Rising incidence of *Pneumocystis jirovecii* pneumonia suggests iatrogenic exposure of immune-compromised patients may be becoming a significant problem

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Against a background of point-source outbreaks of *Pneumocystis* pneumonia (PCP) in renal transplant units in Europe, we undertook a retrospective 3 year observational review of PCP in Northern Ireland. This showed an unexpected increase in incidence, with a mortality rate of 30%. Fifty-one cases were confirmed compared to 10 cases confirmed in the preceding 7 years. Where undiagnosed HIV infection had previously been the main risk factor for PCP, this was now equally matched by chemotherapy for haematological and non-haematological malignancy and immune suppression for a range of autoimmune conditions. Congenital immunodeficiency and transplantation were less common predisposing factors, but renal grafts also showed a rising incidence. Asymptomatic carriage was uncommon. At presentation both upper and lower respiratory samples were of equal use in establishing the diagnosis, and treatment resulted in rapid clearance. These data suggest the need for considering PCP in at-risk patients, reviewing its mode of acquisition and whether iatrogenic colonization is a treatable pre-condition.

**INTRODUCTION**

*Pneumocystis jirovecii*, originally classified as a protozoan, was reclassified in 1999 as a fungus in the phylum *Ascomycota* (Hugot *et al.*, 2003) on the basis of DNA and protein analysis (Stringer *et al.*, 2002). While it is an obligate human parasite of the human respiratory tract, its DNA can be found in airborne fungal spores (Wakefield, 1996), increasing the risk of environmental exposure. Aerosol transmission from an infected patient is also thought to occur and has been demonstrated in animal models (Hughes, 1982; Chabe *et al.*, 2004). Additionally, its detection in hospital air samples up to 8 m from patients with confirmed infection also supports transmission from an infected patient (Choukri *et al.*, 2010). Asymptomatic colonization is thought to be relatively common (Maskell *et al.*, 2003; Wakefield *et al.*, 2003; Vidal *et al.*, 2006) and can progress to overt infection when a patient’s immune status deteriorates (Mori *et al.*, 2009). Co-attendance with other immunocompromised patients in health-care settings may, therefore, be a risk factor for colonization and a later overt infectious episode.

Abbreviations: BAL, bronchoalveolar lavage; PCP, *Pneumocystis* pneumonia; qRT-PCR, quantitative real-time PCR; TRAS, tracheal secretions.

*P. jirovecii* causes *Pneumocystis* pneumonia (PCP), a severe opportunistic infection in immunosuppressed patients. Acute respiratory infection accompanied by diffuse bilateral fine interstitial infiltrates is suggestive of PCP, but a definitive diagnosis requires the microbiological confirmation of *P. jirovecii* or its DNA in respiratory tract secretions (Kameda *et al.*, 2011). PCP is usually confirmed using direct or indirect immunofluorescence or silver staining on bronchoalveolar lavage (BAL) fluid, which have respective sensitivities of 97% and 92% (Cregan *et al.*, 1990). Non-invasive serological fungal markers, in particular 1,3-β-D-glucan, are available but less specific (Morris & Masur, 2011) and less sensitive (Tasaka *et al.*, 2007) for the diagnosis and monitoring of PCP.

PCR is more sensitive, and both single-round and nested assays have been developed (Su *et al.*, 2008; Gupta *et al.*, 2010; Jiancheng *et al.*, 2009; Jarboui *et al.*, 2010). These increase sensitivity but are thought to reduce clinical specificity (Mekinian *et al.*, 2010). They have the potential advantage of a high negative predictive value when considering treatment options (Azoulay *et al.*, 2009). Quantitative real-time PCR (qRT-PCR) can help discriminate carriage from acute infection (Fujisawa *et al.*, 2009; Rohner *et al.*, 2009) but the lack of absolute cut-off values, because of the condition’s immunopathological basis,
means that even low copy number *P. jirovecii* infections can be clinically significant (Alanio et al., 2011). It is therefore critical that an immune suppressed patient not on prophylaxis should be clinically assessed before withholding treatment on the basis of a low-copy qRT-PCR result (Mekinian et al., 2010); a negative qRT-PCR on BAL effectively excludes PCP.

