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Transplantation. Author manuscript; available in PMC 2014 November 15.

Published in final edited form as:

Transplantation. 2013 November 15; 96(9): . doi:10.1097/TP.0b013e3182a1618c.

Outbreak of *Pneumocystis* Pneumonia in Renal and Liver Transplant Patients Caused by Genotypically Distinct Strains of *Pneumocystis jirovecii*[1]

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Abstract

Background—An outbreak of 29 cases of *Pneumocystis jirovecii* pneumonia (PCP) occurred among renal and liver transplant recipients (RTR and LTR) in the largest Danish transplantation centre between 2007 and 2010, when routine PCP prophylaxis was not used.

Methods—*P. jirovecii* isolates from 22 transplant-cases, 2 colonized RTRs and 19 *Pneumocystis*-control samples were genotyped by restriction fragment length polymorphism and multi-locus sequence typing analysis. Contact tracing were used to investigate transmission. Potential risk factors were compared between PCP cases and matched non-PCP transplant patients.

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None of the authors have any conflict of interest.

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Results—Three unique *Pneumocystis* genotypes were shared among 19 of the RTRs, LTRs and a colonized RTR in 3 distinct clusters, two of which overlapped temporally. In contrast, *Pneumocystis*-control samples harbored a wide range of genotypes. Evidence of possible nosocomial transmission was observed. Among several potential risk factors, only CMV viremia was consistently associated with PCP (P = 0.03; P = 0.009). Mycophenolate mofetile was associated with PCP risk only in the RTR population (P = 0.04).

Conclusion—We identified three large groups infected with unique strains of *Pneumocystis* and provide evidence of an outbreak profile and nosocomial transmission. LTRs may be infected in PCP outbreaks simultaneously with RTRs and by the same strains, most likely by inter-human transmission. Patients are at risk several years after transplantation, but the risk is highest during the first 6 months post-transplantation. Since patients at risk cannot be identified clinically and outbreaks cannot be predicted, six months of PCP chemoprophylaxis should be considered for all renal and liver transplant recipients.

Keywords

Pneumocystis jirovecii; *pneumocystis pneumonia*; liver transplant; renal transplant; outbreak

Introduction

Pneumocystis jirovecii pneumonia (PCP) is a common opportunistic infection in immunocompromised patients, and is now believed to result from recent acquisition in most cases [1, 2]. Recipients of solid organ transplants are at increased risk of developing PCP, with substantial variation in risk between transplant types as well as between centers [3–6]. As a consequence, the indications for and duration of PCP prophylaxis have been debated. In many centers, the incidence of PCP after renal or liver transplantation has been very low despite the absence of routine PCP prophylaxis [7].

In PCP outbreaks among renal transplant recipients (RTRs), epidemiological studies with genotyping have suggested inter-human transmission of *Pneumocystis* [3, 8–15]. It remains unclear if such outbreaks are due to the introduction of specific *P. jirovecii* strains into the hospital environment, to changes in immunosuppressive regimens, or to other factors affecting patient susceptibility. The relative importance of asymptomatic carriers and other reservoirs as the source of transmission in these outbreaks remains unclear as well. In this single center report, we describe an outbreak of PCP among both RTRs and liver transplant recipients (LTRs). To examine possible transmission of *Pneumocystis*, we genotyped isolates obtained from RTRs and LTRs during the outbreak (cases) and isolates obtained from other patients with PCP diagnosed at the same hospital and during the same period (*Pneumocystis*-controls). In addition, we compared risk factors for PCP in renal and liver transplanted patients by matching non-infected transplant patients (transplant-controls) 2:1 to PCP cases.

Results

An outbreak of PCP in 29 solid organ transplant patients occurred between 2007 and 2010 (Figure 1), and included 16 renal and 13 liver transplant recipients. The majority were diagnosed by direct immunofluorescence microscopy (DFA), but one RTR (6 %) and four LTRs (29 %) were diagnosed by single-round polymerase chain reaction (PCR). General characteristics of cases are shown in table 1a and 1b. In the preceding period, from 2002 to 2007, only one transplant patient was diagnosed with PCP. This outbreak occurred with no apparent change in the overall use of immunosuppressive regimens. Routine PCP prophylaxis was not utilized between 2002 and 2010. Standard trimethoprim/

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sulfamethoxazole PCP prophylaxis was initiated at the end of the outbreak, in the fall of 2009 for RTRs and in early 2011 for LTRs.

Genotype analysis

To understand the epidemiology of this outbreak, we undertook genotypic analysis of the 22 available *Pneumocystis* isolates with sufficient DNA, using restriction fragment length polymorphism (RFLP) and multi-locus sequence typing (MLST). Twenty-one were successfully analyzed by both methods and one by MLST only; genotyping results were identical by both methods. Three genotype patterns (groups 1, 2 and 3) were observed in 18 of 22 samples (Figure 2a and 2b). Each group was temporally clustered, with groups 2 and 3 overlapping in time (figure 3). Four outbreak cases were not part of the clusters, but resulted from infection with different *Pneumocystis* genotypes. MLST analysis of two additional isolates from *Pneumocystis*-colonized RTRs identified one as a genotype-group 2 isolate and the other as a unique genotype.

Pneumocystis-control samples with sufficient DNA for analysis were available from 19 PCP patients, the majority with hematological malignancies. Nine were successfully analyzed by both methods, and 10 by MLST only. Unlike the transplant isolates, *Pneumocystis*-controls generally had diverse genotypes, though 2 control patients who were hospitalized during the outbreak were infected with group 2 or 3 isolates. The *Pneumocystis*-controls were admitted at the same hospital as the cases, but to different floors, with one exception: one patient with glomerulonephritis was admitted to the same floor as RTRs infected with the same *Pneumocystis* genotype (group 2) and thus appears to be part of the outbreak.

Transmission analysis

To assess possible cross-exposure, contacts between patients were investigated by a transmission map (figure 4). For many but not all cases, we were able to demonstrate exposure, during the period prior to diagnosis of PCP, to a PCP patient infected with the same genotype.

Each major genotype in the outbreak affected both renal and liver recipients. The liver transplant ward is located on the floor above the renal transplant ward and direct contact between renal and liver recipients is limited to the possible brief contact at the entrance hall, elevators or the common out-patient blood sampling unit in the hospital.

Clinical features and risk factors

We conducted a matched case-control study and univariate analysis, to identify potential risk factors associated with development of PCP in the transplant setting. Previous studies have suggested that major risk factors, include high-dose immunosuppression, the use of specific immunosuppressive drugs, graft rejection, cytomegalovirus (CMV) infection and older age [7].

Renal transplant recipients—Four of 16 RTR patients (25%) were diagnosed more than one year after transplantation; the remaining 12 patients presented within ~6 months after transplantation (Table 1a). One case presented with PCP 72 days after cessation of a three month course of post-transplantation prophylaxis; no other patient received prophylaxis.

