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Published in:
Clinical Infectious Diseases

DOI:
10.1086/513198

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Document Version
Publisher's PDF, also known as Version of record

Publication date:
2007

Link to publication in University of Groningen/UMCG research database

Citation for published version (APA):

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An Outbreak of *Pneumocystis jiroveci* Pneumonia with 1 Predominant Genotype among Renal Transplant Recipients: Interhuman Transmission or a Common Environmental Source?

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(See the editorial commentary by Hughes on pages 1150–1)

**Background.** An outbreak of *Pneumocystis jiroveci* pneumonia (PCP) occurred among renal transplant recipients attending the outpatient department at the Leiden University Medical Centre (Leiden, The Netherlands) from 1 March 2005 through 1 February 2006. Clinical, epidemiological, and molecular data were analyzed to trace the outbreak’s origin.

**Methods.** Renal transplant recipients with a clinical suspected diagnosis of PCP were included in the study. The diagnosis had to be confirmed by direct microscopy or real-time polymerase chain reaction of the dihydropteroate synthase gene in a bronchoalveolar fluid specimen. To detect contacts between patients, a transmission map was constructed. A case-control analysis was performed to assess whether infection was associated with certain wardrooms. Genotyping of *Pneumocystis* isolates was performed by sequence analysis of the internal transcribed spacer (ITS) number 1 and 2 gene regions.

**Results.** Twenty-two confirmed PCP cases were identified; approximately 0–1 would have been expected over the same time period. No risk factor was predominantly present, and standard immunosuppressive regimens had not changed. Liver transplant recipients who used the same outpatient facilities had not acquired PCP. The transmission map findings were compatible with interhuman transmission on multiple occasions. The case-control study did not point to wardrooms as a common source. Genotyping by sequencing of the ITS1 and ITS2 gene regions revealed type Ne in 12 of 16 successfully typed samples. Genotype Ne was found in only 2 of 12 reference samples.

**Conclusions.** The clinical data and genotyping results are compatible with either interhuman transmission or an environmental source of infection. More complex models may account for PCP clusters.

*Pneumocystis* pneumonia caused by *Pneumocystis jiroveci* (PCP) remains a substantial cause of morbidity and mortality in immunocompromised individuals [1]. The development of animal models and genotyping methods has contributed to an increased understanding of the complex behavior of this opportunistic pathogen [2, 3]. However, the exact modes of transmission and acquisition of this saprophytic infection are still unclear. Different sources of infection (e.g., the environment or asymptomatic carriers) have been proposed [4, 5]. Recently, the possible role of interhuman transmission between immunocompromised patients was reported [6–8]. In this article, we report an outbreak of PCP in a population of renal transplant recipients attending the outpatient posttransplantation department of the Leiden University Medical Centre (LUMC; Leiden, The Netherlands) during the period from 1 March 2005 through 1 February 2006. PCP was diagnosed in 22 renal transplant recipients (figure 1). In our transplant
program, ~100 patients receive a kidney or kidney-pancreas transplant each year; specialized care is provided for ~1000 renal transplant recipients. In this population, the expected incidence of PCP is 0–1 cases per year, as estimated from registration data from the Departments of Microbiology and the Department of Infectious Diseases from 1995 onwards. Because of the sudden increase in the incidence and because of possible contact between patients when visiting the nephrology outpatient department, either interhuman transmission or a local environmental source was suspected. The clinical, epidemiological, and molecular characteristics of this outbreak were analyzed in 5 separate investigations (a descriptive epidemiology study, statistical analysis of outpatient contacts, a case-control study, air sampling, and genotyping of Pneumocystis strains) to elucidate its origins. We discuss the results and 2 currently proposed models of transmission of P. jiroveci.

METHODS

Patient data. The study included all renal transplant recipients who presented with dyspnea and interstitial pneumonia for which the diagnosis of PCP was considered. The time window of the study ranged from 1 March 2005 through 1 February 2006. After the beginning of the outbreak of PCP, nephrologists and micro­biologists in hospitals that participated in our transplantation program were requested to report patients who had undergone renal transplantation and who had interstitial pneumon­ia. The diagnosis of PCP was regarded confirmed if P. jiroveci was detected by direct microscopy (silver and Giemsa staining) or real-time PCR of the dihydropteroate synthase (DHPS) gene in a bronchoalveolar lavage (BAL) fluid specimen [9]. Data on underlying disease, immunosuppressive medications, use of PCP prophylaxis, dates of hospital visits, and demographic data were obtained from the files. The clinical presentation of PCP was briefly recorded. A transmission map was constructed to detect contacts between patients during admittances to the nephrology unit and visits to the nephrology outpatient department. Two nephrologists (A.G. and S.P.B.) verified that there had been no changes in immunosuppressive regimens. PCP prophylaxis was not prescribed routinely.