There have been a number of recent point-source outbreaks of PCP in renal transplantation units throughout Europe and within the UK, including BelA (de Boer et al., 2007; McCaughan & Courtney, 2012; Neff et al., 2009; Olsson et al., 2001; Yazaki et al., 2009). Against this background, we undertook a 3 year review of PCP since the introduction of a routine qRT-PCR assay in July 2008. The assay had been designed to allow for potential genetic variability in the target genes by using a dual target multiplex recognizing conserved sites in the β-tubulin (Brancant et al., 2005) and dihydrofolate reductase genes (Bandt & Monecke, 2007) (Table 1). Over the 3 year period, all tests, which were undertaken only when requested by the clinical unit, were reviewed. Brief clinical details of patients with confirmed infection were recorded. Details of the qRT-PCR assay and cases tested for PCP are presented.

**METHODS**

*Patients and clinical specimens.* During the period from July 2008 to July 2011, tests for *P. jirovecii* were undertaken only where requested by the clinical unit. Thereafter, all lower respiratory specimens were tested as part of an expanded surveillance program and will be the subject of a separate review. In total, 670 respiratory specimens were received from patients of all age groups in 18 hospitals. The upper airway specimens received included nasal swabs, nasal secretions, throat swabs and combined nasal–throat swabs. Lower airways specimens included sputum, BAL and tracheal secretions (TRAS). Wards with a confirmed infection were contacted to discuss the patient’s clinical presentation, radiological findings, underlying predisposing conditions and treatment.

*Quantitative real-time PCR.* All specimens were extracted without centrifugation or other concentration methods using the QIAamp DNA blood mini-kit in accordance with the manufacturer’s instructions (Qiagen) and eluted in 100 μl of supplied AE buffer. Positive and negative specimens were co-processed. The qRT-PCR was carried out using 1X Superscript III Platinum One step qRT-PCR kit (Invitrogen). To fit with a universally applied laboratory protocol, a final reaction volume of 10 μl was used, comprising 2 μl specimen extract and 8 μl master mix. Final working concentrations in the qRT-PCR were: 0.4 μM primers, 0.2 μM Taqman probes, 0.2 μg μl⁻¹ BSA and 4 mM MgCl₂. All real-time assays were carried out on LightCycler 480 real-time PCR analysers (Roche) using the following cycling conditions: 50 °C for 30 min, 95 °C for 15 min, followed by 45 cycles of 95 °C for 15 s and 60 °C for 1 min. A standard of 1 × 10⁵ copies ml⁻¹ *P. jirovecii* and a negative control were included in each run. All specimens were quantified using a standard curve constructed from serial log dilutions of a NanoDrop-quantified *P. jirovecii* positive control and positive results were expressed as copies ml⁻¹.

*Assay specificity.* Two additional specificity checks were used on the assay. A sequential panel of 126 anonymous lower respiratory specimens, held as part of an infectious diseases bio archive, were selected for testing by the qRT-PCR. These specimens were retained for purposes specified under ethics approval rec. no. 10/NIR01/20. The panel tested contained 24 BAL, 22 TRAS and 80 sputum samples. The assay was also tested against a standard microbiology specificity panel of 50 bacterial, viral and fungal pathogens.

*Data analysis.* Patient demographics were compiled using the relational database Paradox version 4.5 (Corel). The identification of *P. jirovecii* DNA in upper and lower airway secretions at presentation was compared using the χ² statistic; values with *P* < 0.05 were regarded as significant. Epi Info Version 3.5.3 (CDC) was used for statistical analysis.

**RESULTS**

*Patients and clinical specimens.*

The 670 respiratory secretions were received from 476 patients, 278 male and 198 female, and *P. jirovecii* infection was confirmed in 53 patients (11 %), 35 male and 18 female. During 2010 and the first 6 months of 2011 there was a significant increase in the number of patients tested and confirmed with *P. jirovecii* infection (Fig. 1). The highest frequency of test requests was in the 60–70-year-old age group with the highest number of identified infections being in those aged 50–60 years. The median ages of male and female patients tested was 53 years (range: 10 days–87 years) and 55 years (range: 3 days–90 years), respectively, and the median ages of male and female patients with confirmed infection were 54 years (range: 5 months–80 years) and 56 years (range: 6 months–87 years) and 58 years (range: 4 months–90 years).

