Risk factors and new markers of pulmonary fungal infection

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Chapter 2

AN OUTBREAK OF *PNEUMOCYSTIS JIROVECII* PNEUMONIA WITH ONE PREDOMINANT GENOTYPE IN RENAL TRANSPLANT RECIPIENTS: INTERHUMAN TRANSMISSION OR A COMMON ENVIRONMENTAL SOURCE?

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Abstract

Background: An outbreak of *Pneumocystis* pneumonia (PCP) in renal transplant recipients attending the outpatient department occurred in the Leiden University Medical Centre between the first of March 2005 and the first of February 2006; clinical, epidemiological and molecular characteristics were analysed to trace its origin.

Methods: Renal transplant recipients with a clinical suspected diagnosis of PCP were included. The diagnosis had to be confirmed by direct microscopy or real time PCR of the dihydropteroate synthase (DHPS) gene in broncho-alveolar fluid. To detect contacts between patients a transmission map was constructed. A case-control analysis was performed to asses whether infection was associated with certain wardrooms. Genotyping of *Pneumocystis* was performed by sequence analysis of the internal transcribed spacer number 1 (ITS1) and ITS2 gene regions.

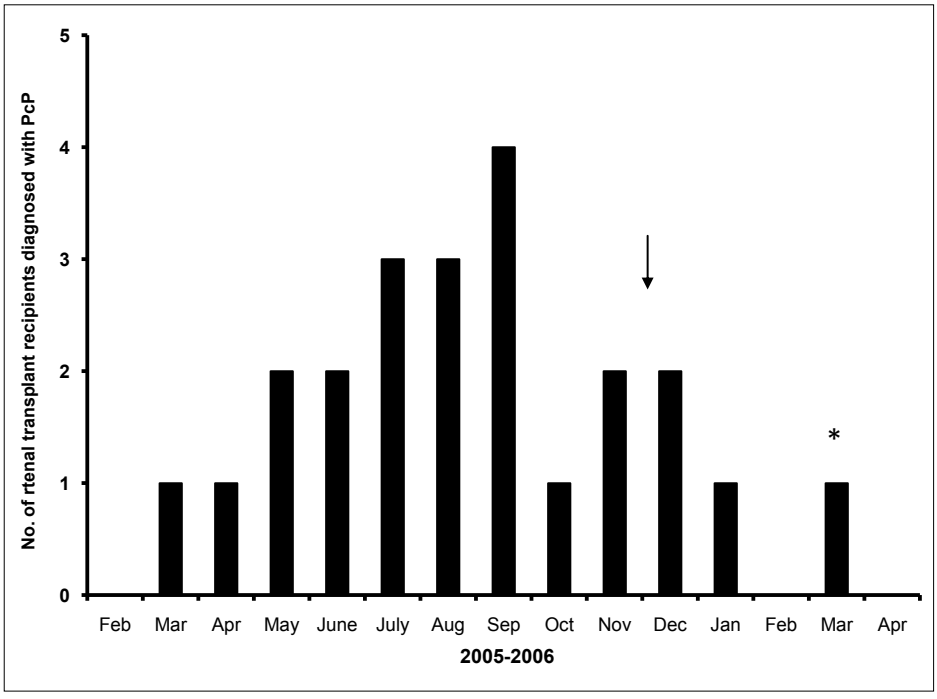
Results: 22 confirmed PCP cases were identified; about 0 to 1 would have been expected over the same time period. No risk factor was predominantly present and standard immune-suppressive regimens had not changed. Liver transplant recipients using the same outpatient facilities had not acquired PCP. The transmission map was 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 showed type 'Ne' in 12 out of 16 successfully typed samples. Genotype 'Ne' was found in only 2 out of 12 reference samples.

Conclusions: The clinical data and genotyping are compatible with either interhuman transmission or an environmental source; more complex models may account for PCP clusters.