Current infection with CMV was associated with PCP (5/16 vs 2/32 for transplant-controls, $P = 0.03$). In addition, a significantly higher fraction of PCP cases received MMF compared to transplant-controls (16/16 vs 24/32, $P = 0.04$). Graft rejection within 6 months was not associated with PCP (4/16 vs 4/32, $P = 0.4$), nor was there a correlation between PCP and

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use of corticosteroids, exposure to other immunosuppressive regimens, the presence of comorbidity, CMV prior to PCP or cold graft ischemia (table 1a).

Liver transplant recipients—All 14 LTRs, including one diagnosed in 2002 who was not part of the outbreak, were diagnosed within 1 year of transplant. Four LTRs were diagnosed exclusively by a positive PCR, but had a clinical course consistent with PCP.

We again observed a statistically significant correlation between current CMV infection and PCP (4/14 vs 0/28 for transplant-controls, $P = 0.009$). Graft rejection within 6 months was not associated with PCP (3/14 vs 7/28, $P = 1.0$). No correlation between PCP and the use of corticosteroids, immunosuppressive regimens, CMV prior to PCP, co-morbidity, blood loss during surgery or biomarkers of liver and bile duct injury was observed (table 1b). In particular, the increased risk of PCP seen in RTRs receiving MMF was not observed in LTRs.

Discussion

This is the first reported outbreak of PCP occurring in both renal and liver transplant recipients. The outbreak was caused by three strains of *Pneumocystis*, as defined by genotyping; the same strains were found in only two cases of the large *Pneumocystis*-control population.

Our results differ from several recent studies which reported outbreaks of PCP among RTRs dominated by a single genotype [3, 8–15]. Surprisingly, LTRs were not infected with a distinct strain of *Pneumocystis*, but were part of clusters dominated by RTRs. This study provides the first molecular evidence linking *Pneumocystis* infection in LTRs to outbreak strains. In a previous report, LTRs were not infected during a single genotype outbreak of PCP among RTRs who attended the same outpatient clinic [12].

Outbreaks such as this one provide the opportunity to better understand the transmission dynamics of *Pneumocystis* infection. Genotyping studies provide strong evidence for recent transmission of *Pneumocystis* within clusters. Detection of *P. jirovecii* DNA by PCR in air samples obtained near PCP patients indicates possible aerosol spread [16]. In the current study, clustering of isolates by time and departments strongly suggests transmission directly between PCP cases or from a common source. If we assume that transmission may happen from 12 weeks prior to PCP symptoms to three weeks after initiation of treatment [17], direct transmission between the majority of our cases could have occurred. We were unable to identify potential exposure events for all cluster cases. However, seven cases were not genotyped and therefore not included in the transmission analysis; these cases may have contributed to transmission. Alternative sources of transmission may potentially include asymptomatic carriers of infection. Our genotype findings suggest that asymptomatic colonized transplant patients (e.g., patient 29) could have acted as one such reservoir. This hypothesis is supported by another recent study [14].

It has been hypothesized that more virulent strains of *P. jirovecii* may play a role in outbreaks: three previous outbreaks in RTRs were associated with a single genotype (ERT strain), but this strain was absent in our patients [13, 15]. The identification of multiple strains in this outbreak suggests that differential virulence is not playing an important role.

Health care workers (HCW) have been suggested as another possible reservoir, though at present there is no direct evidence to support this. Although serologic studies suggest greater exposure of HCW to *Pneumocystis* [18], no colonization of HCW could be demonstrated

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during one PCP outbreak [11]. In our hospital, different HCW care for renal and liver transplant recipients, and shared contacts by physicians are limited.

Analysis of *Pneumocystis* isolates in non-transplanted immunosuppressed patients usually does not show clustering of genotypes, but rather, reveals that broadly diversified strains are responsible for most infections [19, 20]. This suggests unique risk factors for RTRs and LTRs that could be related to exposure or to the underlying immunodeficiency. The reason for the sudden increase in cases of PCP cases in our clinic is unexplained by our risk factor analysis.

Several risk factors have been identified as predisposing transplant patients to PCP, most notably alteration in immunosuppressive therapy, graft rejection, CMV infection and older age [7]. The introduction of immunosuppressive drugs such as MMF and tacrolimus in the 1990s led to decreased rejection rates [21], but targeted T-cell suppressive drugs may predispose patients to PCP. We found a higher use of MMF in RTRs with PCP compared to matched transplant-controls, but this was not seen in LTRs. The association of MMF with PCP was previously reported in a large cohort study and two case-control studies [4, 22, 23]. In our center, MMF has been used as a key component of immunosuppression in RTRs for the past 14 years, yet no outbreaks of PCP were seen in the first 10 years after the introduction of MMF.

The use of corticosteroids is commonly recognized as a significant risk factor for PCP [23]. In one report, among LTRs managed with a corticosteroid-free regimen and no PCP prophylaxis, no PCP cases were observed [6]. Rejection therapy is also regarded as a prominent risk factor for PCP [24, 25]. However, while we observed a non-significant trend towards a higher rejection rate among RTRs with PCP, no clear correlation between corticosteroid exposure and PCP was found, in accordance with other studies [4].

Current infection with CMV was found to be strongly correlated with PCP in both renal and liver transplant recipients. This likely indicates a higher level of immunosuppression, but could also be caused by CMV re-activation secondary to PCP. These results should be interpreted with care, as the cases were suspected of pneumonitis and more likely to be tested. Prior CMV infection did not correlate with PCP.

The use and length of routine PCP prophylaxis for renal and liver transplant recipients has been debated. In our study, most patients developed PCP within 6 months after transplantation, in agreement with other studies [5, 25]. It is noteworthy, however, that 25% of PCP cases in RTRs occurred more than one year after transplantation, similar to the experience of others [4, 8, 9, 11]. In contrast, all LTRs with PCP were diagnosed within one year after transplantation.

Three months of trimethoprim/sulfamethoxazole prophylaxis has been suggested to adequately reduce PCP risk in renal and liver transplants [26–28]. However, studies of a three month prophylaxis should be interpreted with care, due to the risk of PCP outbreaks that have been identified with increasing frequency. Based on our findings, we suggest that PCP prophylaxis should be recommended for all patients 6 months after transplantation and prophylaxis should be considered for other susceptible patients, such as those undergoing rejection treatment, during outbreaks of PCP.

Our study has limitations. The point of contact between cases was difficult to establish, due to the retrospective design. Five cases, four of which were LTRs, were diagnosed solely by PCR; DFA was negative in two of these. However, the clinical course including response to therapy was consistent with a diagnosis of PCP.

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Our study is not powered to rule out risk factors predisposing patients to PCP, in particular rejection therapy, which in other studies was an important risk factor for PCP. Immunosuppressive drugs, especially corticosteroids, could be an important risk factor for PCP that was not identified due to similar high frequency of use between cases and transplant-control subjects. Thus the entire transplant population could have been at risk, but only a minority may have developed it as a result of exposure to the infection.