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### Table 1. Primer and probe sequences used in the multiplex assay

<table>
<thead>
<tr>
<th>Primer/probe</th>
<th>Sequence 5'→3'</th>
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<tbody>
<tr>
<td>β-Tubulin gene</td>
<td>GGCCTGATCAAAGAAGCATGGATA</td>
</tr>
<tr>
<td>PCJ 1A – Forward</td>
<td>CCGCATAAGATATCTGATACCTGTT</td>
</tr>
<tr>
<td>PCJ 1B – Reverse</td>
<td>FAM-TGGCTGAACAGAATCTGGGAGCTTACC-Tamra</td>
</tr>
<tr>
<td>PCJ 1P – Probe</td>
<td>GATCAGAGACATGGTGTGCTATT</td>
</tr>
<tr>
<td>Dihydrofolate reductase</td>
<td>TTAACCCCTTCTCATGGAACACAG</td>
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<tr>
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<td>FAM-TGGTGCAGCGAGTTTTCGCCTTGT-Tamra</td>
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<tr>
<td>PCJ 3B – Reverse</td>
<td>GGCTGATCAAAGAAGCATGGATA</td>
</tr>
<tr>
<td>PCJ 3P – Probe</td>
<td>CCGCATAAGATATCTGATACCTGTT</td>
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</table>

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60 years (range 3 months–79 years), respectively. Of the 53 patients with confirmed *P. jirovecii* infection, 23 (43%) required intensive care and 16 (30%) died. There was no obvious seasonal pattern to the infections.

Of the 53 patients with confirmed *P. jirovecii* infection, 51 (96%) were assessed as being in keeping with PCP by the clinical teams and had an underlying risk factor identified (Table 2). For two patients, after review of the clinical details, the clinical teams decided the findings were not consistent with PCP. The first was a 5-month-old male with a history of recurrent respiratory infections (BAL load $2.50 \times 10^3$ copies ml$^{-1}$) who had already been discharged when the result was available and it was felt that the findings were in keeping with an acute self-limiting *P. jirovecii* infection. The second was a 69-year-old male with a history of alcohol misuse (sputum load $3.17 \times 10^2$ copies ml$^{-1}$). This patient was tested primarily because another patient in the same unit had just been given a confirmed diagnosis of PCP. After review, and after exclusion of a diagnosis of HIV, it was decided not to treat.

Multiple specimens were received from 17 patients as shown in Table 3. While there was no attempt to ascribe absolute values for one-off loads or significant changes in repeat loads, the pattern of response over all suggested rapid response to treatment. Seven patients (patients 1, 2, 6, 10, 11, 12 and 17) had more than one specimen confirmed at diagnosis and 12 patients (patients 3, 4, 5, 7, 8, 9, 11, 13, 14, 15, 16 and 17) had follow-up samples tested. Similar loads were present in both upper and lower respiratory specimens at diagnosis, with no discrepant positive/negative combinations observed. Five patients (patients 5, 7, 8, 15 and 16) showed rapid clearance and became negative when repeated between 2 and 5 days post diagnosis. Three patients (patients 4, 14 and 17) showed significant log reductions when retested between 4 and 7 days post diagnosis. Two patients, 9 and 11, showed persistent high loads in BAL specimens at 9 and 31 days post initial diagnosis, respectively.

Sputum was the most common specimen submitted (302) followed by BAL (186), upper airways secretions (138) and TRAS (44). There was no significant difference between upper and lower airway secretions in diagnosing *P. jirovecii* infection at clinical presentation with 9/105 upper airways secretions and 44/487 lower secretions ($\chi^2 = 0.25$, $P=0.8$) used to confirm infection. At presentation, upper respiratory secretions, sputum and TRAS had similar loads, with median values of $1.13 \times 10^5$, $7.80 \times 10^5$ and $5.63 \times 10^5$ copies ml$^{-1}$, respectively, while higher loads were detected in BAL with a median load of $1.20 \times 10^6$ copies ml$^{-1}$. The loads and respective ranges of the different specimen types are shown in Fig. 2.

**Assay specificity**

The median ages of male and female patients with specimens retrieved from the bio archive were 34 years (range 4 weeks–86 years) and 48 years (range 6 days–86 years), respectively. Of the panel tested, 4/126 (3.2%) were weakly positive for *P. jirovecii* and included a 3-month-old male with $3.33 \times 10^2$ copies ml$^{-1}$ in sputum, a 79-year-old male with $1.31 \times 10^3$ copies ml$^{-1}$ in sputum, a 4-month-old female with $6.96 \times 10^5$ copies ml$^{-1}$ in sputum and an 83-year-old female with $1.14 \times 10^2$ copies ml$^{-1}$ in sputum. All

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**Table 2. Risk factors for PCP**

<table>
<thead>
<tr>
<th>Predisposing condition</th>
<th>No. patients (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HIV</td>
<td>13 (24.5)</td>
</tr>
<tr>
<td>Chemotherapy</td>
<td>13 (24.5)</td>
</tr>
<tr>
<td>Autoimmune conditions on steroids*</td>
<td>12 (22.6)</td>
</tr>
<tr>
<td>Post-transplant†</td>
<td>11 (20.8)</td>
</tr>
<tr>
<td>Other‡</td>
<td>2 (3.8)</td>
</tr>
<tr>
<td>Not clinically significant</td>
<td>2 (3.8)</td>
</tr>
<tr>
<td>Total</td>
<td>53 (100)</td>
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</table>

