**INTRODUCTION**

*Pneumocystis jirovecii* is an opportunistic fungal respiratory pathogen that can cause life-threatening pneumonia in immunocompromised individuals. Pneumocystis pneumonia (PCP) has long been the most common AIDS-defining opportunistic infection (Centers for Disease Control and Prevention, 1999). However, while the incidence of PCP has decreased in human immunodeficiency virus (HIV) patients with access to antiretroviral therapy (ART), it remains a leading cause of disease in HIV patients who are undiagnosed or not on ART (Kaplan et al., 2000). In contrast, PCP is a growing and frequent complication amongst immunocompromised patients following solid organ and stem cell transplant and in oncology patients. *P. jirovecii* infection in these patients is associated with greater severity and mortality (Chabé et al., 2004; Roblot et al., 2002; Pulvirenti et al., 2003; Monnet et al., 2008). The clinical severity of PCP is dictated by the extent of pulmonary inflammation rather than the organism lung burden, with CD4+ T cells playing a central role (Thomas & Limper, 2004). In addition, recent studies also indicate the role of *P. jirovecii* in patients receiving steroid treatment, such as patients with rheumatic pathologies or inflammatory bowel disease, in addition to patients with chronic pulmonary diseases (Matos & Esteves, 2010; Morris et al., 2004, 2008).

The epidemiology and pathogenesis of *P. jirovecii* remain incompletely understood. The epidemiology of *P. jirovecii* infection remains unclear, in particular its mode of acquisition and the frequency and geographical distribution of *P. jirovecii* clones (Hauser et al., 2001). Molecular typing methods, such as single-strand-conformation polymorphism typing and multiple locus sequence typing (MLST), have answered some of the epidemiological issues associated with *P. jirovecii*. Some studies have shown that *P. jirovecii* types exhibit a broad genetic diversity within populations that is independent of HIV status, geographical location or seasonality (Schmoldt et al., 2008). Patients can be infected by single or multiple genotypes with significant recombination between types being observed. However, clonal expansion of a successful MLST genotype can occur and increases rapidly to produce an epidemic clone (Smith et al., 1993).

Historically, reactivation of endogenous latent infection following immune compromise was the preferred model of *Pneumocystis* infection (Thomas & Limper, 2004). However, MLST genotyping supports the potential of *de novo* exposure from the environment or infected individuals as additional sources of *P. jirovecii* acquisition. This has important infection-control implications in hospital and outpatient settings (de Boer et al., 2011; Rabodonirina et al., 2004; Kaneshiro & Maiorano, 1996; Wakefield, 1996).

Outbreaks of PCP have been described in renal transplantation units throughout Asia and Europe (Schmoldt et al., 2008; Gianella et al., 2010; de Boer et al., 2007; Arichi et al., 2009; Yazaki et al., 2009). Two concurrent outbreaks in the North-West of England constituted the largest combined cluster of PCP cases documented amongst renal organ and stem cell transplant and in oncology patients. Amongst immunocompromised patients following solid organ transplantation units throughout Asia and Europe (Schmoldt et al., 2008; Gianella et al., 2010; de Boer et al., 2007; Arichi et al., 2009; Yazaki et al., 2009). Two concurrent outbreaks in the North-West of England constituted the largest combined cluster of PCP cases documented amongst renal
transplant recipients worldwide (Thomas et al., 2011). In Northern Ireland, a PCP cluster amongst 11 renal transplant recipients occurred during a 2 year period (McCaughan & Courtney, 2012). Against this background, a 3 year review of molecular testing of P. jirovecii in Northern Ireland showed a marked increase in clinically significant P. jirovecii infections with an associated mortality of 30% (Coyle et al., 2012). This increase prompted molecular genotyping of previous and current circulating strains of P. jirovecii.