A major strength of our study is the large number of *Pneumocystis*-control samples. For 2009 and 2010 the analyzed samples comprised almost all PCP infections at our hospital.

In conclusion, LTRs and RTRs were simultaneously infected in an outbreak of PCP that was predominantly caused by three unique strains of *Pneumocystis*. Our findings provide strong evidence of an outbreak profile caused by nosocomial transmission.

Materials and Methods

Study population

Rigshospitalet, Copenhagen University Hospital, is a tertiary care hospital in Denmark with a liver transplant program that covers the Danish population of 5.5 million, and a renal transplant program that covers a population of 2.5 million.

By database query, all renal or liver transplant recipients with a positive microscopy or PCR test for *P. jirovecii* between January 1st 2002 and December 31st 2010 were included in the study if the following criteria were met: 1) Symptoms compatible with PCP [29], 2) Radiographic findings and laboratory values compatible with PCP and 3) Detection of *P. jirovecii* by direct immunofluorescence microscopy or PCR. Samples examined for *P. jirovecii* included BAL (n=28), tracheal aspirations (n=1) and oral wash (n=1). Twenty-five of 27 samples tested by direct immunofluorescence microscopy were positive. In five cases a single-round touchdown PCR test, using previously described methods, was the only positive assay [30].

Based on these definitions, two RTRs, who were PCR positive but microscopy negative, and whose clinical course was not consistent with PCP, were excluded from the transplant-control analysis, but included as colonized RTRs in the genotype analysis.

One combined liver and renal transplant recipient is considered a liver transplant in our analysis. Guidelines of the Danish Health and Medicines Authority and the National Institutes of Health (NIH) were followed in the conduct of these studies.

Genotyping

Pneumocystis specimens from cases and *Pneumocystis*-controls were retrieved from stored samples at the Biobank of the *Department of Clinical Microbiology* and *Statens Serum Institut*. *Pneumocystis*-controls were defined before genotyping as patients without renal or liver transplants who were diagnosed with PCP at *Rigshospitalet* during the same years as cases.

Blinded genotype analysis was done at the NIH, Bethesda, MD, USA. The method for RFLP analysis of the *msg* gene has been previously described [13, 19]. Briefly, DNA was extracted using the QIAamp DNA mini Kit (Qiagen), *msg* gene copy number was quantified by a real-time quantitative PCR, and a minimum of approximately 1000 *msg* gene copies per RFLP PCR reaction were amplified using semi-nested PCR with primers GK 472, GK 452 and GK 195, as described [19]. Purified PCR products were treated with restriction enzymes

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Dra1 or Hpy188I. Analysis of the digested fragments was done by agarose gel electrophoresis [13, 19].

MLST analysis was performed by amplifying and sequencing three regions of the *P. jirovecii* genome, ITS, 26S, and mt26S, as described [31]. Sequencing a region of the beta-tubulin gene was not found to have discriminatory utility. Regions with any polymorphism in any of the sequences were identified and utilized to compare the isolates. Isolates with one or more nucleotide differences were considered to be different strains.

Transmission maps

To investigate potential transmission events among patients, all admissions and outpatient visits for PCP cases between 2007 and 2010 were retrospectively recorded and analyzed.

Risk factor analysis - matched case-control selection

We used databases at the transplant units to match two control subjects without PCP (transplant-controls) to each case patient. Each transplant-control was matched by 1) type of organ, 2) age (+/− 10 years) and 3) the transplant-controls were matched according to transplantation date and duration of follow-up at the same hospital *(Rigshospitalet)*; thus patients in the control group were at the same risk of exposure as the PCP cases.

Clinical data and definitions

Selected medical and microbiological patient data variables were recorded in an *Epidata entry form* [32]. Data were cross-validated by repeated recording. Follow up was complete for all patients through December 2010. The interval from transplantation to PCP (time of event), was defined as time to onset of PCP symptoms. For the transplant-controls, we calculated follow up intervals equivalent to the time of event for the matched case (risk date).

Immunosuppression—Standard immunosuppression included induction with basiliximab, daclizumab or thymoglobulin for RTRs and corticosteroids for LTRs, followed by maintenance therapy with calcineurin inhibitors, MMF or azathioprine and corticosteroids. Dosages were adjusted individually according to the unit's protocol.

Rejection—Graft rejection was graded histologically using the BANFF criteria [33, 34]. Borderline rejections were considered as rejections if treated. In RTRs first-line graft rejection therapy consisted of 250–500 mg prednisolone for 3 days. In cases of steroid resistant rejection, anti-thymocyte globuline was subsequently added. If the rejection was antibody mediated, treatment with plasmapheresis and IVIG, and in some cases rituximab, was initiated. In LTRs, high dose pulse prednisolone (500 mg for 5 days) was used for rejection.

Infections and prophylaxis—Standard CMV prophylaxis consisted of valganciclovir for 3–6 months after transplantation. CMV infection was defined as detection of CMV-DNA by routine real time PCR in a blood sample [35]. For transplant-controls, current CMV infection was defined as infection within 2 months before the risk date; prior CMV infection was >2 months.

Co-morbidity—The Charlson co-morbidity index predicts the ten-year mortality for renal and liver transplant patients, based on a wide range of co-morbid conditions (total of 22 conditions) [36, 37]. Co-morbid conditions were scored according to severity and summed for a total Charlson co-morbidity index score. Postoperative complications were not

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included as co-morbidity. Diabetes was included in the score if it was diagnosed and treated prior to transplantation.

Other risk factors and definitions—Other risk factors analyzed included blood loss during the liver transplant procedure, cold ischemia time of renal grafts, neutropenia at the time of PCP diagnosis and among liver transplant recipients biomarkers of liver and bile duct injury.

Statistics

Student's t-test and the Mann-Whitney test were used for comparison of quantitative data for normally and non-normally distributed data, respectively. Differences in proportions were compared using Fisher's exact test. Statistical significance was defined by a two-sided p-value < 0,05. Statistical analyses were done in Stata (Version 11.2, StataCorp, College Station, Texas).

Acknowledgments

We would like to acknowledge and thank Jørgen Skov Jensen *(Statens Serum Institut)* for the work done by retrieving microbiology data and samples from PCP cases diagnosed by PCR.

This work was supported by grants from the Danish Kidney Association's Research Foundation and The Helen and Ejnar Bjørnow Foundation. This research was supported in part by the Intramural Research Program of the National Institutes of Health Clinical Center.