*Autoimmune conditions included rheumatoid arthritis, idiopathic thrombocytopenia, vasculitis, idiopathic pulmonary fibrosis, myasthenia gravis, systemic lupus erythematosus.
†Transplants included eight renal, one liver, one stem cell and one lung transplant.
‡Other conditions included severe combined immune deficiency and haemophagocytic lymphohistiocytosis.
of the bacterial, fungal and viral specimens from the microbial specificity panel were negative by qRT-PCR.

**DISCUSSION**

During the review period, and particularly in the last 18 months, there was a significant increase in the demand for the microbiological investigation of PCP, suggesting a growing cohort of patients with specific risk factors being hospitalized with acute respiratory infections. Detection of *P. jirovecii* was regarded as clinically significant in 51/53 (96%) patients reviewed. Twenty-three of these 51 patients with confirmed PCP required intensive care management and 16 of the patients died. While BAL had the highest bacterial DNA load, unexpectedly, it was found that testing of nasal/throat swabs was a simple and as effective specimen to test for PCP at presentation as other types of samples. This is in keeping with a recent study of PCP in

<table>
<thead>
<tr>
<th>Patient no.</th>
<th>Gender</th>
<th>Risk factor</th>
<th>Specimen</th>
<th>Length of illness (days)</th>
<th><em>P. jirovecii</em> load (copies ml⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>F</td>
<td>Chemotherapy*</td>
<td>Sputum</td>
<td>1</td>
<td>1.06 × 10⁴</td>
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<td>Sputum</td>
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<td>2</td>
<td>F</td>
<td>Immunosuppression†</td>
<td>Tracheal secretions</td>
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<td>4.64 × 10⁴</td>
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<td>Tracheal secretions</td>
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<td>1.23 × 10⁵</td>
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<td>11</td>
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<td>4</td>
<td>M</td>
<td>Transplant</td>
<td>Bronchial lavage</td>
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<td>2.75 × 10⁵</td>
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<td></td>
<td></td>
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<tr>
<td>5</td>
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<td>Sputum</td>
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<td></td>
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<td>Tracheal secretions</td>
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<td>0</td>
</tr>
<tr>
<td>6</td>
<td>F</td>
<td>Chemotherapy*</td>
<td>Nasal/throat swab</td>
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<td>1.52 × 10⁴</td>
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<tr>
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<td>7.22 × 10⁴</td>
</tr>
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<td>Nasal/throat swab</td>
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<td>9</td>
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<td>9.30 × 10⁴</td>
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<td>6.11 × 10⁵</td>
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<td>Nasal/throat swab</td>
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<td>1.50 × 10⁵</td>
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<td>16</td>
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<td>Severe combined immunodeficiency</td>
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<td>Tracheal secretions</td>
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<td>1.55 × 10⁴</td>
</tr>
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</table>

*Patients were receiving chemotherapy for a variety of haematological and solid tumour malignancies.
†Patients were receiving immunosuppressive treatment for a variety of autoimmune conditions, including rheumatoid arthritis, idiopathic thrombocytopenia, vasculitis, idiopathic pulmonary fibrosis, myasthenia gravis and systemic lupus erythematosus.
children (Samuel et al., 2011). Since early diagnosis and treatment of PCP is critical, this is an important finding, allowing earlier investigations of patients with suspected PCP. While there was no obvious reason for the increase in cases, it was felt that it was significant enough to highlight to the local medical community. This was done through an advisory letter from the Public Health Agency in Northern Ireland to all clinical units in the region. In addition, a 12 month extended surveillance program was started in August 2011, with all lower respiratory specimens received being tested for *P. jirovecii*. This will be the subject of further review.

Improved radiological recognition of PCP could be one factor for the increased demand for microbiological testing and while useful it cannot replace a microbiological diagnosis (Kameda et al., 2011; Tasaka et al., 2007). Although radiological findings can indicate PCP with a high degree of accuracy, there is an inevitable overlap between clinical and radiological findings in immune-compromised patients presenting with acute interstitial pneumonitis (Assefa et al., 2011). It is therefore essential that appropriate specimens be submitted for a microbiological assessment in at-risk patients.

