Molecular epidemiology of *Pneumocystis jiroveci* in human immunodeficiency virus-positive and -negative immunocompromised patients in The Netherlands

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*Pneumocystis jiroveci* infections can cause pneumocystis pneumonia (PCP) or lead to colonization without signs of PCP. Over the years, different genotypes of *P. jiroveci* have been discovered. Genomic typing of *P. jiroveci* in different subpopulations can contribute to unravelling the pathogenesis, transmission and spread of the different genotypes. In this study, we wanted to determine the distribution of *P. jiroveci* genotypes in immunocompetent and immunocompromised patients in The Netherlands and determine the clinical relevance of these detected mutations. A real-time PCR targeting the major surface glycoprotein gene (MSG) was used as a screening test for the presence of *P. jiroveci* DNA. Samples positive for MSG were genotyped based on the internal transcribed spacer (ITS) and dihydropteroate synthase (DHPS) genes. Of the 595 included bronchoalveolar lavage fluid samples, 116 revealed the presence of *P. jiroveci* DNA. A total of 52 of the 116 samples were ITS genotyped and 58 DHPS genotyped. The ITS genotyping revealed 17 ITS types, including two types that have not been described previously. There was no correlation between ITS genotype and underlying disease. All ITS- and DHPS-genotyped samples were found in immunocompromised patients. Of the 58 DHPS-genotyped samples, 50 were found to be WT. The other eight samples revealed a mixed genotype consisting of WT and type 1. The majority of the latter recovered on trimethoprim–sulfamethoxazole suggesting no clinical relevance for this mutation.

**INTRODUCTION**

*Pneumocystis jiroveci* was classified as a protozoan until 1988 when DNA analysis demonstrated it to be a fungus (Edman *et al.*, 1988; Stringer *et al.*, 1989). *Pneumocystis jiroveci* frequently causes opportunistic infections in the lower respiratory tract of immunocompromised patients, especially in human immunodeficiency virus (HIV)-infected patients (Phair *et al.*, 1990), causing pneumocystis pneumonia (PCP). However, *P. jiroveci* can also be present in the respiratory tract without causing clinical symptoms of PCP. The detection of *P. jiroveci* in respiratory samples in the absence of clinical infection has been defined as colonization (Morris *et al.*, 2008). The clinical significance of *Pneumocystis* colonization is as yet unknown; however, the presence of low numbers of *P. jiroveci* in the respiratory tract may lead to the development of PCP (Morris *et al.*, 2008). Colonization can also play a role in transmitting *Pneumocystis* (Morris *et al.*, 2008). Furthermore, it can stimulate the host’s immune response, which leads to pulmonary damage and in this way plays a role in the progression of lung diseases (Morris *et al.*, 2008). As *P. jiroveci* is host specific, the route of transmission is thought to be human to human (Helweg-Larsen *et al.*, 1998; Nevez *et al.*, 2008). The results of recent studies based on observations of clusters of PCP

**Abbreviations:** BALF, bronchoalveolar lavage fluid; DHPS, dihydropteroate synthase; HIV, human immunodeficiency virus; ICU, intensive care unit; ITS, internal transcribed spacer; MGG, May–Grunwald–Giemsa; MSG, major surface glycoprotein gene; PCP, pneumocystis pneumonia; TMP-SMX, trimethoprim–sulfamethoxazole.

One supplementary table is available with the online Supplementary Material.
cases combined with *P. jiroveci* typing support inter-human transmission (Rabodonirina et al., 2004; Höcker et al., 2005; de Boer et al., 2007). Moreover, studies investigating the genotype of *P. jiroveci* in colonized patients have suggested that these patients play a role in the circulation and transmission of this micro-organism (Totet et al., 2004). Reports of outbreaks in hospitals among patients at risk (those with HIV, primary immune deficiencies, long-term immunosuppressive regimens, haematological and non-haematological malignancies, and/or severe malnutrition (Barbounis et al., 2005; Resnick et al., 2005; Hui & Kwok, 2006; Mori et al., 2009; Ritter & Pirofski, 2009) have strengthened this hypothesis (de Boer et al., 2011; Sassi et al., 2012). As the fungus cannot be cultured, *in vitro* experiments concerning transmission and epidemiology are difficult. However, some *in vivo* experimental models have been developed that mimic the transmission and epidemiology of *P. jiroveci* in humans (Dei-Cas et al., 1998; Kling et al., 2009, 2010; Shipley et al., 2010).