Introduction

Pneumocystis pneumonia (PCP), caused by *Pneumocystis jirovecii*, remains a substantial cause of morbidity and mortality in immuno-compromised individuals [1]. The development of animal models and genotyping methods has contributed to an increased understanding of the complex behaviour of this opportunistic pathogen [2, 3]. However, the exact mode of transmission and acquisition of this saprophytic infection are still unclear. Different sources of infection have been proposed, e.g. the environment or asymptomatic carriers [4, 5]. Recently, the possible role of interhuman transmission between immuno-compromised patients was described [6-8]. In this article we report an outbreak of PCP in a population of renal transplant recipients attending the outpatient post-transplantation department of the the Leiden University Medical Center (LUMC) between the first of March 2005 and the first of 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 about one thousand renal transplant recipients. In this population the expected incidence of PCP is 0 to 1 case per year as estimated from registration data from the departments of microbiology and infectious diseases from 1995 onwards. Because of the sudden rise in incidence and possible contact between patients when visiting the nephrol-

Figure 1. Number of renal transplant recipients with confirmed PCP in 2005-2006 per month; * marks the last case, reported after February 1st 2006. The arrow indicates the start of antibiotic prophylaxis for PCP.



ogy outpatient department, either interhuman transmission or a local environmental source was suspected. The clinical, epidemiological and molecular characteristics of this outbreak were analysed by conducting five separate investigations (descriptive epidemiology, statistical analysis of outpatient contacts, a case-control study, air sampling and genotyping of *Pneumocystis* strains) to elucidate its origins. We discuss the results along with two currently proposed models of transmission of *P. jirovecii*.

Methods

Patient data

All renal transplant recipients presenting with dyspnoea and interstitial pneumonia in which the diagnosis of PCP was considered, were included. The time window of the study ranged between the first of March 2005 and the first of February 2006. After the beginning of the outbreak, nephrologists and microbiologists in hospitals participating in our transplant program were requested to report patients with a renal transplant and interstitial pneumonia. The diagnosis of PCP was regarded confirmed if *P. jirovecii* was detected by direct microscopy (Silver- and Giemsa staining) or real time PCR of the dihydropteroate synthase (DHPS) gene in bronchoalveolar lavage (BAL) fluid [9]. Data about underlying disease, immune suppressive medication, use of PCP prophylaxis, dates of hospital visits and demographical data were obtained from the files. The clinical presentation of PCP was briefly described. 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 immune-suppressive 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 in the four months preceding the diagnosis in comparison with patients who would become diseased later. Also it was assessed whether a patient that was diagnosed with 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 (Nephrology Unit)

Because the majority of the patients had been hospitalised before the PCP outbreak, we investigated the possibility of transmission via a common source located in – or nearby – rooms of the nephrology unit by means of a case-control analysis. Cases 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 out of renal transplant recipients admitted to the unit in the same time window but who were not diagnosed with PCP later. Data were obtained from the hospital's administrative department. Odds ratios and 95%-confidence intervals were calculated for all rooms.

Air sampling

Air sampling was performed to detect *Pneumocystis* in rooms of the nephrology unit and in the waiting room of the outpatient department. Because this expertise was not available in our institution, the collection of air samples and the procedure of extracting DNA from the filters were carried out by a company specialised in measuring microbiological air quality (Intersave Groeneveld B.V., Dordrecht, The Netherlands). 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). Also 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) were sampled. The outpatient department was sampled overnight when no patients were present. Air sampling was performed by use of Gilair air sampler pumps (Sensidyne Inc., Florida, USA.), creating an airflow over a glass fiber filter with a velocity of 2 liters per minute for ±8 hours on each location. After filtration of approximately 1000 litres of air, the filters were removed and DNA was extracted (Chemagic DNA extraction kit, Chemagen, Baesweiler, Germany). 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 multi-layered filters of the ventilation system of the outpatient department. Two filters passed by inflowing air and one outflow filter were sampled by cutting a 10 cm^2 piece of each filter, which was washed with 500 ml of MilliQ and centrifuged. Both supernatant and residue were subjected to real time PCR (DHPS gene).

