ORIGINAL ARTICLE

Molecular epidemiologic analysis of a *Pneumocystis* pneumonia outbreak among renal transplant patients

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Abstract

Between 18 November and 3 December 2011, five renal transplant patients at the Department of Nephrology, Toho University Omori Medical Centre, Tokyo, were diagnosed with *Pneumocystis* pneumonia (PCP). We used molecular epidemiologic methods to determine whether the patients were infected with the same strain of *Pneumocystis jirovecii*. DNA extracted from the residual bronchoalveolar lavage fluid from the five outbreak cases and from another 20 cases of PCP between 2007 and 2014 were used for multilocus sequence typing to compare the genetic similarity of the *P. jirovecii*. DNA base sequencing by the Sanger method showed some regions where two bases overlapped and could not be defined. A next-generation sequencer was used to analyse the types and ratios of these overlapping bases. DNA base sequences of *P. jirovecii* in the bronchoalveolar lavage fluid from four of the five PCP patients in the 2011 outbreak and from another two renal transplant patients who developed PCP in 2013 were highly homologous. The Sanger method revealed 14 genomic regions where two differing DNA bases overlapped and could not be identified. Analyses of the overlapping bases by a next-generation sequencer revealed that the differing types of base were present in almost identical ratios. There is a strong possibility that the PCP outbreak at the Toho University Omori Medical Centre was caused by the same strain of *P. jirovecii*. Two different types of base present in some regions may be due to *P. jirovecii*'s being a diploid species.

Keywords: Multilocus sequence typing, next-generation sequencer, outbreak, *Pneumocystis* pneumonia, renal transplant patients
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Introduction

Pneumocystis pneumonia (PCP) is an opportunistic infection caused by *Pneumocystis jirovecii* which can become severe and even fatal. Because PCP only occurs in immunocompromised patients, it can be the first indicator of the onset of acquired immunodeficiency syndrome in patients with HIV whose cellular immunity is highly compromised. Because autoimmune disease patients and recipients of haematopoietic stem cell and organ transplants now receive massive amounts of immunosuppressants and steroids, PCP has been on the rise in patient groups other than HIV-infected patients [1]. Outbreaks of PCP have been reported among renal transplant recipients in Japan, France, Germany and the Netherlands [2–11]. It has been reported that 1.5 to 24% of renal transplant patients who were not receiving chemoprophylaxis with sulfamethoxazole/trimethoprim developed PCP [12] and that the PCP mortality rate is 13 to 38% [13].

P. jirovecii is a pathogenic microorganism that still cannot be cultured, and details of its mode of transmission, mode of onset of infection and pathogenic factors remain to be elucidated.

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With regard to its mode of transmission, host-to-host transmission from patients with active PCP, infection from carriers with subclinical PCP and contamination from the environment are considerations. Genetic analyses of renal transplant patients involved in PCP outbreaks [13] and a report of *P. jirovecii* DNA detected in the air surrounding patients with active PCP [14] suggest the possibility of host-to-host transmission from active PCP patients. However, it is known that there are many asymptomatic carriers of *P. jirovecii* [15–17], and the possibility of transmission from these carriers should not be ruled out [9,18,19].

In order to compare the genetic similarity of strains of *P. jirovecii,* which cannot be cultured, DNA directly extracted from samples were used as templates for multilocus sequence typing (MLST). MLST is a method of comparing the genetic similarity of strains by analysing the DNA base sequences of several housekeeping genes determined for each species. MLST for *P. jirovecii* uses loci such as β-tubulin (β-*TUB*), large subunit of the rRNA gene (26S), large subunit of the mitochondrial rRNA gene (mt26S), internal transcribed spacer 1 (ITS1), dihydropteroate synthase (DHPS), dihydrofolate reductase (DHFR), superoxide dismutase (*SOD*) and cytochrome *b* (*CYB*) [20].