Genomic typing of *P. jiroveci* can give more insight into the spread and transmission as well as the virulence of the different genotypes. Different genes [major surface glycoprotein (MSG), internal transcribed spacer 1 (ITS1), ITS2, mitochondrial large subunit rRNA, \(\beta\)-tubulin, 26S RNA and dihydropteroate synthase (DHPS)] have been used previously to identify *P. jiroveci* genotypes (Banerji et al., 1995; Edman et al., 1996; Tsolaki et al., 1996; Lane et al., 1997; Denis et al., 2000; Nahimana et al., 2000; Kutty et al., 2001; Ma et al., 2002b). The genes most frequently used are ITS1 and ITS2 (Lu et al., 1994; Tsolaki et al., 1996; Lee et al., 1998; Helweg-Larsen et al., 2001; Nimri et al., 2002; Nevez et al., 2003; Totet et al., 2003) and DHPS (Lane et al., 1997; Armstrong et al., 2000; Takahashi et al., 2000). Several molecular techniques for the typing of *P. jiroveci* have been described. These are all based on PCR amplification and sequencing or restriction fragment analyses (Beard et al., 2000, 2004; Hauser et al., 2001; Höcker et al., 2005; Gupta et al., 2010). ITS typing is currently the method of choice (Lu et al., 1994; Lee et al., 1998; Tsolaki et al., 1998; Hosoya et al., 2000; Valerio et al., 2007; Esteves et al., 2008; van Hal et al., 2009; Matsumura et al., 2011).

Point mutations at codons 55 and 57 of the DHPS gene change the active site of the enzyme, causing resistance to trimethoprim–sulfamethoxazole (TMP-SMX) (Beard et al., 2000; Huang et al., 2000; Navin et al., 2001). As the drug of choice for treatment of PCP is TMP-SMX, this mutation could have clinical relevance. Evidence indicates that *P. jiroveci* strains are developing resistance to TMP-SMX (Beard et al., 2000; Huang et al., 2000; Navin et al., 2001).

The aim of this study therefore was to determine the distribution of *P. jiroveci* genotypes in immunocompetent and immunocompromised patients in The Netherlands. In addition, the clinical relevance of mutations found in both the ITS and DHPS gene of detected *P. jiroveci* was determined.

**METHODS**

**Patients and specimens.** This retrospective study was conducted at the Maastricht University Medical Centre, a 715-bed hospital in the southern part of The Netherlands. Patients who underwent bronchoalveolar lavage (BAL) between February 1997 and August 2010 were eligible for inclusion. Samples obtained from three different patient groups were included: 1, patients admitted to the general intensive care unit (ICU) with suspected ventilator-associated pneumonia; 2, immunocompromised patients; and 3, outpatients with a suspected pulmonary disease (Table 1). Immunocompromised patients admitted to the ICU were classified as category 2.

**Sampling technique.** Bronchoscopy with subsequent lavage was performed as described previously (Linssen et al., 2008). BAL fluid (BALF) samples were transported to the laboratory within 15 min of collection and were processed immediately upon arrival at the microbiology laboratory.

**Laboratory processing.** The first fraction of BALF representing the bronchial fraction was not used in the present study. The remaining three fractions (alveolar fractions) were pooled. BALF work-up was performed as described previously (Linssen et al., 2008) and included a total cell count, differential cell count and quantitative bacterial culture. From each sample, 6 ml was centrifuged (250 g, 10 min), dividing the sample into cells and supernatant. The supernatant was stored in 1 ml tubes at −80°C. The cells were resuspended in 6 ml of a mixture of Eagle’s minimal essential medium with 2% DMSO and stored in 1 ml tubes at −80°C.

**Microscopy.** Cytocentrifuged preparations (De Brauwer et al., 2000) were prepared and stained with May–Grünewald–Giemsa (MGG) stain and Grocott stain to detect both trophozoite and cyst forms of *P. jiroveci*. A total of six cytospin preparations (three MGG and three Grocott stained) were evaluated by an experienced technician. A sample was regarded positive if even a single *P. jiroveci* trophozoite or cyst was present.

**Nucleic acid extraction.** A total of 200 μl of the stored cell fraction was used for DNA extraction using a Wizard Genomic DNA Purification kit (Promega). The purified DNA was suspended in a final volume of 120 μl nuclease-free water.

**Real-time PCR for the MSG gene.** An in-house real-time PCR targeting the MSG gene was performed (Linssen et al., 2006), with a sensitivity of 100% and a specificity of 81%. Details of the PCR are given in Table S1, available in the online Supplementary Material. The primers and probes used for the PCR are shown in Table 2.

