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Multilocus Sequence Typing of *Pneumocystis jirovecii* from Clinical Samples: How Many and Which Loci Should Be Used?

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***Pneumocystis jirovecii* pneumonia (PCP) is an opportunistic infection with airborne transmission and remains a major cause of respiratory illness among immunocompromised individuals. In recent years, several outbreaks of PCP, occurring mostly in kidney transplant recipients, have been reported. Currently, multilocus sequence typing (MLST) performed on clinical samples is considered to be the gold standard for epidemiological investigations of nosocomial clusters of PCP. However, until now, no MLST consensus scheme has emerged. The aim of this study was to evaluate the discriminatory power of eight distinct loci previously used for the molecular typing of *P. jirovecii* (internal transcribed spacer 1 [ITS1], cytochrome *b* [*CYB*], mitochondrial rRNA gene [mt26S], large subunit of the rRNA gene [26S], superoxide dismutase [*SOD*], β-tubulin [β-*TUB*], dihydropteroate synthase [*DHPS*], and dihydrofolate reductase [*DHFR*]) using a cohort of 33 epidemiologically unrelated patients having respiratory samples that were positive for *P. jirovecii* and who were admitted to our hospital between 2006 and 2011. Our results highlight that the choice of loci for MLST is crucial, as the discriminatory power of the method was highly variable from locus to locus. In all, the eight-locus-based scheme we used displayed a high discriminatory power (Hunter [H] index, 0.996). Based on our findings, a simple and alternative MLST scheme relying on three loci only (mt26S, *CYB*, and *SOD*) provides enough discriminatory power (H-index, 0.987) to be used for preliminary investigations of nosocomial clusters of PCP.**

Pneumocystis jirovecii is an opportunistic fungal pathogen with humans as its only host (1, 2). *P. jirovecii* can be responsible for a severe pulmonary disease known as *P. jirovecii* pneumonia (PCP) in immunocompromised subjects, such as HIV-infected patients with CD4 cell counts of <200 cells/mm^3, hematopoietic stem cell or solid organ transplant recipients, or those receiving high doses of corticosteroids for several months (3, 4). In recent years, intense research has been conducted, leading to a better understanding of *Pneumocystis* biology and epidemiology (5, 6). As shown in several studies, *P. jirovecii* is commonly recovered from the respiratory tracts of immunocompetent subjects in the general population, with a prevalence rate ranging from 20% to 65% (7–9). Importantly, Choukri et al. (10) recently provided the first demonstration of *P. jirovecii* that was spread through the surrounding air of infected patients, supporting the risk of direct interhuman transmission. Recently, the role of colonized patients as potential reservoirs of *P. jirovecii* has been nicely illustrated by Le Gal and coworkers (11). Since the first putative description of interhuman transmission of *P. jirovecii* in 1967, a large number of nosocomial outbreaks of PCP (sometimes referred to as clusters) have been reported in the literature, most of them being described in kidney transplant recipients (12, 13). Usually, epidemiological investigations of PCP outbreaks rely on the study of patient encounters together with molecular typing to search for a single *P. jirovecii* clone infecting distinct patients (11, 14–16). Although numerous typing methods have been developed, multilocus sequence typing (MLST) is now considered to be the gold standard (16–18). Moreover, it offers many advantages over other methods, such as reproducibility and the possibility of exchanging data from different laboratories. Up to 17 coding and noncoding DNA regions of the *P. jirovecii* genome have been explored for their allelic polymorphisms: mitochondrial rRNA gene (mt26S; also referred to as mtLSU rRNA), internal transcribed spacer 1 (ITS1), *ITS2*, β-tubulin (β-*TUB*), large subunit of the rRNA gene (26S rRNA), mitochondrial small subunit (mtSSU) rRNA, superoxide dismutase (*SOD*), cytochrome *b* (*CYB*), thymidylate synthase (*TS*), 5.8S rRNA, *AROM*, *TRR1*, *UCS*, *MSG*, *KEX1*, dihydrofolate reductase (*DHFR*), and dihydropteroate synthase (*DHPS*) (18–20). Unfortunately, and despite the increasing number of studies reporting nosocomial clusters of PCP, no consensus MLST scheme has yet emerged. As a consequence, various schemes have been developed relying on two, three, or four to eight loci (11, 16–18, 21–24). Therefore, data exchangeability and comparisons between studies are not possible. Moreover, as the levels of allelic polymorphisms clearly differ between loci, the question of the performance of each of these typing schemes can be raised (23, 25). In the present study, our aim was to evaluate the performance, in terms of discriminatory power, of a multilocus sequence typing method relying on eight loci that were previously investigated for the molecular typing of *P. jirovecii*.