At the Toho University Omori Medical Centre we had only four cases of PCP among renal transplant patients between January 2007 and October 2011, a period of 4 years and 10 months, but in a span of 16 days in November and December 2011, there were five cases. We conducted phylogenetic analysis of *P. jirovecii* using residual specimens of bronchoalveolar lavage fluid (BALF) from these five patients and from another 20 PCP patients with various underlying illnesses who were treated at the centre between 2007 and 2014.

Materials and methods

Clinical specimens

Phylogenetic analysis was conducted using residual specimens of BALF obtained during regular examinations of the five PCP patients who were outpatients at the Department of Nephrology in the 16 days between 18 November and 3 December 2011 and of the 20 patients who developed PCP in the 8 years between 2007 and 2014 (Table 1).

Before they were analysed, the BALF specimens were stored at −80°C in a freezer at the Department of Microbiology and Infectious Diseases, Toho University School of Medicine. This study was approved by the Toho University School of Medicine internal review board (approval 26061).

Case definition

Patients were diagnosed with PCP if they met all of the following criteria: (a) compromised cellular immunity due to HIV infection, steroid use or immunosuppressant use; (b) appearance of symptoms such as dry cough, dyspnea or fever; (c) diffuse, bilateral interstitial infiltrates observed in plain chest radiographs; (d) 1,3-β-D-glucan higher than the cutoff value; and (e) *P. jirovecii* cysts recognized by Grocott silver staining of BALF specimens or by positive detection of PCP by PCR amplification of mt26S [21]. Other bacteria- or fungus-positive cases were excluded from this study.

Phylogenetic analysis by *P. jirovecii* MLST

Phylogenetic analysis by *P. jirovecii* was based on PCR amplification and sequencing of housekeeping genes at four loci (β-*TUB,* mt26S, *SOD, CYB*) in DNA extracted from BALF. A 1 mL sample of the residual BALF specimen that had been stored in a freezer at −80°C and thawed at room temperature was centrifuged (13 000 × *g,* 5 minutes, 20°C), and DNA was extracted from the pellet using a QIAamp DNA Mini Kit (Qiagen, Limburg, the Netherlands). The extracted DNA was stored in a freezer at −20°C until PCR analysis. β-*TUB,* mt26S and *CYB* locus PCR were performed as described elsewhere [20]. *SOD* locus PCR was performed with new primers (forward, GGCACTTGAACCTTATC; reverse, CCAAGAATAACTTTGCCTTGAG) and with the following conditions (94°C for 5 minutes; 35 cycles at 94°C for 30 seconds, 55°C for 45 seconds, 72°C for 1 minute; 72°C for 7 minutes). The PCR amplification products were separated by electrophoresis on a 2% agarose gel, stained with ethidium bromide and visualized under ultraviolet irradiation. After the PCR amplification products were confirmed to be of the known fragment size, DNA fragments were purified using a QIAquick PCR Purification Kit (Qiagen). DNA base sequencing was conducted using the conventional Sanger method (FASMAC, Kanagawa, Japan). The resulting DNA base sequence information of the four housekeeping genes was combined, and phylogenetic analysis was carried out [6] using CLUSTALW [22] and MEGA6 [23]. To be specific, we first used CLUSTALW to carry out multiple alignment of the DNA base sequences obtained; the results were then phylogenetically analysed using the neighbour-joining method of MEGA6. Also, sequences were compared to the following reference sequences (accession numbers): AF170964 (β-*TUB*), AF320344 (*CYB*), M58605 (mt26S) and AF146753 (*SOD*) [20].