Genotyping of Pneumocystis strains

Genotyping of *P. jirovecii* was performed by sequence analysis of the internal transcribed spacer number 1 (ITS1) and ITS2 of the nuclear rRNA operon. Reference data reflecting the distribution of *P. jirovecii* genotypes in this region was obtained by genotyping 11 samples obtained from patients with PCP admitted to the LUMC between January 2003 and January

2005 and 3 samples containing *P. jirovecii* 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. was shortened and used with the sequence 5'-GCGGGTGATCCTGCCT-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 approximately 540 bp. DNA was extracted from BAL samples using the total nucleic acid protocol with the MagNA pure LC nucleic acid isolation system (Roche Diagnostics, Almere, The Netherlands). Each sample was eluted in 100 µl of buffer and stored at –80°C until processing. 5 µl of DNA-extract was added to 45 µl reaction mix containing 25 µl of 2x Hotstar mastermix (Qiagen, Venlo, The Netherlands) and 25 pmol of each primer. Cycling conditions: 15 min at 95°C, 50 cycles of 30 s at 92°C, 30 s at 62°C, and 30 s at 72°C respectively, followed by a 5 min. hold at 72°C. The PCR product was analysed with agarose gel electrophoresis. In case of aspecific amplification, the correct product was cut out and purified using the Qia-quick 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; 16 with positive microscopy and PCR, 1 with microscopy and 6 with PCR only. Twelve (55%) were male; age ranged from 36 to 72 years (median 57). No geographic clustering according to postal code was noted. All patients (including the 6 patients reported from other hospitals) except one had visited the nephrology outpatient department of the LUMC. The cause of original renal disease was heterogeneous; 3 out of 22 cases had received a kidney-pancreas transplant and 11 cases had received their graft within 1 year prior to the diagnosis of PCP. Immune-suppressive regimens contained mofetyl mycofenolate and prednisone (7.5 to 20 mg once daily) in all but one patient. Ten patients also used cyclosporine. No changes in routine immune-suppressive regimens had been implemented in the past 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 the very low incidence of PCP so far, the benefits were not considered to outweigh the side effects.

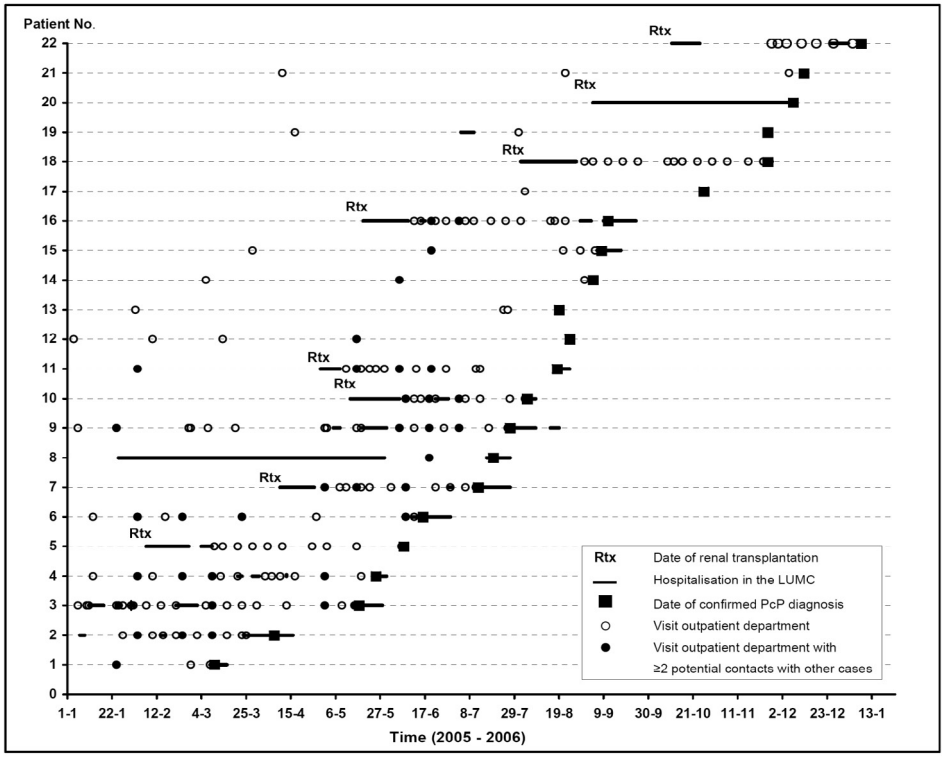
Cytomegalovirus (CMV) replication was present in 10 of 19 patients with known CMV status; only one received anti-viral medication at the time of diagnosis. Five patients had received treatment for graft rejection within 12 months before the diagnosis of PCP.

One patient became critically ill and died due to pulmonary and cardiac failure; none of the other patients was transferred to an intensive care unit.