Statistical analysis of outpatient department contacts.
A separate analysis was performed on the transmission map data to assess whether a patient who had received the diagnosis of PCP on a particular day had more often visited the outpatient department during the 4 months preceding the diagnosis, compared with patients who would become diseased later. We also determined whether a patient who had received a diagnosis of PCP on a particular day had more frequently encountered other future patients (i.e., potentially contagious patients) in the outpatient department in comparison to patients who would only become diseased later. These analyses were performed with a Cox model wherein the time varying exposure were the number of visits and the number of potentially infected patients with whom a patient had contact before the onset of disease.

Case-control study for inpatient rooms in the nephrology unit. Because the majority of the patients had been hospitalized before the PCP outbreak, we investigated the possibility of transmission via a common source located in (or near) rooms in the nephrology unit by means of a case-control analysis. Case patients were defined as renal transplant recipients with confirmed PCP in 2005 who had stayed in the nephrology unit earlier in 2005 (i.e., before the diagnosis of PCP). The control group consisted of renal transplant recipients admitted to the unit in the same time window but who did not later receive a diagnosis of PCP. Data were obtained from the hospital’s administrative department. ORs and 95% CIs were calculated for all rooms.

Air sampling. Air sampling was performed to detect Pneumocystis species in rooms of the nephrology unit and in the waiting room of the outpatient department. Because this sampling expertise was not available in our institution, the collection of air samples and the procedure of extracting DNA from the filters were performed by a company specialized in measuring microbiological air quality (Intersave Groeneveld). The following locations were sampled: a wardroom of the nephrology unit, the nurse post of the nephrology unit, and the outpatient department waiting room (twice). Samples were also obtained from a room in the hospital that was never used for patient care and a room that was used by a patient with PCP (a supposed negative and positive control room). Samples were obtained from the outpatient department overnight, when no patients were present. Air sampling was performed by use of Gilair air sampler pumps (Sensidyne), creating an airflow over a glass fiber filter with a velocity of 2 L per minute for ~8 h.
at each location. After filtration of ~1000 L of air, the filters were removed, and DNA was extracted (Chemagic DNA extraction kit; Chemagen). Specimens were transported to the laboratory of the microbiology department of the LUMC for analysis by real-time PCR (DHPS gene). Further investigations included the analysis of multilayered filters of the ventilation system of the outpatient department. Samples were obtained from 2 filters passed by inflowing air and 1 outflow filter by cutting a 10-cm² piece of each filter, washing it with 500 mL of MilliQ, and subjecting it to centrifugation. Both supernatant and residue were subjected to real-time PCR (DHPS gene).

**Genotyping of Pneumocystis strains.** Genotyping of *P. jiroveci* was performed by sequence analysis of the internal transcribed spacer (ITS) numbers 1 and 2 of the nuclear rRNA operon. Reference data reflecting the distribution of *P. jiroveci* genotypes in this region was obtained by genotyping 11 samples obtained from patients with PCP admitted to the LUMC during the period from January 2003 through January 2005 and 3 samples containing *P. jiroveci* from other Dutch hospitals (all not related to this outbreak).

The forward primer (ITS1F) was described previously by Lu et al. [10, 11]. The reverse primer (ITS2R1) from Lu et al. [10, 11] was shortened and used with the sequence 5′-GCGGGTGA-TCTTGCCCT-3′ to lower the melting temperature. The formed PCR product consists of the ITS1, 5.8S gene, and the ITS2 gene region and has a total length of ~540 bp. DNA was extracted from BAL samples using the total nucleic acid protocol with the MagNA pureLC nucleic acid isolation system (Roche Diagnostics). Each sample was eluted in 100 µL of buffer and stored at −80°C until processing. Five µL of DNA-extract was added to 45 µL of reaction mix containing 25 µL of 2X Hotstar mastermix (Qiagen) and 25 pmol of each primer. Cycling conditions were as follows: 15 min at 95°C; 50 cycles of 30 s at 92°C, 30 s at 62°C, and 30 s at 72°C; followed by a 5-min hold at 72°C. The PCR product was analyzed with agarose gel electrophoresis. In case of aspecific amplification, the correct product was cut out and purified using the Qiaquick gel-extraction kit (Qiagen). Sequencing was performed on an ABI3100 automatic sequencer (Applied Biosystems) using a sequencing ready reaction kit (ABI). Sequence types were designated according to the method of Lee et al. [12].