Analysis of DNA base sequences where overlap was recognized

When the DNA base sequences of the four housekeeping genes were determined by the Sanger method, the DNA bases in some areas overlapped and could not be defined. In these

TABLE 1. Pneumocystis pneumonia patient characteristics and clinical features

Case no.	Age (years) (Sex)	Date of diagnosis	Clinical status	Microscopy positive	PCR positive
1	43 (M)	18 Nov 2011	Renal transplantation	Yes	Yes
2	37 (M)	25 Nov 2011	Renal transplantation	No	Yes
3	57 (F)	29 Nov 2011	Renal transplantation	No	Yes
4	56 (F)	2 Dec 2011	Renal transplantation	No	Yes
5	26 (F)	3 Dec 2011	Renal transplantation	No	Yes
6	35 (M)	9 Feb 2007	HIV	Yes	Yes
7	39 (M)	8 July 2007	HIV	Yes	Yes
8	56 (M)	17 Oct 2007	HIV	Yes	Yes
9	63 (F)	22 Nov 2008	HIV	No	Yes
10	42 (M)	15 Jan 2009	HIV	Yes	Yes
11	36 (M)	21 May 2009	HIV	Yes	Yes
12	38 (M)	10 Mar 2010	HIV	Yes	Yes
13	34 (M)	5 Oct 2010	HIV	Yes	Yes
14	86 (F)	5 Jan 2012	Autoimmune disorder	No	Yes
15	70 (M)	12 Feb 2012	Cholesterol crystal embolization	Yes	Yes
16	32 (F)	21 Mar 2013	Renal transplantation	No	Yes
17	41 (F)	6 Apr 2013	Autoimmune disorder	No	Yes
18	66 (M)	8 Apr 2013	Renal transplantation	No	Yes
19	78 (F)	15 June 2013	Autoimmune disorder	No	Yes
20	60 (M)	26 June 2013	Malignant tumour	No	Yes
21	75 (F)	23 Aug 2013	Autoimmune disorder	No	Yes
22	39 (M)	28 Jan 2014	HIV	Yes	Yes
23	83 (F)	31 Mar 2014	Malignant tumour	No	Yes
24	74 (M)	2 June 2014	Autoimmune disorder	No	Yes
25	65 (F)	17 Oct 2014	Renal transplantation	No	Yes

cases, we were able to identify the type of each of the overlapping bases and to calculate their ratios by using a next-generation sequencer (MiSeq; Illumina, San Diego, CA, USA). Using the primers used for PCR of the BALF, we designed primers (overhang primers) that included the following sequences, which were compatible with the Illumina index sequence: forward, TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG; reverse, GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG) [24]. Using these indexed primers, we conducted another PCR with DNA extracted from BALF and analysed the resulting PCR amplification products using the MiSeq sequencer.

Results

Phylogenetic analysis by *P. jirovecii* MLST

The results of MLST of the four housekeeping genes of *P. jirovecii* found in the BALF from 25 PCP cases are presented in Table 2. Among patients 1 to 5 (i.e. patients who developed PCP at the Department of Nephrology, Toho University Omori Medical Centre, between 18 November and 7 December 2011), the DNA base sequences of β-*TUB,* mt26S and *CYB* of *P. jirovecii* showed a perfect match to that of patients 1, 2, 3 and 4. In the case of *SOD,* however, the 188th base was a T or C in patient 1 but a T in patients 2, 3 and 4. The four genes from *P. jirovecii* in the BALF of these four patients were highly homologous: patients 2, 3 and 4 had matches in all 14 variable base regions, and patient 1 had matches in 13 of 14 regions. Furthermore, the DNA base sequences of β-*TUB,* mt26S and *CYB* of *P. jirovecii* from the BALF of patients 16 and 18 who developed PCP at the Department of Nephrology in 2013 completely matched those of patients 2, 3 and 4. Further, whereas the 83rd base of *SOD* was a T or C in patient 18, it was identified as a C in patients 1, 2, 3, 4 and 16. The base sequences of the four types of housekeeping genes of *P. jirovecii* isolated from BALF obtained from patients 16 and 18 in 2013 were highly homologous with those of patients 1, 2, 3 and 4 isolated in 2011 (patient 16 had matches with patients 2, 3 and 4 in all 14 variable base regions, and patient 18 had matches with patients 2, 3 and 4 in 13 of 14 regions). The DNA base sequences of β-*TUB,* mt26S and *CYB* of *P. jirovecii* from the BALF of patient 5, who developed PCP in 2011, showed a perfect match, but the 83rd and 188th bases of *SOD* were T and C, respectively, and had low homology compared to patients 1, 2, 3, 4, 16 and 18. Patients 2, 3, 4 and 16 had 12 of 14 matches in variable base regions.