Transmission map

The transmission map (figure 2) showed 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 in to account, multiple possibilities of transmission exist. When each case is regarded as a possible index case, a combination of patient No.3 and No.9 suffices to trace potential contacts with all but one case with type 'Ne' (the predominant genotype). Both patients had received multiple treatments for rejection and had a higher load of *Pneumocystis* in BAL fluid (microscopy 3+, Ct values 34.4 and 27.5 respectively) in comparison to other patients.

Figure 2. Transmission map.



Legend: Genotyping of *Pneumocystis* showed ITS type 'Ne' in cases 2,3,6,7,9-13,16-18. Only ITS2 could be determined for case 14: 'e'. Determination of ITS genotypes failed in case 1,4,5 and 20-22. Genotype 'Bi' was found in case 8. PCP was diagnosed in this case after a long stay on the hematology ward due to treatment for malignant lymphoma.

Statistical analysis of outpatient department contacts

An analysis of the frequency of visits to the outpatient department, and the encounter of other future patients over the four months preceding the PCP diagnosis was performed on the transmission chart data (i.e. on cases only). The frequency of visit was more strongly associated with disease development than encounter of 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 or more patients of this cluster had been admitted to the nephrology unit at the same time before they had developed PCP. In the case-control study on inpatient rooms, a total of 24 and 257 hospitalisations of 10 case patients and 139 control patients were analysed (data not shown). The odds ratios for individuals rooms and for combinations of rooms varied from 0.75 to 1.89 with 95% confidence intervals including 1.00.

Air sampling

This part of the study was conducted in January and February 2006. The mean total amount of filtered air on each location was 1000 liters (range 861-1216 liters). In none of the 6 samples derived from the pump filters *Pneumocystis* was detected by real time PCR. The supposedly positive control room was found to be negative. The negative results were not due to inhibition of the samples since the internal controls (phocine herpes virus) were positive. A specimen derived from one of the inlet-filters of a ventilation shaft tested positive for PCP. Subsequent ITS genotyping failed probably due to 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 in 16 of the 22 available BAL samples from 22 different patients. Sequence analysis showed type 'Ne' in 12 out of 16 successfully analysed samples, type 'Bi' was present in 1 sample. In 3 samples only the ITS2 genotypes could be determined: type 'e' once and 'g' twice. Genotyping failed in 6 samples due to a weak signal or the presence of >2 strains. Interestingly, of the 12 successfully genotyped reference samples (i.e. unrelated to the present outbreak) only 2 (17%) showed 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. jirovecii* genotype. From the first of December 2005 - the 10th month of the outbreak - trimethoprim-sulfamethoxazole or an alternative form of prophylaxis was prescribed for patients within their first year post transplantation as well as for patients treated for graft rejection. Despite increased alertness among physicians, only one new case was reported since February 2006. We found no indication that the incidence of PCP had increased due to changes in immune-suppressive medication. The risk of PCP is associated with the treatment for rejection and CMV reactivation [14, 15], but none of these risk factors was predominantly present or changed over time and thus unlikely to have played a major role. We discuss the results of this outbreak investigation along with two hypothetical models of transmission of *P. jirovecii*.

The environmental source hypothesis

This thesis is based on the assumption that inhaled forms of *P. jirovecii* that cause PCP do directly originate from a niche in the environment. No environmental source has been discovered to harbour *P. jirovecii* in previous outbreaks. Air sampling studies indicated that the route of transmission is by air [16, 17]. However, the source from which *P. jirovecii* becomes airborne remains to be specified: either environmental or human. Attempts to isolate *Pneumocystis* by air sampling were unsuccessful in this study; the sensitivity and specificity of the methods used are unknown. The positive PCR analysis of one of the *inlet* filters is compatible with more than one hypothesis about transmission, but 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 equally indicate that they acquired PCP through interhuman transmission as 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 encounter of other future patients in the outpatient department – showed the strongest association with the number of times 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 time makes it uncertain whether these calculations can reliably discriminate between interhuman transmission and an environmental source.