**ITS genotyping.** Genotyping was based on sequence analysis of the ITS1 and ITS2 regions as previously performed (Lu et al., 2000, 2004) and included a total cell count, differential cell count and quantitative bacterial culture. Details of the PCR are given in Table S1, available in the online Supplementary Material. The primers and probes used for the PCR are shown in Table 2.

**ITS genotyping.** Genotyping was based on sequence analysis of the ITS1 and ITS2 regions as previously performed (Lu et al., 1994). Specifics about the PCR are depicted in Table S1. The primers and probes used for the PCR are shown in Table 2.

**DHPS genotyping.** Genotyping was based on RFLP of the DHPS regions described by Montes-Cano et al. (2004). Details of the PCR are shown in Table S1. The primers and probes used for the PCR are shown in Table 2. The purified DNA was digested using the restriction enzymes AccI and HaeIII. For each restriction reaction, 5 μl purified DNA, 12 μl nuclease-free water, 2 μl 10× buffer and 0.5 μl restriction enzyme were incubated at 37°C for 1 h. Detection of restriction fragments was carried out using a 2% agarose gel with a 100 bp marker (Qiagen). Using the restriction enzymes AccI and HaeIII, four DHPS genotypes were possible; WT, type 1, type 2 and
type 3. A sample fully restricted by both enzymes indicated WT, a sample not restricted by AccI but fully restricted by HaeIII indicated type 1, a sample fully restricted by AccI but not restricted by HaeIII indicated type 2 and the indications for type 3 were that the sample would not be restricted by AccI or HaeIII (Montes-Cano et al., 2004; Totet et al., 2004; Tyagi et al., 2008; Jarboui et al., 2011).

### RESULTS

#### General

A total of 595 BAL specimens from 532 patients were included in this study: 115 patients from category 1 (with

<table>
<thead>
<tr>
<th>Target</th>
<th>Primer/probe</th>
<th>Sequence (5′→3′) (name)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MSG*</td>
<td>Forward</td>
<td>CAAAATAACAYTSACATCAACRAGG (PCPFor)</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>AAATCATGAACGAAATAACCATTGC (PCPRev)</td>
</tr>
<tr>
<td></td>
<td>Probe</td>
<td>FAM-TGCAAACCAACCAAGTGTACGACAGG-TAMRA (PCPProbe)</td>
</tr>
<tr>
<td>ITS1 and -2†</td>
<td>Forward 1</td>
<td>AAGTTGATCAAATTTGGTC (1724F)</td>
</tr>
<tr>
<td></td>
<td>Reverse 1</td>
<td>GAACCGGTCGATAGTGCAC (3454R)</td>
</tr>
<tr>
<td></td>
<td>Forward 2</td>
<td>CGTAGGTAACCTGCACAACAAAAGATCG (ITS1F)</td>
</tr>
<tr>
<td></td>
<td>Reverse 2</td>
<td>GTTCACGGCGCTGATCTGCCTG (ITS2R1)</td>
</tr>
<tr>
<td>DHPS‡</td>
<td>Forward</td>
<td>GGGCCTACACATTTATGCCCACCTTTAATC (DHPS-3)</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>GGAACCTTCAACTTGCAACCCAC (DHPS-4)</td>
</tr>
</tbody>
</table>

*The sequences for the MSG gene were obtained from Larsen et al. (2002).
†The sequences for ITS1 and -2 genes were obtained from Lu et al. (1994).
‡The sequences for the DHPS gene were obtained from Montes-Cano et al. (2004).
suspected ventilator-associated pneumonia), 370 patients from category 2 (immunocompromised patients) and 47 patients from category 3 (outpatients). The demographic data are shown in Table 1.

Of the 595 included samples, 116 (from 107 patients, 19.5%) revealed *P. jiroveci* DNA and were subsequently ITS and DHPS genotyped. An ITS genotype could be detected in 52/116 (44.8%) *P. jiroveci*-positive samples, of which 35 could be genotyped for both ITS and DHPS. A DHPS genotype was found in 59/116 (50.8%) *P. jiroveci*-positive samples. One sample showed multiple bands in the ITS PCR. The genotypes of the patients who contributed multiple positive samples did not differ among samples. The remaining 40 samples were negative in both the ITS and the DHPS PCR. In 68/116 (58.6%) PCR-positive samples, *P. jiroveci* cysts or trophozoites were also detected during microscopy.