The DNA base sequence results of β-*TUB,* mt26S, *CYB* and *SOD* in the BALF of the remaining 18 PCP patients were markedly different from those of the seven renal transplant patients discussed above. The phylogenetic tree constructed by the neighbour-joining method using genes of the four regions revealed that, compared with the genotypes of the other patients, the *P. jirovecii* genotype in patients 1, 2, 3, 4, 16 and 18 was highly homologous (Fig. 1).

Analysis of DNA base sequences where overlap was recognized

When analysing the DNA base sequences by the Sanger method, there were 14 places where two types of base overlapped and could not be determined. This was resolved by using next-generation sequencing. Table 3 shows results of the next-generation sequencing.

TABLE 2. Results of multilocus sequence typing by Sanger method and PCR

	β-*TUB*	*CYB*						mt26S			*SOD*		
Case no.	**282**[a]	**36**[b]	**115**[b]	**162**[b]	**273**[b]	**589–590**[b]	**595**[b]	**48**[c]	**211**[c]	**216**[c]	**83**[d]	**152**[d]	**188**[d]
Reference	A	C	A	G	C	GG	C	G	G	G	N	N	N
1	G	C	A	G	C	TT	T	G	A	G	C	T	T or C[e]
2	G	C	A	G	C	TT	T	G	A	G	C	T	T
3	G	C	A	G	C	TT	T	G	A	G	C	T	T
4	G	C	A	G	C	TT	T	G	A	G	C	T	T
5	G	C	A	G	C	TT	T	G	A	G	T	T	C
6	A or G[e]	C	A	G	C	TT	C	A or G[e]	G	G	C	T	C
7	A	C	A	G	C	TT	C	A	G	G	T	T	C
8	A	T	A	G	C	TT	C	G	T	G	C	T	T
9	A	T	A	G	C	TT	C	G	TTT	G	T	T	C
10	G	C	A	T	C	TT	C	A	G	G	T	T	C
11	G	C	A	G	C	TT	T or C[e]	A or G[e]	G	G	T	T	T or C[e]
12	G	C	A	G	C	TT	C	G	T	G	T	T	C
13	A	C	A	G	C	TT	C	G	G	G	T	T	C
14	G	T	A	G	C	TT	C	G	G	A	C	T	T
15	G	T	A	G	C	TT	C	A	G	G	C	T	C
16	G	C	A	G	C	TT	T	G	A	G	C	T	T
17	G	C	G	G	C	TT	C	A	G	G	C	T	T
18	G	C	A	G	C	TT	T	G	A	G	T or C[e]	T	T
19	G	C	A	G	T or C[e]	TT	C	G	G	G	T	T	C
20	A	C	A	G	T or C[e]	TT	C	A or G[e]	T or G[e]	G	C	T	T
21	G	T	A	G	T	TT	C	G	T	G	T	T	T or C[e]
22	G	C	A	G	C	TT	C	A	G	G	T	T	C
23	A or G[e]	C	A	G	C	TT	C	G	T	G	C	T	T
24	G	C	A	G	C	TT	T or C[e]	G	G	G	C	T	T
25	A	C	A	G	C	TT	T	A	G	G	C	T	T

[a]Nucleotides in β-*TUB* locus at position (bp).
[b]Nucleotides in *CYB* locus at position (bp).
[c]Nucleotides in mt26S locus at position (bp).
[d]Nucleotides in *SOD* locus at position (bp).
[e]DNA bases overlapped and could not be identified.