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MATERIALS AND METHODS

Clinical samples. Thirty-three respiratory samples that were positive for *P. jirovecii* obtained from 33 epidemiologically unrelated patients who were admitted to our hospital between 2006 and 2011 were included in this study. Most were bronchoalveolar lavage fluid (BAL) samples. *P. ji-*

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TABLE 1 Nucleotide sequences of primers used in this study

Locus	Forward or reverse primer	Nucleotide sequence	Product size (bp)	Reference(s)
mt26S	mt26S-F	GATGGCTGTTTCCAAGCCCA	347	45
	mt26S-R	GTGTACGTTGCAAAGTACTC		
26S rDNA	26S-F	GAAGAAATTCAACCAAGC	426	17
	26S-R	ATTTGGCTACCTTAAGAG		
ITS1	ITS1-F	CTGCGGAAGGATCATTAGAAA	204	17
	ITS1-R	CGCGAGAGCCAAGAGATC		
β-*TUB*	β-Tubulin-F	TCATTAGGTGGTGGAACGGG	309	17
	β-Tubulin-R	ATCACCATATCCTGGATCCG		
SOD	MnSODFw	GGGTTTAATTAGTCTTTTTAGGCAC	652	43
	MnSODRw	CATGTTCCCACGCATCCTAT		
CYB	CytbFw	CCCAGAATTCTCGTTTGGTCTATT	638	43
	CytbRw	AAGAGGTCTAAAAGCAGAACCTCAA		
DHPS	Ahum	GCGCCTACACATATTATGGCCATTTTAAATC	318	46, 47
	Bhs′	ACCTTCCCCCACTTATATC		
DHFR	FR208	GCAGAAAGTAGGTACATTATTACGAGA	610	26
	FR1018	AAGCTTGCTTCAAACCTTGTGTAACGCG		

rovecii was detected in each sample by microscopic examination following Gomori-Grocott staining and/or using a specific real-time PCR assay targeting the mtLSU rRNA gene on a Rotor-Gene 3000 instrument (Qiagen, Courtaboeuf, France). Thirty-one of these patients (94%) fulfilled the criteria for PCP diagnosis (1). The remaining two patients (patients 28 and 30 [6%]) were considered to be being colonized by *P. jirovecii*, as both had a positive PCR for *P. jirovecii* without clinical symptoms. HIV infection was the main underlying disease in these patients ($n = 15$ [45%]), followed by hematological malignancies or cancer ($n = 5$ [15%]), solid organ transplantation ($n = 5$ [15%]), or immune disorders ($n = 8$ [24%]). Except for three patients receiving trimethoprim-sulfamethoxazole (patients 10 and 11) or pentamidine (patient 16), most of the remaining patients were not being given anti-*Pneumocystis* chemoprophylaxis at the time of the recovery of *P. jirovecii* ($n = 29$ [88%]; data were unavailable for one patient). This study was approved by the Comité de Protection des Personnes, Ouest IV, France.