Abbreviations

CMV	Cytomegalovirus
DFA	Direct fluorescens antibody
LTR	Liver transplant recipient
MLST	Multi locus sequence typing
MMF	Mycophenolate mofetil
NIH	National Institutes of Health
PCP	*Pneumocystis* Pneumonia
PCR	Polymerase chain reaction
RFLP	Restriction fragment length polymorphism
RTR	Renal transplant recipient

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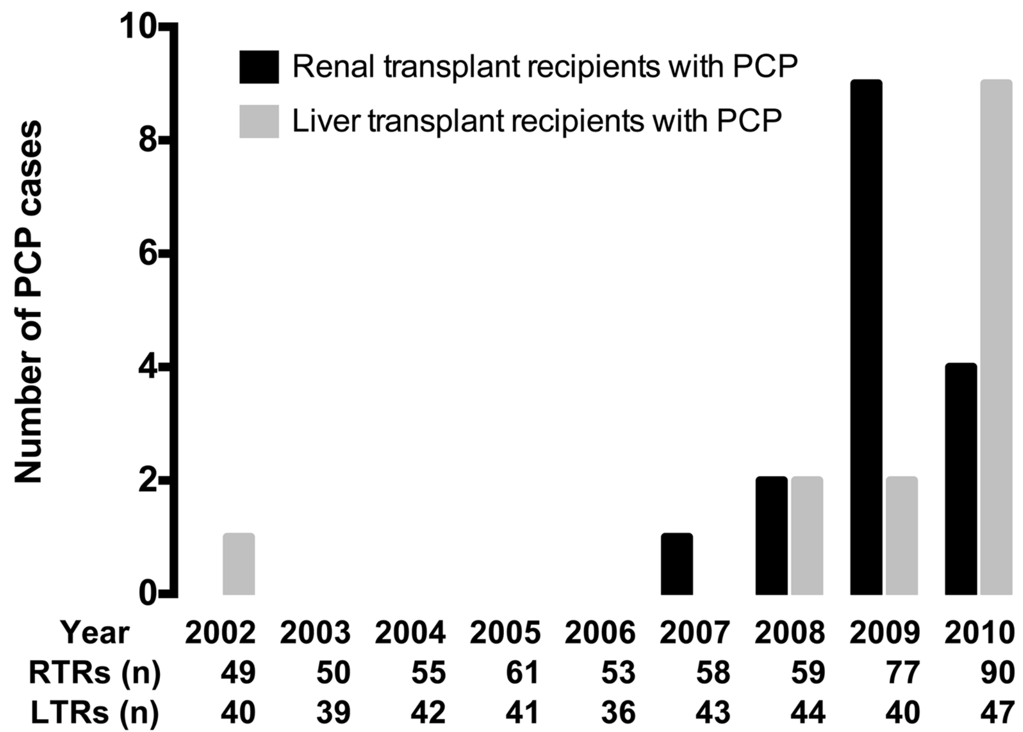
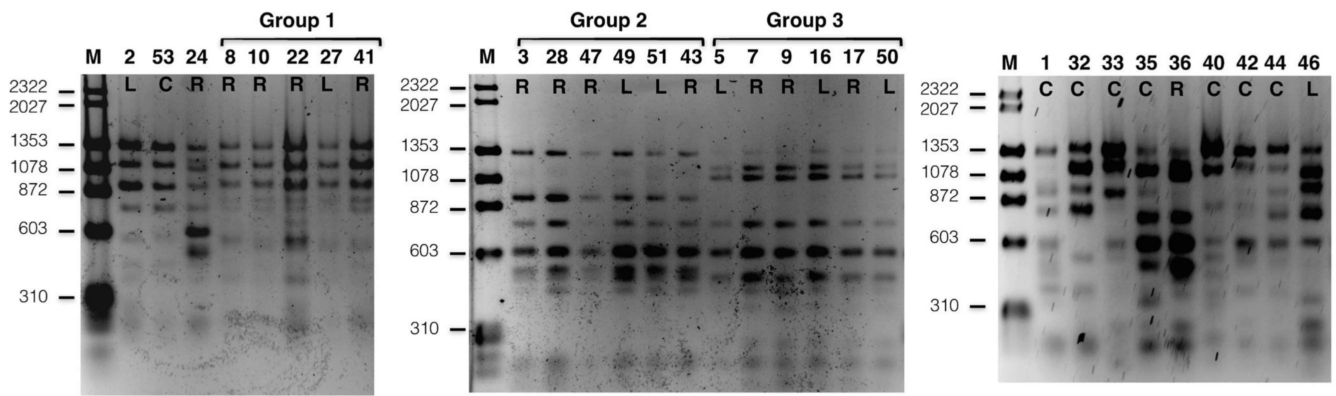


Figure 1.
Incidence of PCP and number of transplants performed, 2002 to 2010.