There was little evidence of asymptomatic carriage of *P. jirovecii*, which was only detected and felt to be not significant in 2/476 (0.4%) patients tested. As only those with suspected PCP were tested, this cohort might have been expected to have a higher colonization rate than average. The findings are at odds with reports of colonization rates of 10–46% in both immune-compromised and immune-competent individuals (Vidal et al., 2006; Wakefield et al., 2003; Maskell et al., 2003; Mori et al., 2009). However, some of these results were based on nested protocols, which often suffer from poor specificity. The findings of Oz & Hughes (2000), however, mirror our observations with no evidence of carriage in immune-competent patients or asymptomatic immune-compromised patients. This is in contrast to patients with PCP where both upper and lower airway specimens had detectable levels of *P. jirovecii*. While the negative results in this review do not exclude carriage, they do support a high specificity for PCP using the qRT-PCR assay. Assay insensitivity could also explain these findings, but the sharp rise in confirmed cases of PCP, including a significant point-source outbreak, would indicate an appropriate clinical sensitivity for the current assay configuration. The confirmation of *P. jirovecii* in 4/126 (3%) patients tested through the bio archive, in two infants and two elderly patients, also supports a high clinical specificity for the assay. The low copy numbers detected and lack of a test request for *P. jirovecii* would suggest that PCP was not likely in these patients and detection in the extremes of age concurs with current evidence. Self-limiting infection in children below 4 months of age, following acute upper respiratory tract infection, has been reported (Larsen et al., 2007) and a carriage rate of 12.5% has been detected in elderly patients (Vargas et al., 2010). While we had evidence of infection and carriage in the extremes of age, we had no evidence for it in the age bands we most commonly tested and confirmed for PCP during the 3 year period.

However, asymptomatic colonization may be putting patients at risk for the later development of PCP. Mori found evidence of carriage in 9/82 (11%) of patients with rheumatoid arthritis on low dose methotrexate, three of whom subsequently developed PCP. Colonization may, therefore, have a pathological potential that might require pre-emptive therapy (Mori et al., 2009). Therapeutically immune-suppressed patients are increasing in all health-care settings and, if colonized, may be at risk of PCP and a source of infection to other at-risk patients. The rise in PCP noted in this study would be in keeping with this scenario and certainly underlines a need to review routes of transmission. The point-source outbreaks in renal transplant settings throughout Europe would specifically suggest iatrogenic exposure and a potential for pathological colonization leading to overt infection. Post-transplant monitoring for cytomegalovirus infection is routine and can detect infection several weeks before clinical presentation; it is likely that patients with PCP may have similar detectable and treatable levels of *P. jirovecii* infection. There is a clear need for research in this area and the scope for pre-emptive monitoring and treatment is one that should be considered.

Targeting two genetic regions in the multiplex qRT-PCR was designed to address potential genetic variability. The assay performed reliably during the period of study and confirmed PCP in 51/476 (10.7%) of patients investigated. Surprisingly, there was no difference in the detection rate of *P. jirovecii* in upper and lower airway specimens from patients with PCP at clinical presentation. While loads were highest in BAL specimens, this did not significantly influence the diagnosis of PCP. Where upper and lower airway samples were co-tested from the same patient, there were no conflicting results, with *P. jirovecii* being detectable
in both sample types. Patients that were started on treatment showed rapid reduction or elimination, and only two patients, both with HIV, showed sustained DNA loads while on treatment (Table 3).

The risk factors for PCP, as shown in Table 2, have changed compared to those seen in the previous 7 years where undiagnosed HIV infection was responsible for 7/10 (70%) cases of PCP. Undiagnosed HIV is still a common risk factor and underlines the need for early recognition of asymptomatic HIV infection, in line with recent calls for more proactive testing for HIV in both primary and secondary care settings (NICE, 2011). However, the majority of patients now appear to be in specific iatrogenic risk groups, including patients on chemotherapy, those receiving immunosuppression as part of treatment for a range of autoimmune conditions, and transplant recipients. In the transplant cohort, only renal transplant recipients showed an increased incidence, which appeared to be caused by a single genotype, based on multi-locus sequence typing. Two paediatric cases were confirmed, one with undiagnosed severe combined immunodeficiency and one with haemophagocytic lymphohistiocytosis.

**Conclusions**

In conclusion there has been an increase in PCP in Northern Ireland over the period July 2008–July 2011, with most new cases in patients under iatrogenic immune suppression with undiagnosed HIV still being a significant factor. At presentation, both upper and lower respiratory samples were found to be of equal use in confirming a microbiological diagnosis. These data suggest a need to consider this condition in at-risk patients presenting with respiratory symptoms as well as an urgent need to reassess modes of acquiring this infection.

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