**RESULTS**

**Patient characteristics and outcome.** Twenty-six patients presenting with symptoms and radiological signs compatible with PCP were identified. The diagnosis of PCP was confirmed in 22 cases (by microscopy and positive PCR results for 15, by microscopy alone for 1, and by PCR alone for 6). Twelve patients (55%) were male. The patients’ ages ranged from 36 to 72 years (median age, 57 years). No geographic clustering according to postal code was noted. All patients (including the 6 patients reported from other hospitals) but 1 had visited the nephrology outpatient department of the LUMC. The cause of original renal disease was heterogeneous; 3 of 22 patients had received a kidney-pancreas transplant, and 11 patients had received their graft within 1 year prior to the diagnosis of PCP. Immunosuppressive regimens contained mofetyl mycofenolate and prednisone (7.5–20 mg once daily) for all patients but 1. Ten patients also used cyclosporine. No changes in routine immunosuppressive regimens had been implemented in the previous 5 years. Although aware of the recommendation of the European guidelines [13], it was the nephrology department’s policy—prior to this outbreak—not to prescribe trimethoprim-sulfamethoxazole prophylaxis; because of the very low incidence of PCP thus far, the benefits were not considered to outweigh the adverse effects.

Cytomegalovirus replication was present in 10 of 19 patients with known cytomegalovirus infection status. Only 1 of these patients received antiviral medication at the time of diagnosis.

One patient became critically ill and died of pulmonary and cardiac failure. None of the other patients were transferred to an intensive care unit.

**Transmission map.** The transmission map (figure 2) revealed that interhuman transmission of *Pneumocystis* might have been possible on multiple occasions during outpatient department visits. The map does not allow to define a moment that all patients were in contact. However, if time windows are taken into account, multiple possibilities of transmission exist. When each case is regarded as a possible index case, a combination of patients 3 and 9 suffices to trace potential contacts with all but 1 patient with type Ne (the predominant genotype). Both patients had received multiple treatments for rejection and had higher *Pneumocystis* loads in BAL fluid specimens (microscopy 3+ [i.e., >10 microorganisms/field at magnification ×400]; cycle time values, 34.4 and 27.5), compared with other patients.

**Statistical analysis of outpatient department contacts.** An analysis of the frequency of visits to the outpatient department and of encounters with other future patients over the 4 months preceding the PCP diagnosis was performed on the transmission chart data (i.e., on cases only). Frequency of visits was more strongly associated with disease development than were encounters with other patients who developed PCP at a later time (i.e., potentially contagious patients).

**Case-control analysis.** There were several time windows in which ≥2 patients from this cluster had been admitted to the nephrology unit at the same time before they had developed PCP. In the case-control study of inpatient rooms, a total of 24 and 257 hospitalizations (affecting 10 case patients and 139 control patients) were analyzed (data not shown). The ORs for
individuals rooms and for combinations of rooms varied from 0.75 to 1.89, with 95% CIs including 1.00.

**Air sampling.** This part of the study was conducted in January and February 2006. The mean total amount of filtered air at each location was 1000 L (range, 861–1216 L). In none of the 6 samples derived from the pump filters were *Pneumocystis* organisms detected by real-time PCR. The supposedly positive control room was found to yield negative results. The negative results were not associated with the inhibition of PCR samples, because the internal controls (phocine herpes virus) yielded positive results. A specimen derived from one of the inlet filters in a ventilation shaft tested positive for *P. jiroveci*. Subsequent ITS genotyping failed to yield a definitive result, probably because of sequence homology with ITS eukaryotic plant material. The outlet filters tested negative.