		ITS1											ITS2										26S											mt26S		
Patient no.	Category	6	39795	15	21	22-23	26	50-51	58-66	74-75	83-88	108-111	39449	27-29	47-48	50-51	52-53	55-56	59	63-64	107-108	150-154	3	34	78	119	136	181-183	190	212	223	244-249	296-306	85	248	288
REF		C	TTT	A	T	TC	T	TC	9xT	GG	6xT	TTTA	TTA	AAG	AT	TT	AG	AA	A	TA	CT	TATAA	A	A	A	G	C	ATA	A	A	G	ATTTTC	TTTTTTACTCT	C	C	G
D031	Renal	T	TT	A	T	TC	T	TC	10xT	GAGG	6xT	TTTA	TTA	AAG	AT	TAATT	AG	ATAAAA	A	TA	CT	TATAA	A	A	A	G	C	ATA	A	A	G	ATTTTC	TTTTTTACTCT	A	C	A
D001	Control	T	TT	A	T	TC	T	TC	10xT	GAGG	6xT	TTTA	TTA	AAG	AT	TAATT	AG	ATAAAA	A	TA	CT	TATAA	A	A	A	G	C	ATA	A	A	G	ATTTTC	TTTTTACTCTT	T	C	A
D013	Control	T	TT	A	T	TC	T	TC	10xT	GAGG	6xT	TTTA	TTA	AAG	AT	TAATT	AG	ATAAAA	A	TA	CT	TATAA	A	A	A	G	C	ATA	A	A	G	ATTTTC	TTTTTACTCTT	M	C	A
D026	Control	T	TT	A	T	TC	T	TC	10xT	GAGG	6xT	TTTA	TTA	AAG	AT	TAATT	AG	ATAAAA	A	TA	CT	TATAA	A	A	A	G	C	ATA	A	A	G	ATTTTC	TTTTTACTCTT	M	C	A
D044	Control	T	TT	A	T	TC	T	TC	10xT	GAGG	6xT	TTTA	TA	AAG	AT	TAATT	AG	ATAAAA	T	TA	CT	TATATAG	A	A	A	G	C	ATA	A	A	G	ATTTTC	TTTTTTACTCT	C	C	A
D046	Liver	T	TT	A	T	TC	T	TC	10xT	GAGG	6xT	TTTA	TA	AAG	AT	TAATT	AG	ATAAAA	T	TA	CCT	TATATTTATATAG	A	A	A	G	C	ATA	A	A	G	ATTTTC	TTTTTTACTCT	C	C	A
D012	Control	T	TT	A	T	TC	T	TC	10xT	GAGG	6xT	TTTA	TA	AAG	AT	TAATT	AG	ATAAAA	T	TA	CCT	ATATAG	A	A	A	G	C	ATA	A	A	G	ATTTTC	TTTTTTACTCT	A	C	A
D020	Control	T	TT	A	T	TC	T	TC	10xT	GAGG	6xT	del	TTTTA	AG	AAAT	TAATT	AG	ATAAAA	A	TA	CT	TTTATAA	A	A	A	G	C	ATA	A	A	G	ATTTTC	TTTTTACTCTT	A	C	A
D014	Control	T	TT	A	T	TC	T	TC	10xT	GAGG	6xT	del	A	AG	AAAT	TAATT	AG	ATAAAA	A	TA	CT	TTATAA	A	A	A	G	C	ATA	A	A	G	ATTTTC	TTTTTACTCTT	T	C	A
D037	Renal	T	TT	A	T	TTTC	C	TC	10xT	GAGG	5xT	TTTA	TTA	AAG	AAT	TAATT	ATAG	ATAAAA	T	TA	CT	TAA	A	A	A	G	C	ATA	A	A	G	ATTTTC	TTTTTACTCTT	A	C	A
D029	Renal	T	TT	A	T	TTTC	C	TC	10xT	GAGG	5xT	TTTA	TTA	AAG	AAT	TAATT	ATAG	ATAAAA	T	TA	CT	TAA	A	A	A	A	C	ATGAGTA	T	A	T	AATTTTTC	TTTACTC	C	T	A
D030	Control	T	TT	A	T	TTTC	C	TC	10xT	GAGG	5xT	TTTA	TTA	AAG	AAT	TAATT	ATAG	ATAAAA	T	TA	CT	TAA	A	A	A	A	C	ATGAGTA	T	A	T	AATTTTTC	TTTACTC	C	T	A
D003	Renal	T	TT	A	T	TTTC	C	TC	10xT	GAGG	5xT	TTTA	TTA	AAG	AAT	TAATT	ATAG	ATAAAA	T	TA	CT	TAA	A	A	A	A	C	ATGAGTA	T	A	T	AATTTTTC	TTTACTC	C	T	A
D043	Renal	T	TT	A	T	TTTC	C	TC	10xT	GAGG	5xT	TTTA	TTA	AAG	AAT	TAATT	ATAG	ATAAAA	T	TA	CT	TAA	A	A	A	A	C	ATGAGTA	T	A	T	AATTTTTC	TTTACTC	C	T	A
D047	Renal	T	TT	A	T	TTTC	C	TC	10xT	GAGG	5xT	TTTA	TTA	AAG	AAT	TAATT	ATAG	ATAAAA	T	TA	CT	TAA	A	A	A	A	C	ATGAGTA	T	A	T	AATTTTTC	TTTACTC	C	T	A
D028	Renal	T	TT	A	T	TTTC	C	TC	10xT	GAGG	5xT	TTTA	TTA	AAG	AAT	TAATT	ATAG	ATAAAA	T	TA	CT	TAA	A	A	A	A	C	ATGAGTA	T	A	T	AATTTTTC	TTTACTC	C	T	A
D049	Liver	T	TT	A	T	TTTC	C	TC	10xT	GAGG	5xT	TTTA	TTA	AAG	AAT	TAATT	ATAG	ATAAAA	T	TA	CT	TAA	A	A	A	A	C	ATGAGTA	T	A	T	AATTTTTC	TTTACTC	C	T	A
D051	Liver	T	TT	A	T	TTTC	C	TC	10xT	GAGG	5xT	TTTA	TTA	AAG	AAT	TAATT	ATAG	ATAAAA	T	TA	CT	TAA	A	A	A	A	C	ATGAGTA	T	A	T	AATTTTTC	TTTACTC	C	T	A
D033	Control	T	TT	A	T	TTTC	C	TC	10xT	GAGG	5xT	TTTA	TTA	AAG	AAT	TAATT	AG	ATAAAA	T	TA	CT	TAA	A	A	A	A	C	ATGAGTA	T	A	T	AATTTTTC	TTTACTC	T	C	A
D018	Control	T	TT	A	T	TTTC	C	TC	10xT	GAGG	5xT	TTTA	TTA	AAG	AAT	TAATT	ATAG	ATAAAA	T	TA	CT	TAA	A	A	A	A	C	ATGAGTA	T	A	T	AATTTTTC	TTTACTC	A	C	A
D048	Control	T	TT	A	T	TTTC	C	TC	10xT	GAGG	5xT	TTTA	TA	G	AAT	TAATT	AG	ATGAAA	T	TA	CT	AA	G	A	A	G	T	ATA	A	A	G	ATTTTC	TTTTTACTCTT	T	C	A
D008	Renal	C	TTT	A	T	TC	T	TC	10xT	GG	6xT	TTTA	TTA	AAG	AT	TT	AG	AA	A	TA	CT	TATAA	A	A	A	G	C	ATA	A	A	G	ATTTTC	TTTTTACTCTT	C	T	A
D027	Liver	C	TTT	A	T	TC	T	TC	10xT	GG	6xT	TTTA	TTA	AAG	AT	TT	AG	AA	A	TA	CT	TATAA	A	A	A	G	C	ATA	A	A	G	ATTTTC	TTTTTACTCTT	C	T	A
D010	Renal	C	TTT	A	T	TC	T	TC	10xT	GG	6xT	TTTA	TTA	AAG	AT	TT	AG	AA	A	TA	CT	TATAA	A	A	A	G	C	ATA	A	A	G	ATTTTC	TTTTTACTCTT	C	T	A
D022	Renal	C	TTT	A	T	TC	T	TC	10xT	GG	6xT	TTTA	TTA	AAG	AT	TT	AG	AA	A	TA	CT	TATAA	A	A	A	G	C	ATA	A	A	G	ATTTTC	TTTTTACTCTT	C	T	A
D041	Renal	C	TTT	A	T	TC	T	TC	10xT	GG	6xT	TTTA	TTA	AAG	AT	TT	AG	AA	A	TA	CT	TATAA	A	A	A	G	C	ATA	A	A	G	ATTTTC	TTTTTACTCTT	C	T	A
D040	Control	C	TTT	A	T	TC	T	TC	10xT	GG	6xT	TTTA	TTA	AAG	AT	TT	AG	AA	A	TA	CT	TATAA	A	A	A	G	C	ATA	A	A	G	ATTTTC	TTTTTACTCTT	A	C	A
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D002	Liver	T	TT	A	T	TC	T	TC	9xT	GAGG	6xT	TTTA	TTA	AAG	AT	TAATT	AG	ATAAAA	A	TA	CT	TATAA	A	A	A	G	C	ATA	A	A	G	ATTTTC	TTTTTTACTCT	C	T	A
D053	Control	T	TT	A	T	TC	T	TC	9xT	GAGG	6xT	TTTA	TTA	AAG	AT	TAATT	AG	ATAAAA	A	TA	CT	TATAA	A	A	A	G	C	ATA	A	A	G	ATTTTC	TTTTTACTCTT	C	T	A
D024	Renal	T	TT	A	A	TC	T	TC	10xT	GAGG	6xT	TTTA	TTA	AAG	AT	TAATT	AG	ATAAAA	T	TA	CT	ATAG	A	A	A	G	C	ATA	A	A	G	ATTTTC	TTTTTACTCTT	C	T	A
D023	Control	T	TT	A	A	TC	T	TC	10xT	GAGG	6xT	TTTA	TTA	AAG	AT	TAATT	AG	ATAAAA	T	TA	CT	ATAG	A	A	A	G	C	ATA	A	A	G	ATTTTC	TTTTTTACTCT	T	C	A
D009	Renal	T	TT	A	A	TC	T	TC	10xT	GAGG	6xT	TTTA	TTA	AAG	AT	TAATT	AG	ATAAAA	T	TA	CT	ATAG	A	A	A	G	C	ATA	A	A	G	ATTTTC	TTTTTTACTCT	T	C	A
D017	Renal	T	TT	A	A	TC	T	TC	10xT	GAGG	6xT	TTTA	TTA	AAG	AT	TAATT	AG	ATAAAA	T	TA	CT	ATAG	A	A	A	G	C	ATA	A	A	G	ATTTTC	TTTTTTACTCT	T	C	A
D050	Liver	T	TT	A	A	TC	T	TC	10xT	GAGG	6xT	TTTA	TTA	AAG	AT	TAATT	AG	ATAAAA	T	TA	CT	ATAG	A	A	A	G	C	ATA	A	A	G	ATTTTC	TTTTTTACTCT	T	C	A
D036	Renal	T	TT	A	A	TC	T	TC	10xT	GAGG	6xT	TTTA	TTA	AAG	AT	TAATT	AG	ATAAAA	T	TA	CT	ATAG	A	A	A	G	C	ATA	A	A	G	ATTTTC	TTTTTTACTCT	T	C	A
D005	Liver	T	TT	A	A	TC	T	TC	10xT	GAGG	6xT	TTTA	TTA	AAG	AT	TAATT	AG	ATAAAA	T	TA	CT	ATAG	A	A	A	G	C	ATA	A	A	G	ATTTTC	TTTTTTACTCT	T	C	A
D007	Renal	T	TT	A	A	TC	T	TC	10xT	GAGG	6xT	TTTA	TTA	AAG	AT	TAATT	AG	ATAAAA	T	TA	CT	ATAG	A	A	A	G	C	ATA	A	A	G	ATTTTC	TTTTTTACTCT	T	C	A
D016	Liver	T	TT	A	A	TC	T	TC	10xT	GAGG	6xT	TTTA	TTA	AAG	AT	TAATT	AG	ATAAAA	T	TA	CT	ATAG	A	A	A	G	C	ATA	A	A	G	ATTTTC	TTTTTTACTCT	T	C	A
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D035	Control	T	TT	C	T	TC	C	TATC	9xT	GAGG	6xT	TTTA	TTA	AAG	AT	TAATT	AG	ATAAAA	T	TTA	CT	TATAA	A	G	A	G	C	ATA	A	A	G	ATTTTC	TTTTTACTCT	T	C	A
D032	Control	T	TT	A	A	TC	T	TC	11xT	GAGG	6xT	TTTA	TTA	AAG	AT	TAATT	AG	ATAAAA	T	TA	CT	TATAR	A	A	A	G	C	ATA	A	A	G	ATTTTTC	TTTTTACTCTT	AAA	C	A