Discussion

Although eight housekeeping genes have so far been reported for MLST of *P. jirovecii,* three or four of those would be sufficient for discriminating among strains [20]. The following schemes are reported to have shown high discriminatory power in MLST: (ITS1, mt26S, *CYB*), (26S, mt26S, ITS1, β-*TUB*), (mt26S, *CYB, SOD*) [20]. Many PCR amplification failures have been reported with ITS1. We therefore conducted phylogenetic analysis of *P. jirovecii* with four types of housekeeping genes: the combination (mt26S, *CYB, SOD*), which was the only set among the three

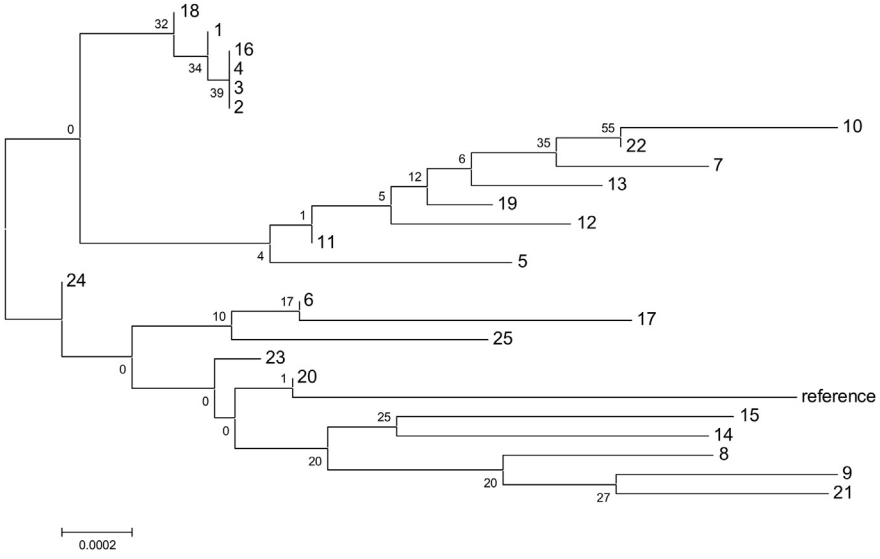

FIG. 1. Tree diagram of Pneumocystis jirovecii MLST. PCR was conducted on four housekeeping genes (β-TUB, mt26S, SOD, CYB). Their DNA base sequence data were combined, and multiple alignments of the DNA base sequences were conducted with CLUSTALW. Phylogenetic analysis was then carried out by neighbour-joining method using MEGA6. Of the five patients diagnosed with PCP in November–December 2011 at the Department of Nephrology, patients 1, 2, 3 and 4 showed high homology with DNA base sequences of P. jirovecii isolated from patients 16 and 18, diagnosed in 2013 in our department. Scale bar = 0.0002. β-TUB, AF170964; CYB, AF320344; mt26S, M58605; SOD, AF146753.

TABLE 3. Result of NGS

Locus	bp	Patient number	Result of NGS, *n* (%) of read		Result of Sanger method
β-*TUB*	282[a]		A	G	
		7 (control case)	25364 (99.9%)	23 (0.1%)	A
		6	15867 (54.6%)	13178 (45.4%)	A or G
		23	10355 (44.8%)	12759 (55.2%)	A or G
CYB	273[b]		C	T	
		7 (control case)	8095 (>99.9%)	4 (<0.1%)	C
		19	648 (32.4%)	1349 (67.6%)	C or T
		20	5575 (54.4%)	4668 (45.6%)	C or T
	595[b]		C	T	
		7 (control case)	7978 (99.8%)	14 (0.2%)	C
		11	4296 (49.9%)	4311 (50.1%)	C or T
		24	499 (56.4%)	386 (43.6%)	C or T
mt26S	48[c]		A	G	
		7 (control case)	22055 (>99.9%)	20 (<0.1%)	A
		6	7983 (43.6%)	10321 (56.4%)	A or G
		11	11555 (46.0%)	13587 (54.0%)	A or G
		20	5086 (37.3%)	8543 (62.7%)	A or G
	211[c]		G	T	
		7 (control case)	21913 (>99.9%)	4 (<0.1%)	G
		20	8524 (55.6%)	6795 (44.4%)	G or T
SOD	83[d]		T	C	
		7 (control case)	10914 (100%)	0 (0%)	T
		18	15753 (36.3%)	27697 (63.7%)	T or C
	188[d]		C	T	
		7 (control case)	10949 (>99.9%)	9 (<0.1%)	C
		1	7732 (43.3%)	10132 (56.7%)	C or T
		11	35718 (49.1%)	36995 (50.9%)	C or T
		21	1025 (38.0%)	1672 (62.0%)	C or T