**Genotyping of Pneumocystis strains.** Identification of *Pneumocystis* strains by genotyping of the ITS1 and ITS2 gene regions was accomplished for 16 of 22 available BAL samples, which were obtained from 22 different patients. Sequence analysis revealed type Ne in 12 of 16 successfully analyzed samples; type Bi was present in 1 sample. In 3 samples, only the ITS2 genotypes could be determined (type e once and type g twice). Genotyping failed for 6 samples because of a weak signal or the presence of >2 strains. Interestingly, of the 12 successfully genotyped reference samples (i.e., those that were unrelated to the present outbreak), only 2 (17%) were determined to be type Ne.

**DISCUSSION**

Unique aspects of this outbreak of PCP are the relatively large number of cases, the high probability of contact between cases, and the observation of one predominant *P. jiroveci* genotype. From the first of December 2005—the tenth month of the outbreak—trimethoprim-sulfamethoxazole or an alternative form of prophylaxis was prescribed for patients within the first year after transplantation, as well as for patients treated for graft rejection. Despite increased alertness among physicians, only 1 new case of PCP has been reported since February 2006. We found no indication that the incidence of PCP had increased in association with changes in immunosuppressive therapy. The risk of PCP is associated with the treatment for rejection and cytomegalovirus reactivation [14, 15], but none of these risk factors was predominantly present or changed over time and
Table 1. Studies of recent clinical clusters of *Pneumocystis jiroveci* pneumonia (PCP) in which genotyping was performed.

<table>
<thead>
<tr>
<th>Reference</th>
<th>Year of publication</th>
<th>No. of patients</th>
<th>Clinical background</th>
<th>Genotyping method</th>
<th>Authors’ conclusions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Helweg-Larsen et al. [18]</td>
<td>1998</td>
<td>14 (in clusters of 8, 3, and 3)</td>
<td>8 Clustered cases among patients with a hematological malignancy and PCP, plus 2 small clusters among HIV-infected individuals</td>
<td>DNA sequence analysis of the ITS1 and ITS2 gene regions</td>
<td>Interhuman transmission may have occurred but did not constitute the major route of transmission</td>
</tr>
<tr>
<td>Olsson et al. [29]</td>
<td>2001</td>
<td>17 (in clusters of 3, 7, and 7)</td>
<td>2 Clusters among renal transplant recipients and 1 cluster in patients with a hematological malignancy</td>
<td>DNA sequence analysis of the mtLSU-rRNA locus</td>
<td>Interhuman transmission unlikely</td>
</tr>
<tr>
<td>Rabodonirina et al. [8]</td>
<td>2004</td>
<td>10</td>
<td>A cluster among renal transplant recipients who encountered HIV-infected patients during hospitalization</td>
<td>Multitarget SSCP method of 4 genomic regions (ITS1, 26S, mt26, and β-tubulin)</td>
<td>Possible nosocomial, interhuman transmission</td>
</tr>
<tr>
<td>Hocker et al. [27]</td>
<td>2005</td>
<td>6 (in clusters of 3 and 3)</td>
<td>Sudden increase in the incidence of PCP in a pediatric transplant unit; 3 cases were found to be related to 1 index case on the basis of clinical and molecular findings</td>
<td>Multitarget SSCP method of 4 genomic regions (ITS1, 26S, mt26, and β-tubulin)</td>
<td>Possible nosocomial, interhuman transmission</td>
</tr>
</tbody>
</table>

**NOTE.** β-Tubulin, region surrounding intron 6 of the β-tubulin gene; ITS1, internal transcribed spacer 1; mtLSU-rRNA, mitochondrial large subunit rRNA locus; mt26, variable region of the mitochondrial 26S rRNA gene; SSCP, single-strand confirmation polymorphism; 26S, intron of the nuclear 26S rRNA gene.
thus were unlikely to have played a major role. We discuss the results of this outbreak investigation along with 2 hypothetical models of transmission of \textit{P. jiroveci}.

**The environmental source hypothesis.** This thesis is based on the assumption that inhaled forms of \textit{P. jiroveci} that cause PCP directly originate from a niche in the environment. No environmental source has been discovered to harbor \textit{P. jiroveci} in previous outbreaks of infection. Air sampling studies indicated that the route of transmission is by air \cite{16, 17}. However, the source from which \textit{P. jiroveci} becomes airborne remains to be specified; it is either environmental or human. Attempts to isolate \textit{Pneumocystis} organisms by air sampling were unsuccessful in this study. The sensitivity and specificity of the methods used are unknown. The positive PCR results for one of the inlet filters is compatible with \(\geq 1\) hypothesis about transmission, but it suggests an environmental source.