Figure 2.

Figure 2a. Restriction fragment length polymorphism (RFLP) analysis of *Pneumocystis* samples from outbreak patients and PCP controls. Group number refer to the one of the three major genotypes shared among 18 of 22 RTRs and LTRs. PCR products were digested with Dra1 and analyzed by agarose gel electrophoresis. Samples from the same group were run in adjacent lanes to facilitate visualization. Digestion with Hpy188I confirmed the groupings (data not shown). The numbers represent the patient number, and the letters underneath represent the clinical group: R, RTR; L, LTR; C, control. Sample 36 is part of group 3 (confirmed on other gels). Sample 2 is from an LTR and 24 is from an RTR with non-outbreak genotypes. Sample 48 were analyzed by RFLP, but not included due to space limitations. Molecular weight markers (M) are located in the left lane of each gel.

Figure 2b. Multi-locus sequence typing (MLST) analysis of *Pneumocystis* samples from outbreak patients and PCP controls. A shows results for ITS1 and ITS2, and B for 26S and mt26S sequences. Only those sites that showed a polymorphism in any sequence are included; numbering is based on the reference sequence, which is shown in the top row. Groups 1–3 are indicated on the left and color coded. Category refers to classification of patients as RTR (Renal), LTR (Liver) and controls. D029 and D031 are considered colonized RTRs. Two pairs of control samples that had an identical MLST pattern are also color coded (D013 and D026; D040 and D006). Samples D002 (LTR) and D053 (control)

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had an identical RFLP pattern by two enzymes but differed at a single locus in the 26S gene. All sequences have been deposited with GenBank, and have been assigned GenBank numbers; ITS1 and ITS2 KC470767-KC470809; 26s, KC470724-KC470766; mt26s, KC470810-KC470852. GenBank numbers for the reference sequences are as follows: ITS1 and ITS2, U07220; 26S, L13615; mt26S, M58605. For ITS2, position 1 in the figure corresponds to nucleotide 313 of U07220.