Genotyping showed that 12(75%) of genotyped strains were *P. jirovecii* type 'Ne'. Analysis of the reference strains indicates that type 'Ne' is less frequent in this region. However, in Denmark type 'Ne' was the second most prevalent strain in a study of randomly selected *P. jirovecii* positive BAL samples, but was not found in a cluster reported from that country [12,

18]. Data from Thailand and England confirm a regional dependent distribution of *P. jirovecii* ITS types [19, 20]. Since the frequency of different ITS types of *P. jirovecii* in the Leiden region is unknown, definite interpretation of the genotyping results is not possible. Genotype 'Ne' has not been reported as a more virulent type in human disease [21].

The interhuman transmission hypothesis

In this model PCP is considered a transmittable disease; i.e. the source is another infected (or colonized) individual [22]. Accumulating evidence from animal models, the host specificity of *P. jirovecii* and the phenomenon of carriage of *P. jirovecii* in the respiratory tract of healthy and immuno-compromised individuals all support the idea that humans may constitute the reservoir themselves [3, 23-26]. Transmission would then occur by spreading and subsequent inhalation of air or aerosols containing infectious forms of *P. jirovecii*. Four previous studies attempted to elucidate the possible role of interhuman transmission in small clinical clusters of PCP by genotyping (summarized in table 1). The methods used and the clinical evidence of contact between patients differs between these studies. In the two more recent investigations, genotyping and clinical data suggest the possibility of interhuman transmission [8, 28].

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

Table 1. Studies of recent clinical clusters of *Pneumocystis* pneumonia in which genotyping was performed.

Authors (ref.)	Year of publication	No. of patients (clustered)	Clinical background	Genotyping method	Authors' conclusions
Helweg-Larsen J. et al. [17]	1998	14 (8+4+3)	8 Clustered cases of patients with an haematological malignancy and PCP plus 2 small clusters of HIV infected individuals	DNA sequence analysis ITS1, ITS2	Interhuman transmission may occur but may not constitute the major route of transmission.
Ollson et al. [27]	2001	17 (3+7+7)	3 Clusters, 2 in renal transplant recipients, 1 in patients with a haematological malignancy.	DNA sequence analysis mtLSU-rRNA	Interhuman transmission unlikely
Rabodorina M. et al. [8]	2004	10	A cluster of PCP in renal transplant recipients who encountered HIV+ patients during hospital admittance	multitarget PCR-SSCP ITS1, 26S, mt26, beta-tubulin	Possible nosocomial interhuman transmission
Hocker B. et al. [26]	2005	6 (3+3)	Sudden rise in incidence of PCP in a paediatric transplant unit; 3 cases on a clinical and molecular basis related to one index case	multitarget PCR-SSCP ITS1, 26S, mt26, beta-tubulin	Possible nosocomial interhuman transmission

SSCP denotes single strand confirmation polymorphism, ITS1: internal transcribed spacer 1, 26S intron of the Nuclear 26S rRNA gene, mt26: variable region of the Mitochondrial 26S rRNA gene, beta tubulin: region surrounding intron 6 of the beta-tubulin gene, mtLSU-rRNA: mitochondrial large subunit ribosomal RNA locus, HIV: Human Immuno-deficiency Virus, PCP: *Pneumocystis* pneumonia.

could not be reconstructed, it accurately describes the presence of one or more cases within a limited waiting area (16m^2) within a limited time period. In view of the fact that the actual mechanism of transmission of *P. jirovecii* is unknown, the thesis that one or two 'hyper-spreaders' (case No.3 and No.9) caused this outbreak remains speculative.

Interestingly, the outbreak was restricted to renal transplant recipients. No case of PCP was 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 one morning per week. Because the liver transplant recipients use comparable immune-suppressive drugs and do not receive PCP prophylaxis, a proportional incidence of up to 4 cases of PCP would be expected in case of an environmental source in the outpatient department [29]. The interpretation of the genotyping results - potentially supportive for both hypotheses - is discussed in the previous paragraph.

Interpretation and conclusions

In this outbreak the evidence for clinical clustering as well as the presence of one 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 from an environmental source. Hence, the need for further elucidation of the general mode of transmission of *P. jirovecii* seems evident. This requires sophisticated methods to investigate possible environmental sources and clinical research aimed at understanding the role of carriers of *P. jirovecii*. More complex transmission models may account for clusters of PCP. Progress in understanding the source of PCP may permit to undertake effective action to prevent and control future outbreaks.

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