### ITS genotyping

Using the scoring positions of Lee *et al.* (1998) and Totet *et al.* (2004), 9 ITS1 alleles and 10 ITS2 alleles were found, resulting in a total of 17 *P. jiroveci* ITS types. All samples that could be genotyped were from patients belonging to the immunocompromised category (category 2) and not to category 1 or 3. No correlation was found between the cycle threshold (Ct) value (MSG PCR) of the sample and the possibility or not of it being ITS genotyped. However, all microscopy-positive samples could be detected with the PCR and were all genotyped. The most frequently detected type was Eg (n=14), followed by Ne (n=7) and Bi (n=6) (Table 3).

A correlation was not found between the ITS genotype and the year or month of admittance, or between the genotype and admittance to the ICU or other specific wards. Furthermore, a correlation was also lacking between the ITS genotype and underlying disease. The genotypes were equally distributed between HIV-positive and -negative patients (Table 3). However, of the 52 ITS-genotyped samples, two sets of samples (set 1: patients 1 and 2; set 2: patients 3 and 4) seemed to suggest clustering (Table 4). No link could be found between patients 1 and 2, whilst patients 3 and 4 shared a room for 1 week in the period they experienced respiratory symptoms.

In the present study, one genotype was found that has not been described previously, namely Bj (an HIV-negative patient with a haematological malignancy). Furthermore, one sample showed the presence of genotype Hc (an HIV-negative rheumatological patient), which has been described only once before in a study conducted in Australia (van Hal *et al.*, 2009).

One sample contained multiple bands on the ITS PCR. However, after sequencing the multiple bands, this turned out to be a cross-reaction of the ITS PCR with *Candida albicans*. This cross-reaction also occurred in five other samples in which the ITS genotype could not obtained. This cross-reactivity had no influence on the MSG PCR and therefore none on the detection of *P. jiroveci* with this PCR.

### DHPS genotyping

A total of 59 samples (from 55 patients) could be DHPS genotyped. Of these 59 samples, 51 samples (from 47 patients) showed the DHPS WT. The remaining eight samples (from eight patients) showed the presence of WT *P. jiroveci* combined with type 1. These patients were equally distributed among the patient categories, without signs of clustering (Table 5).

The DHPS genotypes did not change over time, with type 1 ranging from 0 to 25%, depending on the year of collection. All patients in this study were treated with TMP-SMX, the standard therapy for patients with PCP. Of the eight patients, seven responded well to the therapy and the remaining patient died 4 days after the BALF sample was collected.

### DISCUSSION

#### Detection of *P. jiroveci* in BALF samples by PCR and microscopy

Diagnosis of PCP usually relies on microscopic demonstration of *P. jiroveci* in BALF samples by the use of different staining methods. However, in non-HIV-infected patients, a low burden of *P. jiroveci* in BALF samples decreases the sensitivity of the staining techniques. Therefore, molecular techniques based on PCR have been used for the diagnosis and detection of *P. jiroveci* in BALF samples. Compared with conventional staining, PCR techniques have a superior sensitivity for the diagnosis of *P. jiroveci* in BALF specimens (Flori *et al.*, 2004; Alanio *et al.*, 2011; Botterel *et al.*, 2012).

In this study a total of 19.5% (116/595) of BALF samples showed the presence of *P. jiroveci* DNA. Of these positive samples, in 68 samples *P. jiroveci* could also be detected by microscopy. Of the 116 MSG-positive samples, only 52 could be ITS genotyped and only 58 could be DHPS genotyped. A possible explanation is that the MSG gene is a multi-copy target, whilst both the ITS and DHPS regions are single-copy targets. Therefore, PCR for the MSG gene can detect very small amounts of DNA, whilst both the ITS and the DHPS PCR may miss these low numbers of DNA. However, one would consequently expect a correlation between the Ct value of the MSG gene and whether or not a genotype could be determined. A low Ct value corresponds to a high load of *P. jiroveci* in the sample, which should lead to the detection of a genotype. A high Ct value corresponds to a low load of *P. jiroveci* in the sample and would therefore lead to an indeterminate result. In the present study, such a correlation was not found. Therefore, it is hypothesized that the *P. jiroveci* load is not the only explanation for whether or not a sample can be genotyped.
Interestingly, all samples that resulted in an ITS genotype were from patients belonging to the immunocompromised category and all were diagnosed with clinical PCP. Furthermore, all microscopy-positive samples could be typed. Based on these findings, it is hypothesized that the presence of viable cysts and trophozoids results in a qualitatively better sample for DNA isolation compared with the microscopy-negative samples. An additional explanation for this observation is that the microscopy-negative samples were derived from patients already being treated for PCP, resulting in destroyed cysts and trophozoids, including damage to the DNA of *P. jiroveci*.