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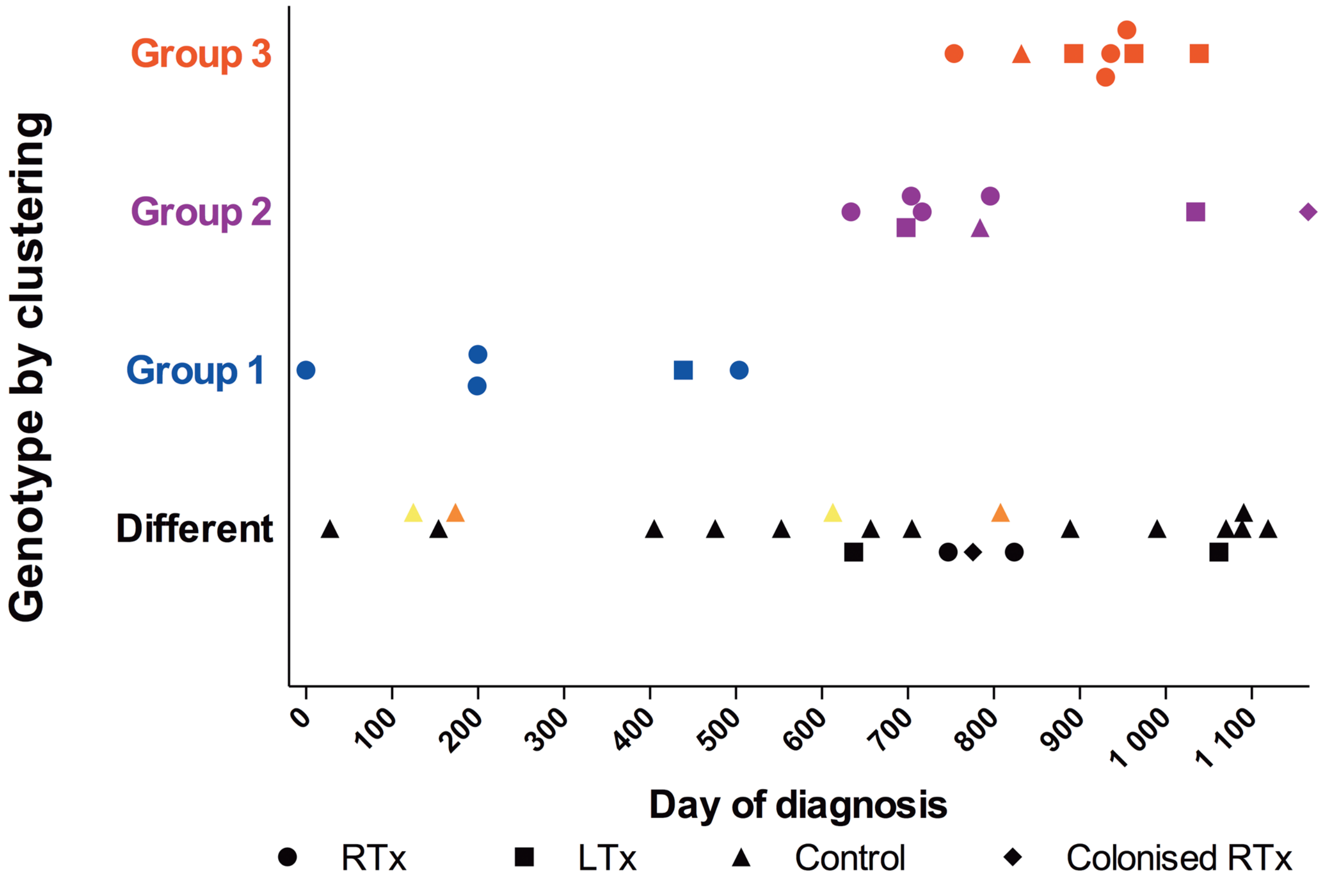

Figure 3.

Pneumocystis cases and *Pneumocystis*-controls plotted by genotype-group and date of diagnosis. Day "0": Diagnosis of first outbreak case, September 21, 2007. The three groups are color coded. Control patients were admitted at the same hospital as the cases. Two pairs of *Pneumocystis*-controls had the same genotype and were colored accordingly. Two controls were infected with outbreak strains. One, a patient treated with immunosuppressants for glomerulonephritis was admitted to the same ward as the RTRs; the other was a patient with myelodysplastic syndrome admitted to the Department of Hematology in another building at the hospital.

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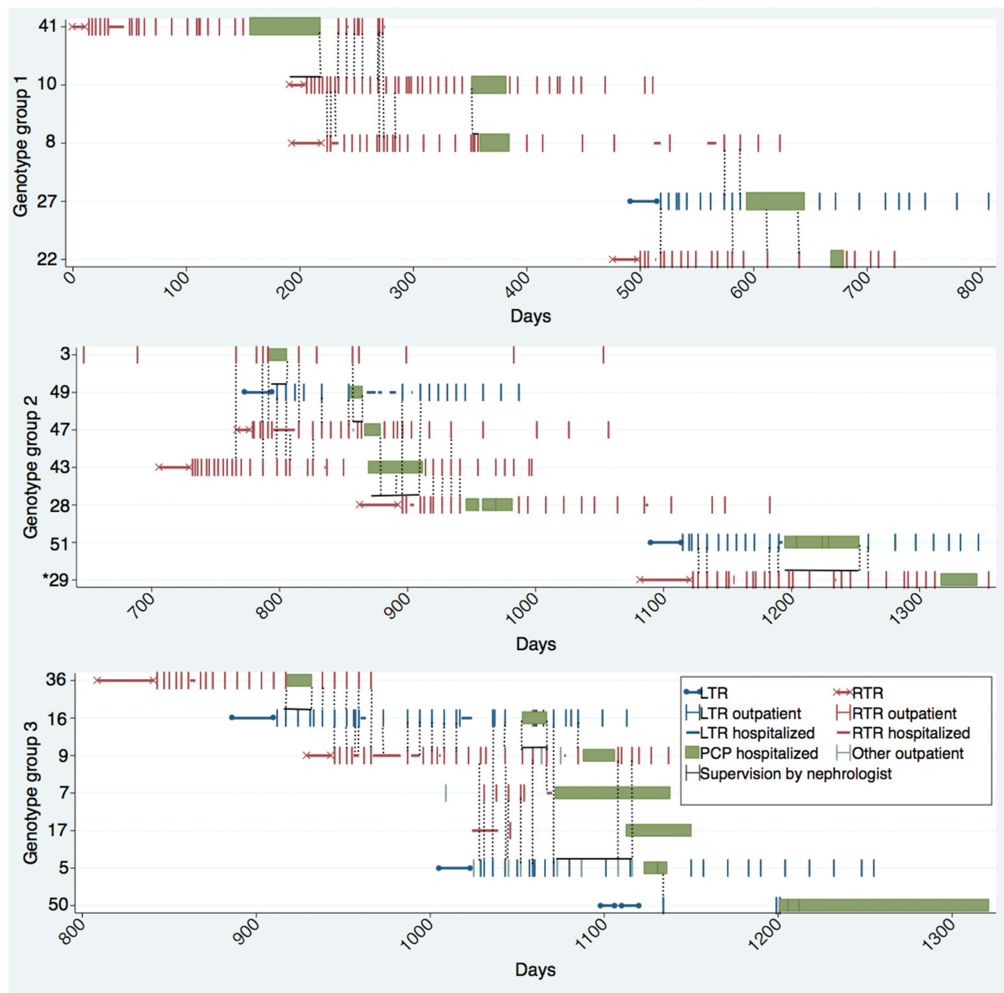

Figure 4.

Transmission map by case id. The transmission map is split according to genotype-group. Day "0" is April 9, 2007. The * indicates a colonized renal transplant recipient, the green bar indicates admission for pneumonia when PCP were suspected.

Group 1: Cases 2 and 3 developed PCP 19–20 weeks after the discharge of case 1. Cases 4 and 5 were transplanted a minimum of 13 weeks after the discharge of case 3.

Group 2: The interval from the discharge of case 10 to the transplantation of case 11 was 15 weeks.

Group 3: The interval between the discharge of case 13 to the transplantation of case 14 was 20 weeks.

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Table 1a

Risk factor analysis for renal transplant recipients compared to transplant-controls.