The epidemiological data neither exclude nor suggest the presence of an environmental source. The communal presence of patients in the outpatient department can indicate that they acquired PCP through interhuman transmission just as easily as it can indicate that they were infected by a local environmental source. No geographic clustering by postal code was noted, making one or multiple regional environmental source(s) outside the hospital less likely.

The statistical approach—the analysis of outpatient visit frequency and frequency of encounters of other future patients in the outpatient department—showed the strongest association with the number of times that a patient visited the outpatient department. This points to an environmental source. However, the almost constant presence of several future patients and the unknown incubation times make it uncertain whether these calculations can reliably discriminate between interhuman transmission and an environmental source.

Genotyping showed that 12 genotyped strains (75\%) were \textit{P. jiroveci} type Ne. Analysis of the reference strains indicates that type Ne is less common in this region. However, in Denmark, type Ne was the second most prevalent strain in a study of randomly selected \textit{P. jiroveci}–positive BAL samples, but it was not found in a cluster reported from that country \cite{12, 18}. Data from Thailand and England confirm a region-dependent distribution of \textit{P. jiroveci} ITS types \cite{19, 20}. Because the frequency of different ITS types of \textit{P. jiroveci} in the Leiden region is unknown, definite interpretation of the genotyping results is not possible. Genotype Ne has not been reported to be a more virulent type in human disease \cite{21}.

**The interhuman transmission hypothesis.** In this model, PCP is considered to be a transmittable disease (i.e., the source is another infected (or colonized) individual) \cite{22}. Accumulating evidence from animal models, the host specificity of \textit{P. jiroveci}, and the phenomenon of carriage of \textit{P. jiroveci} in the respiratory tract of healthy and immunocompromised individuals all support the idea that humans may constitute the reservoir themselves \cite{3, 23–26}. Transmission would then occur by spreading and subsequent inhalation of air or aerosols containing infectious forms of \textit{P. jiroveci}.

Four previous studies attempted to elucidate the possible role of interhuman transmission in small clinical clusters of PCP by genotyping (table 1). The methods used and the clinical evidence of contact between patients differ between these studies. In the 2 more recent investigations, genotyping and clinical data suggest the possibility of interhuman transmission \cite{8, 27}.

The transmission map from our study shows that contact between cases was possible on multiple occasions in this outbreak. Although details of interactions between patients could not be reconstructed, it accurately describes the presence of \(\geq 1\) case within a limited waiting area (16 m\(^2\)) within a limited time period. In view of the fact that the actual mechanism of transmission of \textit{P. jiroveci} is unknown, the thesis that 1 or 2 “hyperspreaders” (patients 3 and 9) caused this outbreak remains speculative.

Interestingly, the outbreak of PCP was restricted to renal transplant recipients. No cases of PCP were found in the population of 200 liver transplant recipients in our hospital, despite the fact that these patients wait in the same waiting room as the renal transplant recipients. The number of hospital visits in both populations is proportional, but visiting hours overlap just 1 morning per week. Because the liver transplant recipients use comparable immunosuppressive drugs and do not receive PCP prophylaxis, a proportional incidence of up to 4 cases of PCP would be expected in the event of an environmental source in the outpatient department \cite{28}.

The interpretation of the genotyping results—potentially supportive for both hypotheses—is discussed in the previous paragraph.

**Interpretation and conclusions.** In this outbreak of PCP, the evidence of clinical clustering and the presence of a single predominant genotype are compatible with either an environmental source or interhuman transmission. From published data, one can not assess whether clustering of PCP usually starts with index patients, comes from a temporary increased reservoir of carriers in the general population, or comes from an environmental source. Therefore, the need for further elucidation of the general mode of transmission of \textit{P. jiroveci} seems evident. This requires sophisticated methods to investigate possible environmental sources and clinical research aimed at understanding the role of carriers of \textit{P. jiroveci}. More complex transmission models may account for clusters of PCP. Progress in understanding the source of PCP may permit us to undertake effective action to prevent and control future outbreaks.

**Acknowledgments**

We thank Dr. Kate Templeton (Royal Infirmary Hospital; Edinburgh, United Kingdom) for her contribution to ITS typing of \textit{P. jiroveci}.
Potential conflicts of interest. All authors: no conflicts.

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