Genotype 1 n=6	Genotypes 1 or 4 n=3
Fungal genotype percentage			50,0	23,5	17,6	8,8
<i>Patient characteristics</i>						
Male sex	39 (68)	24 (71)	9 (53)	6 (75)	6 (100)	3 (100)
Mean age ± SD (range)	55±17 (31-87)	50±15 (31-87)	59±17 (28-87)	59±10 (40-74)	55±15.4 (35-82)	43±9 (31-52)
<i>Underlying disease</i>						
AIDS	17 (29,8)	14 (24,6)	5 (29,4)	4 (50,0)	2 (33,3)	3 (100,0)
COPD	9 (15,8)	6 (10,5)	3 (17,6)	1 (12,5)	2 (33,3)	0 (-)
Lymphoma	7 (12,3)	1 (1,8)	1 (5,9)	0 (-)	0 (-)	0 (-)
AML	1 (1,8)	1 (1,8)	1 (5,9)	0 (-)	0 (-)	0 (-)
ALL	1 (1,8)	0 (-)	0 (-)	0 (-)	0 (-)	0 (-)
Myeloma	1 (1,8)	0 (-)	0 (-)	0 (-)	0 (-)	0 (-)
Myelodysplasia	1 (1,8)	1 (1,8)	1 (5,9)	0 (-)	0 (-)	0 (-)
HSCT	1 (1,8)	1 (1,8)	1 (5,9)	0 (-)	0 (-)	0 (-)
Solid neoplasia	5 (8,8)	1 (1,8)	1 (5,9)	0 (-)	0 (-)	0 (-)
Cirrhosis	1 (1,8)	1 (1,8)	1 (5,9)	0 (-)	0 (-)	0 (-)
Autoimmune disease	2 (3,5)	2 (3,5)	1 (5,9)	1 (12,5)	0 (-)	0 (-)
Liver transplantation	2 (3,5)	1 (1,8)	1 (5,9)	0 (-)	0 (-)	0 (-)
Kidney transplantation	1 (1,8)	0 (-)	0 (-)	0 (-)	0 (-)	0 (-)
Chronic renal failure	2 (3,5)	2 (3,5)	1 (5,9)	1 (12,5)	0 (-)	0 (-)
Other	5 (8,8)	3 (5,3)	0 (-)	1 (12,5)	2 (33,3)	0 (-)

If not specified, in parenthesis, relative percent values are shown.

AIDS = Acquired immune deficiency syndrome, COPD = chronic obstructive pulmonary disease, AML = acute myeloid leukemia, ALL = acute lymphoblastic leukemia, HSCT = hematopoietic stem cell transplantation.

recovery was 82% (14 out of 17 processed BAL samples) in AIDS patients and 50% (20 out of 40 processed BAL samples) in non-AIDS patients. Finally, as detailed in the *Table 1*, genotype 3 was the most prevalent in the studied population (50% of the cases; 17 out of 34 PCR positive BAL samples), followed by genotype 2 and genotype 1 that were detected in 23.5% and 17.6% of the cases. Uncertain genotypes, 1 or 4, were recorded in 8.8% of the BAL positive samples.

DISCUSSION

The present study depicts the *P.jirovecii* genotype distribution in a previously unstudied area of Northern Italy, focusing on patients admitted to a tertiary hospital (Modena University Hospital Trust) over a six-year. According to the study design, the BAL specimens from PJP cases were retrospectively selected, being IFA or PCR positive (routine diagnostic laboratory) and having enough BAL leftover available for further genotypic studies. Thus, although dealing with a relatively small number of cases, the study offers an accurate picture of the epidemiological situation occurring in our area in terms of circulating *P.jirovecii* genotypes within a relative long time period. Compared to other recent data concerning *P.jirovecii* molecular epidemiology, our findings show a distribution of *P.jirovecii* genotypes unique to our area with respect to that observed in other parts of Italy. In particular, genotype 3 appears the most prevalent in Modena (being observed in 50% of the cases), while in a study from Turin (Volpe *et al.*, 2001), genotype 1 and genotype 3 were detected as equally prevalent (both at 36%). Conversely, in Rome (Dimonte *et al.*, 2013), genotype 2 was the most prevalent (39%). Taken together, previous and present data highlight the occurrence of regional variations in terms *P.jirovecii* genotype circulation in Italy, thus encouraging large-scale molecular analysis for a more in-depth definition of both general and local *P.jirovecii* epidemiology in Italy. In this respect, it is worth noting that such information has numerous epidemiological implications, including the choice and application of measures to prevent airborne community or nosocomial transmission of *P.jirovecii* (Gits-Muselli *et al.*, 2015).

We are aware of the internal bias related to the low recovery rate of fungal DNA (60%). This may be due to the low quality of the leftover samples used in the present study. If so, future molecular studies should only use carefully handled/dedicated samples in order to enhance the efficacy of such analysis. Furthermore, it should be noted that the *P.jirovecii* DNA recovery rate was approximately double in AIDS with respect to non-AIDS patients (82% vs 50%). A possible explanation for this finding may be found in the pathogenesis of PJP that evolves differently in these two distinct patient populations. Indeed, it has been reported that the *P.jirovecii* burden is much higher in AIDS than in non-AIDS individuals (Morris and Norris 2012). In any case, similar rates of fungal DNA recovery have commonly been observed in other laboratories (Di Cave, personal communication).

In conclusion, this pilot study adds novel insights on the epidemiology of *P.jirovecii* in a previously unexplored area of Northern Italy, and provides an original contribution to a consolidated international network of scientists involved in *P.jirovecii* genotype distribution and circulation on the European continent (Alanio *et al.*, 2016).

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