**METHODS**

**Patient selection.** During July 2008 to July 2011, testing for P. jirovecii was undertaken where clinically requested. During this period 670 specimens (302 sputa, 186 bronchoalveolar lavages, 138 upper respiratory specimens and 44 tracheal secretions) from 476 patients from all age groups in 18 hospitals across Northern Ireland were tested and P. jirovecii was confirmed in 53 patients (11%) (35 male and 18 female). The range in P. jirovecii load ranged from 270 copies ml⁻¹ to 5.45 x 10⁶ copies ml⁻¹. Fifty-one P. jirovecii positive patients (96%) were assessed clinically as having PCP and an underlying risk factor. These included HIV (13/53), chemotherapy (13/53), autoimmune conditions and steroid treatment (12/53), post-transplant (11/53) and other conditions (severe combined immunodeficiency and haemophagocytic lymphohistocytosis) (2/53). Upon clinical assessment two patients were considered to be colonized with no underlying risk factor and were not treated (Coyle et al., 2012).

**PCR.** Infection was established by detection of P. jirovecii DNA using a duplex quantitative real-time PCR assay targeting both the dihydrofolate reductase and β-tubulin genes (Coyle et al., 2012). Specimens with a Ct value of <40 were regarded as positive for P. jirovecii.

**P. jirovecii MLST PCR and sequencing.** P. jirovecii-positive respiratory specimens were stored frozen at −20°C. Sputum was liquefied before DNA extraction in an equal volume of Sputasol (Oxoid). DNA was extracted using the QIAamp DNA Blood Mini Kit on the QIAasympohemy automated extractor (Qiagen).

Genotyping of P. jirovecii-positive specimens was performed using the MLST method previously described (Hauser et al., 1997). Fragments of four known variable regions of the P. jirovecii genome (ITS1, 26S, mt26S, and β-tubulin) were amplified using 23 μl of Master Mix consisting of 4 mM MgCl₂ (26S reaction contained 3 mM), 0.4 μM of the relevant forward and reverse primer, 1X Platinum qPCR Supermix-UDG (Life Technologies) and 2 μl of extracted specimen. PCR conditions were as follows: 94°C for 3 min, followed by 40 cycles of 94°C for 30 s, 56°C for 1 min for ITS1, 54°C for 26S, 52°C for mt26S and 62°C for β-tubulin, and 72°C for 1 min. This was followed by a final extension step of 72°C for 5 min.

Following electrophoresis on a 3% gel, the relevant-sized DNA amplicon (ITS1 204 bp, 26S 426 bp, mt26S 347 bp and β-tubulin 309 bp) was purified using QIAquick Gel Extraction Kit (Qiagen) and eluted in 40 μl of AE buffer. Purified products were sequenced on a 3300 capillary sequencer (Life Technologies) using the Big Dyes v3.1 sequencing kit (Life Technologies).

Sequence analysis was performed with SeqMan software v5.08 (DNASTAR). The obtained sequences were compared with P. jirovecii reference gene sequences for each gene (Accession No. U07220 for ITS1, M58605 for mt26S, L13615 for 26S and AF170964 for β-tubulin) and alleles determined.

Following analysis the sensitivities of ITS1 and mt26S PCR sequencing assays were superior to those of 26S and β-tubulin assays and only the discriminatory power of ITS1 and mt26S alleles allowed the final genotype determination used previously (Thomas et al., 2011).

**RESULTS**

**MLST types**

An MLST genotype (based on ITS1 and mt26S) was obtained for 31 P. jirovecii-positive patients and these are summarized in Table 1. All patients had a single P. jirovecii genotype detected. Fifteen different MLST genotypes of P. jirovecii were found to be circulating in Northern Ireland with similar MLST types circulating amongst the different risk groups, including the identification of a strain with a previously unreported ITS1 allele. These genotypes were found throughout the region, which incorporates eight different hospitals, including a number of outpatient clinics for patients receiving immunosuppressive treatments and chemotherapy. Variant A3 (in 13 patients) was the predominant ITS1 type while variant 8 (in 21 patients) was the predominant mt26S type identified.

An MLST type was determined for 11/15 (73%) of P. jirovecii-positive HIV patients. This group showed relatively large diversity in circulating MLST types with eight different genotypes, the most common being B,8. No obvious difference in circulating genotypes amongst HIV patients was observed between the different hospital sites. Amongst the eight renal transplant patients within the PCP cluster, a single type, A3,8, was confirmed. However, this type was not exclusive to these patients, being also detected in three non-transplant patients, one of whom had HIV, one with rheumatoid arthritis receiving steroid treatment and one with nephrotic syndrome. Four P. jirovecii MLST types were found circulating in the five patients within the malignancy and chemotherapy risk group. Five different MLST types were prevalent amongst those patients classified as having other risk factors. Of the five patients, two had severe combined immunodeficiency, one had nephrotic syndrome and two had unknown risk factors. Two different MLST types were obtained for two patients, one with rheumatoid arthritis and the other myasthenia gravis; both were receiving steroid treatment for their conditions.