NGS, next-generation sequencing.
[a]Nucleotides in β-*TUB* locus at position (bp).
[b]Nucleotides in *CYB* locus at position (bp).
[c]Nucleotides in mt26S locus at position (bp).
[d]Nucleotides in *SOD* locus at position (bp).

above that did not include ITS1, plus β-*TUB,* which had shown a high PCR success rate in our experiments.

Among the outpatients of the Department of Nephrology, there was one PCP patient who developed PCP in September 2011 and who was confirmed to have been in the outpatient clinic with the patients of the PCP outbreak in November and December. The patient complained of respiratory symptoms on 10 September and was examined at our department. On a second visit to the outpatient clinic on 13 September, the patient was suspected of having contracted PCP and was hospitalized in a private ward. We could not conduct a genetic analysis because we had not preserved a specimen from this patient's BALF, but we found that patients 2, 3 and 4 had come to the department and had shared the outpatient booth with the patient on 10 September, and that patients 1 and 5 had done the same on 13 September (Fig. 2). If patients 1, 2, 3 and 4—whose *P. jirovecii* genotypes showed a strong homology—had contracted PCP from this active patient (the index patient), the latency period would have been 11 to 12 weeks, which would coincide with reported latencies of 3 to 12 weeks [25], or an average of 53 days (range, 7–188 days) [26]. Patients 16 and 18, who were diagnosed 2 years later in 2013, had genotypes highly homologous with those of patients 1, 2, 3 and 4 but did not share an appointment date at the department with any of those patients. It had been assumed that patients 16 and 18 had been infected with the same strain of *P. jirovecii* as that in 2011 through contact with asymptomatic carriers such as medical personnel or from the waiting room environment, or that they were infected through contact with patients who were carriers but did not exhibit symptoms. However, we believe that their mode of onset was *de novo,* not reactivation [27], so it is considered highly likely that they were infected immediately before onset in 2013.

Among the DNA base sequences of the genes that we studied, the Sanger method revealed 14 places where two different types of base overlapped and could not be identified (Table 2). Analysis with the next-generation sequencer showed that for ten of these 14 places, the percentages were similar for each of the two overlapping bases (Table 3). Furthermore, in the case of patient 7 (a control patient in whom overlap was not observed), for all four types of gene used in MLST in this study, almost all the reads by the next-generation sequencer matched results obtained by the conventional Sanger method. These results attest to the accuracy of the next-generation sequencer analyses (Table 3). Two studies have also reported on the overlap of differing bases [28,29]. They suggest that in such cases, the same patient may have had multiple infections of *P. jirovecii.* From these results, it was apparent that two different types of base were present in the same ratio in many genes. These results also suggest that because *P. jirovecii* is a diploid species, there is a possibility that the genes in each pair of chromosomes are constructed of different bases. Although *P. jirovecii* from the BALF of patient 5 is of low homology compared to that of patients 1, 2, 3, 4, 16 and 18 by phylogenetic analysis, the 83rd and 188th bases of *SOD* were T and C, respectively, in patient 5, which is similar to that of patient 18 (the 83rd base of *SOD* was T or C), and that of patient 1 (the 188th base of *SOD* was T or C). Therefore, patient 5 has high homology if *P. jirovecii* is a diploid species.