Renal transplant recipients			
Variable	**Cases n = 16**	**Transplant-controls n = 32**	**P-value**
Male	8 (50 %)	21 (66 %)	0,4
Length of admission after RTx, days (mean)	21	19	0,6
Age at PCP symptoms/risk date, years (mean)	46	46	0,9
PCP admission, days (median)	25 (10–95)	-	-
Death in relation to PCP	0 (0 %)	-	-
PCP prophylaxis	1 (6 %)	6 (19 %)	0,4
Immunosuppression			
Tacrolimus	5 (31 %)	9 (28 %)	1
Cyclosporine	7 (44 %)	20 (63%)	0,2
Everolimus	2 (13 %)	3 (9 %)	1
Sirolimus	0	1 (3%)	1
MMF	16 (100 %)	24 (75 %)	**0,04**
Azathioprine	0	7 (22 %)	0,08
Corticosteroids	16 (100 %)	32 (100 %)	1
Corticosteroid dosage[1], mg/day (median)	10	10,5	0,8
High dose corticosteroids[2]	0	1 (3%)	1
250 mg prednisolone/day for > 2 days	3 (19 %)	4 (13 %)	0,7
Induction therapy	**Cases n = 15**[3]	**Transplant-controls n = 32**	
Thymoglobulin	4 (27 %)	7 (22 %)	0,7
Simulect	5 (33 %)	15 (47 %)	0,5
Zenapax	4 (27 %)	8 (25 %)	1
Rituximab + IVIG	1 (7 %)	0 (0 %)	0,3
ATGAM	1 (7 %)	2 (6 %)	1
Rejection			
Graft rejection, total	6 (38 %)	6 (19 %)	0,2
Graft rejection <6 months	4 (25 %)	4 (13 %)	0,4
Last graft rejection to PCP/risk date[4], days (median)	149 (62–4868)	131 (33–4911)	0,7
CMV			
CMV infection prior to PCP/risk date	2 (13 %)	3 (9 %)	1
Time from last positive CMV-PCR to PCP/risk date, days (median)	49 (25–73)	29 (16–2154)	1
CMV prophylaxis at PCP symptoms/risk date[5]	5 (31 %)	8 (25 %)	0,7
CMV infection concurrent/current with PCP/risk date[6]	5 (31 %)	2 (6%)	**0,03**
Other			
Diabetes type 1	2 (13 %)	1 (3 %)	0,3

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Renal transplant recipients			
Variable	**Cases n = 16**	**Transplant-controls n = 32**	**P-value**
Diabetes type 2	1 (6 %)	2 (6 %)	1
Hepatitis C	1 (6 %)	1 (3 %)	1
Charlson co-morbidity index			0,8
1	5 (31 %)	14 (44 %)	
2	2 (13 %)	3 (9 %)	
3	0 (0 %)	1 (3 %)	
Neutropenia	1 (6 %)	1 (3%)	1
ABO incompatibility	1 (7 %)[3]	0	0,3
Graft cold ischemia, hours (median), n = 10 and 22	14 (1–22)	17,5 (2–29)	0,1

Variables expressed as medians are tested by a Mann-Whitney-Wilcoxon test. RTx = renal transplantation

[1]From 30 days prior to presentation of clinical symptoms/risk date.

[2]Prednisolone or equivalent corticosteroid dosage of 20 mg/day given for at least 1 month prior to clinical symptoms.

[3]One patient was transplanted in another country and not included.

[4]From the time of biopsy.

[5]Prescription of valganciclovir within 7 days before PCP/risk date.

[6]For transplant-controls, current CMV infection was defined as infection within 2 months before the risk date.

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Table 1b

Risk factor analysis for liver transplant recipients compared to transplant-controls.

Liver transplants recipients			
Variable	**Cases n = 14**[1]	**Transplant- controls n = 28**	**P-value**
Male	10 (71 %)	18 (64 %)	0,7
Length of admission after LTx, days (mean)	27	42	0,2
Age at PCP symptoms/risk date, years (mean)	51	49	0,8
PCP admission, days (median)	24 (8–120)	-	-
Death in relation to PCP	1 (7 %)	-	-
PCP prophylaxis	0	4 (14 %)	0,3
Immunosuppression			
Tacrolimus	10 (71 %)	19 (68 %)	1
Cyclosporine	4 (29 %)	8 (29 %)	1
Everolimus	0 (0 %)	1 (4%)	1
MMF	12 (86 %)	26 (93 %)	0,6
Corticosteroids	14 (100 %)	28 (100 %)	1
Corticosteroid dosage[2], mg/day (median)	15	15,5	0,9
High dose corticosteroids[3]	4 (29 %)	10 (36 %)	0,7
250 mg prednisolone/day for >2 days	3 (21 %)	7 (25 %)	1
Anti-lymphocyte	1 (7 %)	1 (4%)	1
Rejection			
Graft rejection, total	4 (29 %)	7 (25 %)	1
Graft rejection < 6 months	3 (21 %)	7 (25 %)	1
Last graft rejection to PCP/risk date[4], days (median)	122 (26–226)	97 (75–135)	0,3
CMV			
CMV infection prior to PCP/risk date	3 (21 %)	2 (7 %)	0,3
Time from last positive CMV- PCR to PCP/risk date, days (median)	48	76	0,6
CMV prophylaxis at PCP symptoms/risk date[5]	9 (64 %)	16 (57 %)	0,7
CMV infection concurrent/current with PCP/risk date[6]	4 (29 %)	0 (0 %)	**0,009**
Other			
Diabetes type 1	0	0	-
Diabetes type 2	5 (36 %)	6 (21 %)	0,5
Hepatitis C	1 (7 %)	1 (4 %)	1
Charlson co-morbidity index			0,1
1	3 (21 %)	10 (36 %)	
2	6 (43 %)	3 (11 %)	
6	0 (0 %)	1 (4 %)	
Neutropenia	3 (21 %)	1 (4%)	0,1

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Liver transplants recipients			
Variable	**Cases n = 14[1]**	**Transplant- controls n = 28**	**P-value**
Blood loss, liters (median), n =14 and 24	7,8	8,5	0,2
Fulminant liver failure	2 (14 %)	5 (18%)	1
Partial organ transplantation	0 (0 %)	3 (11 %)	0,5
ALT (median), n = 13 and n = 25	28 (9–172)	52 (16–287)	0,2
Alkaline phosphatase (median), n = 13 and 25	82 (45–1050)	163 (34–792)	0,6

Variables expressed as medians are tested by a Mann-Whitney-Wilcoxon test. LTx = liver transplantation

[1]One LTR was diagnosed in 2002 and was not part of the outbreak, but is included in the risk factor analysis.

[2]From 30 days prior to presentation of clinical symptoms/risk date.

[3]Prednisolone or equivalent corticosteroid dosage of 20 mg/day given for at least 1 month prior to clinical symptoms.

[4]From the time of biopsy.

[5]Prescription of valganciclovir within 7 days before PCP/risk date.

[6]For transplant-controls, current CMV infection was defined as infection within 2 months before the risk date.