**DISCUSSION**

P. jirovecii remains an important opportunistic pathogen amongst HIV patients, particularly those who are not on HAART (highly active antiretroviral therapy) or who do not know they have HIV. However, in more recent years, the number of non-HIV immunosuppressed patients at risk of P. jirovecii has been increasing (Morris & Norris, 2012). The number of P. jirovecii infections has dramatically increased throughout the UK and Europe, in particular amongst renal transplant patients for whom a number of outbreaks have been characterized (Thomas et al., 2011).
Likewise, a similar rise in cases in Northern Ireland following a 3 year review of molecular testing was recently documented and highlighted the expanding population at risk of *P. jirovecii* infections (Coyle et al., 2012).

Epidemiological investigation of *P. jirovecii*-positive specimens in this 3 year period using MLST typing found a diverse population of *P. jirovecii* types circulating, with 15 different genotypes identified, including the identification of a strain with a previously unreported ITS1 allele. There was no association of specific genotypes with geographically distinct hospital sites (this study included specimens from eight hospitals). Circulation of a large number of *P. jirovecii* genotypes in the Northern Irish patient population is consistent with similar studies reported in other European countries, although only single genotype infections were found in this population (Esteves et al., 2008; Latouche et al., 1997; Lu et al., 1994; Volpe et al., 2001; Tsolaki et al., 1996). Indeed, one study characterized *P. jirovecii* genotypes from 10 European hospitals involving 212 patients over an 8 year period and demonstrated a broad diversity of stable and ubiquitous types, which was independent of time, geography or HIV status (Hauser et al., 2001). Interestingly 10 of the 15 types prevalent in Northern Ireland were also found in other studies where the same MLST typing scheme was utilized (Volpe et al., 2001; Thomas et al., 2011). Some studies have shown genotype diversity based on larger geographical location. In one US study, genotype distribution patterns differed in HIV patients in five cities, with differences correlating with the place of diagnosis (Beard et al., 2000; Matos & Esteves, 2010). However, a number of studies have failed to demonstrate any geographical difference in circulating *P. jirovecii* genotypes (Hauser et al., 2001; Esteves et al., 2008).

The findings in this study and previous population studies provide strong evidence that *P. jirovecii* infection is non-clonal. However, characterization of numerous recent *P. jirovecii* outbreaks amongst renal transplant patients in Europe and Asia has shown that one clonal genotype tends to prevail. This suggests that different genotypes are involved in local clonal outbreaks. In Northern Ireland, MLST typing of *P. jirovecii* from eight patients in this cluster demonstrated an association with a single genotype (A3,8). In the North of England, renal units at two different hospitals where concurrent *P. jirovecii* outbreaks were occurring demonstrated a predominant but different clone in each hospital: type B2,8 in 10 of 17 cases attending the Royal Liverpool University Hospital and B1,3 in seven of ten cases attending Salford Royal Hospital (Thomas et al., 2011). In a German study, a single genotype (B,7) was found to be responsible for a cluster of PCP amongst 14 renal transplant patients (Schmoldt et al., 2008). The predominance of a single clonal genotype suggests person-to-person transmission or environmental exposure within these units. Previous studies have established that nosocomial transmission of *P. jirovecii* that occurs amongst immunocompromised patients may be directly and also indirectly transmitted through colonization of asymptomatic carriers such as healthcare workers (Gianella et al., 2010; Miller et al., 2001). Additionally, the detection of *P. jirovecii* DNA in environmental samples, such as air samples, suggests the strong possibility of acquisition from the environment (Bartlett et al., 1997; Olsson et al., 1998). This nosocomial risk has been significantly reduced in the Northern Ireland renal transplant patients due to the extension of post-transplant prophylaxis to 6 months in all transplant patients.