Eight renal transplant patients developed PCP on average 7 years (range, 2–12 years) from the time of transplantation, and all patients did not receive sulfamethoxazole/trimethoprim as a prophylactic agent. McCaughan and Courtney [30] recommended providing a prophylactic agent to transplant recipients at high risk of PCP infection.

After the PCP outbreak at Toho University Omori Medical Centre, we identified all renal transplant patients who were receiving immunosuppressants or who were receiving more than 20 mg of prednisolone [31] and who had come to the outpatient examinations on the same dates as the PCP index patient. For these patients, we prescribed a prophylactic regimen of sulfamethoxazole/trimethoprim to be administered regularly for 2 to 3 months. As a result, there were only four cases of PCP at the Department of Nephrology in the 3 years from January 2012 to December 2014.

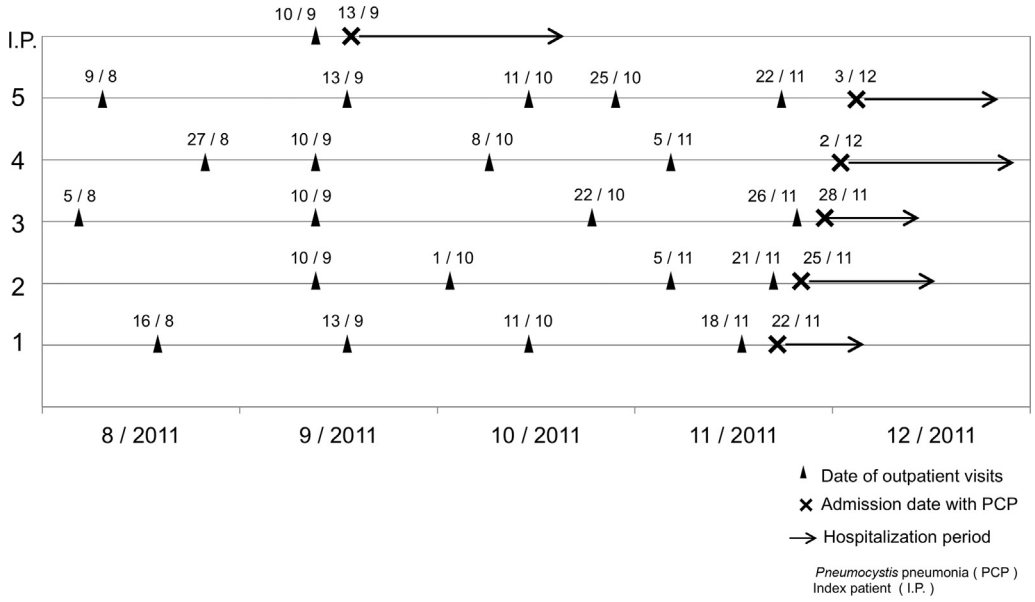

FIG. 2. Transmission map. Index patient (IP) who developed PCP in September 2011 and who visited the Department of Nephrology outpatient clinic at the same time as patients 1 to 5, who were diagnosed in November–December 2011. x-axis represents chronology, y-axis patient numbers. IP shared outpatient booth with patients 2, 3 and 4 on 10 September and with patients 1 and 5 on 13 September.

Through the phylogenetic analysis of gene base sequences, we found a high level of genetic homology in the *P. jirovecii* obtained from patients at the Toho University Omori Medical Centre who had each developed PCP within a short span of time. This indicated a strong possibility that the PCP outbreak was caused by a single strain. Analysis by a next-generation sequencer revealed the presence of genes with two differing types of base in almost identical ratios. These findings may be due to *P. jirovecii* being a diploid species.

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Transparency declaration

All authors report no conflicts of interest relevant to this article.

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