### ITS genotyping

Previous studies have shown that many *P. jiroveci* genotypes exist. To date, a total of 105 genotypes have been reported (Lee *et al.*, 1998; Nevez *et al.*, 2003; Totet *et al.*, 2003, 2004; Beser *et al.*, 2007; Valerio *et al.*, 2007; Gupta *et al.*, 2010). In the present study, 17 ITS subtypes were found. The most frequently detected subtype was Eg (29%). This is in line with previous studies conducted where this subtype was found in up to 50 % of cases (Nevez *et al.*, 2003; Beser *et al.*, 2007; Gupta *et al.*, 2010).

The majority of studies have been performed in HIV-positive patients (Tsolaki *et al.*, 1999; Beard *et al.*, 2000; Hauser *et al.*, 2001; Visconti *et al.*, 2001; Ma *et al.*, 2002a; Nahimana *et al.*, 2003; Rabodonirina *et al.*, 2004; Valerio *et al.*, 2007; Esteves *et al.*, 2008). One could speculate that HIV-negative patients are more frequently infected with different ITS subtypes compared with HIV-positive patients. However, in a recent study conducted in Iran, no difference was shown in the most prevalent subtype in patients either infected with HIV, or with an underlying malignancy or with an acute exacerbation of chronic obstructive pulmonary disease (Sheikholeslami *et al.*, 2013). A recent French study (Le Gal *et al.*, 2013) showed a high percentage of Ec and Ai (8.9 % each) in HIV-negative patients either colonized with *P. jiroveci* or suffering from PCP. In our study, both HIV-positive and HIV-negative patients were included. Only two patients with genotype Ec and one patient with genotype Ai were detected. Of these three patients, two were HIV positive. The fact that the French study shows a different genotype in a large portion of

### Table 3. ITS genotypes of *P. jiroveci* distributed over the different patient categories

<table>
<thead>
<tr>
<th>Genotype</th>
<th>HIV positive</th>
<th>HIV negative</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Haematological malignancy</td>
<td>Rheumatological disease</td>
</tr>
<tr>
<td>Ai</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Be</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>Bi</td>
<td>4</td>
<td>1</td>
</tr>
<tr>
<td>Bj</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Eb</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Ec</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Ee</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Eg</td>
<td>7</td>
<td>4</td>
</tr>
<tr>
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<td>1</td>
</tr>
<tr>
<td>Ej</td>
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<td>1</td>
</tr>
<tr>
<td>Em</td>
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<td>1</td>
</tr>
<tr>
<td>Gg</td>
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<tr>
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<tr>
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<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Je</td>
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<td>1</td>
</tr>
<tr>
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<td>1</td>
</tr>
<tr>
<td>Ne</td>
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<td>2</td>
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</table>

### Table 4. Clustering of ITS-genotyped samples

<table>
<thead>
<tr>
<th>Cluster</th>
<th>Patient</th>
<th>Gender</th>
<th>Age</th>
<th>Underlying disease</th>
<th>Sample collected</th>
<th>ITS genotype</th>
<th>C&lt;sub&gt;i&lt;/sub&gt; value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cluster 1</td>
<td>1</td>
<td>M</td>
<td>72</td>
<td>M. Wegener</td>
<td>09-05-2001</td>
<td>Eg</td>
<td>30</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>M</td>
<td>33</td>
<td>Non-Hodgkin’s lymphoma</td>
<td>18-05-2001</td>
<td>Eg</td>
<td>33</td>
</tr>
<tr>
<td>Cluster 2</td>
<td>3</td>
<td>M</td>
<td>28</td>
<td>Acute myeloid lymphoma</td>
<td>31-05-2004</td>
<td>Eg</td>
<td>34</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>M</td>
<td>68</td>
<td>Brain tumour</td>
<td>18-06-2004</td>
<td>Eg</td>
<td>29</td>
</tr>
</tbody>
</table>
patients compared with the rest of Europe may point towards a local distribution pattern of a certain genotype. All the samples collected in the present study were derived from patients residing in the south of The Netherlands (approx. 2100 km²) in an area next to the border with Belgium and Germany. Therefore, the genotypes here may be comparable to those found in the adjacent countries. To our knowledge, no large studies have previously investigated *P. jiroveci* subtypes in The Netherlands or neighbouring countries. However, genotyping yielded comparable results in other northern European countries such as the UK and Denmark (Lee et al., 1998; Miller & Wakefield, 1999; Helweg-Larsen et al., 2001). The most commonly found genotypes in this study were equally distributed between HIV-negative and HIV-positive patients and no correlation was found between infection or colonization with a *P. jiroveci* genotype. In our study, one previously undescribed subtype was found, ITS type Bj. None of the ITS typed samples suggested mixed infection, in contrast to a large percentage of mixed infections found in other studies (Matsumura et al., 2011; Le Gal et al., 2013; Rabodonirina et al., 2013; Sheikholeslami et al., 2013).