<table>
<thead>
<tr>
<th>Predisposing conditions (no. of patients with condition)</th>
<th>Genotypes (ITS1, mt26S)</th>
<th>No. of patients</th>
</tr>
</thead>
<tbody>
<tr>
<td>HIV (11)</td>
<td>A3,3</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>A3,8</td>
<td>1</td>
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<tr>
<td></td>
<td>B,3</td>
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<td></td>
<td>B,8</td>
<td>3</td>
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<td></td>
<td>B2,8</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>B3,8</td>
<td>1</td>
</tr>
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<td></td>
<td>D,8</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>Novel, 7</td>
<td>1</td>
</tr>
<tr>
<td>Renal transplant (8)</td>
<td>A3,8</td>
<td>8</td>
</tr>
<tr>
<td>Malignancy and chemotherapy (5)</td>
<td>B,2</td>
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<tr>
<td></td>
<td>B,8</td>
<td>1</td>
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<tr>
<td></td>
<td>B1,8</td>
<td>1</td>
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<tr>
<td></td>
<td>B2,7</td>
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<tr>
<td>Other/not known (5)</td>
<td>A3,2</td>
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<tr>
<td></td>
<td>A3,8</td>
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<tr>
<td></td>
<td>B,8</td>
<td>1</td>
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<tr>
<td></td>
<td>D,2</td>
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<tr>
<td></td>
<td>Novel, 2</td>
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<td>Autoimmune conditions on steroids (2)</td>
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<td></td>
<td>B,7</td>
<td>1</td>
</tr>
</tbody>
</table>

Table 1. MLST genotypes of *P. jirovecii* circulating in Northern Ireland
This study has demonstrated a diverse number of *P. jirovecii* genotypes amongst patients with different predisposing conditions (Table 1). While A3,8 was the only genotype in the renal transplantation group, there was no other restriction of specific genotypes to specific patient risk factor groups. Indeed, the A3,8 genotype was also circulating amongst patients within other risk factor groups, namely HIV, those on steroids for autoimmune conditions and in those with other or unknown predisposing risk factors. This predominance of a local clone rather than the emergence of a particularly virulent or resistant clone is in keeping with other studies investigating *P. jirovecii* outbreaks. Interestingly, genotypes such as B7 and B2,8, responsible for outbreaks in renal transplant patients in Germany and England, respectively, were also found circulating in Northern Ireland (Schmoldt et al., 2008, Thomas et al., 2011). Clearly, the circulation of highly diverse genotypes amongst these patients indicates multiple sources of *P. jirovecii* and represents a significant risk for colonization for patients receiving immunosuppressive and biological therapies in hospital wards and outpatient settings. However, the requirement of prophyaxis in these colonized at-risk patients is unknown, but it would seem appropriate to consider a ‘decolonization regime’ not only to prevent progression to PCP, but also to eliminate onwards transmission from colonized reservoirs to other ward and outpatient clinic patients. Whether colonization is also associated with a higher prevalence of respiratory infections than seen with non-colonized patients should also be the subject of further study.

Genotypic investigation of *P. jirovecii* presents a unique challenge. MLST of other micro-organisms can be undertaken on cultured isolates allowing sequencing of seven or more loci. However, the inability to isolate the fungus on solid media or in cell culture restricts the number of loci that can be sequenced successfully and hence the discriminatory power of the typing. Unlike for many other organisms, no core *P. jirovecii* MLST sequence database exists, resulting in a lack of a single comparable typing method and limiting access to *P. jirovecii* sequences. Despite these limitations the diversity of genotypes detected in this and other studies highlights the importance of ongoing genotypic surveillance of *P. jirovecii*. This would allow prompt detection of different local types emerging as dominant local clones in outbreaks. While infections with single *P. jirovecii* genotypes were detected in this study, other outbreak investigations in Europe and the UK have demonstrated patients infected with multiple *P. jirovecii* genotypes. Therefore, continued routine surveillance is important to detect the emergence of new genotypes through recombination events associated with the more widespread transmission now being recognized.

**CONCLUSION**

This study demonstrates that a relatively large number of *P. jirovecii* genotypes are circulating within Northern Ireland, including a strain with a previously unreported ITS1 allele identified by the study. Multiple genotypes were found to circulate within each patient risk group; however, among the renal transplant patients one local clonal genotype prevailed, suggesting that a person-to-person transmission or environmental exposure may have occurred in these patients.

**REFERENCES**