### ITS clustering

In four patients, there was an indication of clustering based on ITS genotypes; however, in only two patients could a connection be assessed as they shared a room for 1 week. Previous studies have shown that *P. jiroveci* isolates are genetically identical within hospital outbreaks, although rare (de Boer et al., 2011; Sassi et al., 2012). However, as the infection was caused by genotype Eg, the most commonly found genotype in the present study, the presence in both patients 3 and 4 in this study could be just a coincidence, suggesting that the infections may be community acquired.

### DHPS genotyping

Of the 58 DHPS-genotyped samples (Montes-Cano et al., 2004), 86.2% showed the presence of the WT, whilst the remaining 13.8% samples suggested mixed infection of the WT and type 1. The genotypes do not change over time. Mutation in the DHPS gene can result in TMP-SMX resistance. Therefore, we believe that an increase in DHPS type 1 can be the result of an increase in TMP-SMX exposure. If the exposure of *P. jiroveci* to TMP-SMX does not increase over the years, the genotypes will not change over time. As point mutations in the DHPS gene can result in resistance to TMP-SMX, this mutation may lead to clinical failure in the patients treated with TMP-SMX. All eight patients with mixed infections with both WT and type 1 were treated with TMP-SMX. Seven recovered and were discharged from hospital. The remaining patient died within 4 days of being diagnosed with clinical PCP. Unfortunately, no post-mortem material was obtained. Therefore, it cannot be concluded with certainty that the patient had died from a PCP infection.

### Clinical recovery

There are several possible reasons why the patients infected with type 1 *P. jiroveci* nevertheless recovered from their infection. First, it may be that only a small percentage of the *P. jiroveci* organisms present had the mutation and therefore the majority of cysts and trophozoites (without mutation) were killed by TMP-SMX treatment, resulting in clinical recovery. However, it may also indicate that the type 1 mutation has only limited clinical relevance. Previous studies have reported contradictory results, with a negative influence of DHPS mutations on prognosis in some (Helweg-Larsen et al., 1999; Takahashi et al., 2000; van Hal et al., 2009), but no influence in the majority of studies (Navin et al., 2001; Ma et al., 2002a; Alvarez-Martinez et al., 2008). A study conducted by Rabodonirina et al. (2004) evaluated DHPS mutations in *P. jiroveci* in HIV-positive patients. The majority of patients harboured WT *P. jiroveci*. However, in 33% of these cases, patients were infected with a *P. jiroveci* strain with a mutated DHPS gene. A multivariate analysis showed that patients on mechanical ventilation had an increased risk of death associated with PCP. They found no association between mutations in the DHPS gene and outcome in the patient group. Few studies have been conducted in HIV-negative patients. A study by Matsumura et al. (2011) investigated HIV-negative patients diagnosed with PCP. They showed that the general condition and the respiratory conditions of the patients at diagnosis were independent predictors of mortality. As previous studies have focused mainly on HIV-positive patients, additional studies are needed to show the extent of the spread of DHPS mutations among *P. jiroveci* strains and the associated clinical relevance with regard to clinical failure when patients are treated with TMP-SMX, especially in HIV-negative patients.

### CONCLUSION

No correlation was found between ITS genotype and underlying disease, and the genotypes were equally distributed between immunocompromised and immunocompetent patients. In eight patients, a *P. jiroveci* strain with a type 1 mutated DHPS gene was present. However, the majority of these patients still recovered on TMP-SMX, suggesting that this mutation may not have clinical relevance.

<table>
<thead>
<tr>
<th>Patient category</th>
<th>WT</th>
<th>WT + type 1</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>2</td>
<td>46</td>
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</tr>
<tr>
<td>3</td>
<td>3</td>
<td>1</td>
</tr>
</tbody>
</table>

Table 5. Number of DHPS genotypes of *P. jiroveci* distributed over the different patient categories
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