Multilocus sequence typing of *P. jirovecii* from clinical samples. DNA extraction was performed on an iPrep instrument (Invitrogen, Groningen, The Netherlands) with the iPrep PureLink reagent, as recommended by the manufacturer. Briefly, 1 ml of each respiratory sample was centrifuged at 3,000 rpm for 10 min. Two hundred microliters of the pellet was subjected to DNA extraction. DNA extracts were stored at −20°C until PCR analysis. Genotyping was performed at the eight following loci: large subunit of the mitochondrial rRNA gene (mt26S), large subunit of the rRNA gene (26S), internal transcribed spacer 1 (ITS1), β-tubulin (β-*TUB*), superoxide dismutase (*SOD*), cytochrome *b* (*CYB*), dihydrofolate reductase (*DHFR*), and dihydropteroate synthase (*DHPS*). All these loci have been previously reported in molecular investigations of nosocomial clusters of *P. jirovecii* (18). To avoid cross-contamination between samples, only single-round PCRs were performed (no nested PCRs). The nucleotide sequences of each primer are given in Table 1. PCRs were carried out in a 25-μl final volume using *Premix Ex Taq* (perfect real-time) (TaKaRa Bio, Inc., Otsu, Shiga, Japan) and 5 μl of each DNA extract. The final concentration of each primer was 0.5 μM. Amplification was conducted on an Applied GeneAmp 9700 (Applied Biosystems, Foster City, CA) under the following conditions: 7 min at 94°C followed by 35 cycles, including 30 s at 94°C, 45 s at 60°C, 30 s at 72°C, and a final elongation step at 72°C for 7 min. PCR products were purified and sequenced on a 3130xl genetic analyzer (Applied Biosystems). Nucleotide sequences were analyzed using the SeqScape software (Applied Biosystems). Sequences were compared to the following reference sequences with the accession numbers U07220 (ITS1), AF320344 (*CYB*), M58605 (mt26S), L13615 (26S), AF146753 (*SOD*), AF170964 (β-*TUB*), AY628435 (*DHPS*), and AF090368 (*DHFR*). When available, genotypes were named according to the previous published nomenclature (17, 23, 26–28). Each new mutation was confirmed with a second round of amplification and sequencing.

Discriminatory power can be defined as the ability of a typing method to differentiate between any strains chosen at random. Here, the discriminatory power of each locus was determined by the Hunter index (H-index), with an index value of 0.95 being considered suitable for discrimination between isolates (29, 30). Briefly, an H-index of 0.95 means that there is a 95% chance that any two random unrelated samples will be different with respect to the DNA sequences observed. Mixed infections (i.e., distinct *P. jirovecii* genotypes in a single clinical sample) were not considered for the analysis of discriminatory power (30). The Hunter index was determined for the full MLST scheme (eight loci) and for several combinations, including some previously reported in the literature, to propose a simple and efficient MLST scheme that is useful for preliminary investigations of PCP outbreaks.

RESULTS

Amplification and sequencing of each locus were achieved for most of the clinical samples and loci (Table 2). In all, *CYB*, mt26S, β-*TUB*, *SOD*, and *DHPS* could be examined for most samples and patients. Amplification failures were mainly observed for the ITS1 locus (five samples could not be analyzed). Several new alleles and genotypes were identified at some loci (Table 3). For example, three new ITS1 genotypes (named A4, B5, and B6) were observed among the 33 patients. As expected from previous studies, the level of allelic polymorphisms and therefore the performance of each MLST scheme clearly differed between the eight loci. ITS1, *CYB*, and mt26S all exhibited higher discriminatory power (H-indices, 0.828, 0.794, and 0.751, respectively), being able to identify nine, seven, and four genotypes, respectively, among the 33

TABLE 2 Results of genotyping of *P. jirovecii* at the eight loci[a]

Patient no.	Sample type[b]	Genotype determined in each locus								Multilocus genotype
		ITS1	*CYB*	mt26S	*SOD*	26S	β-*TUB*	*DHFR*	*DHPS*	
1	BAL	B	*CYB* 1	8	*SOD* 2	5	β-*TUB* 3	WT[d]	WT	A
2	BAL	B1	*CYB* 2	7	*SOD* 1	1	β-*TUB* 3	WT	WT	B
3	BAL	B5	*CYB* 1	8	*SOD* 2	1	β-*TUB* 3	WT	WT	C
4	BAL	B	*CYB* 9	7	*SOD* 2	8	β-*TUB* 3	*DHFR* 312	WT	D
5[f]	BAL	A5	*CYB* 1	8	*SOD* 2+ *SOD* 1	5	β-*TUB* 1	*DHFR* 312	WT	Mixed[e]
6	BAL	B	*CYB* 1	2	*SOD* 2	5	β-*TUB* 3	*DHFR* 201	WT	E
7	BAL	B2	*CYB* 1	7	*SOD* 1	1	β-*TUB* 1	WT	WT	F
8	BAL	B1	*CYB* 2	3	*SOD* 1	ND[c]	β-*TUB* 1	*DHFR* 312	WT	G
9	BAL	ND	*CYB* 7	8	*SOD* 2	5	β-*TUB* 3	*DHFR* 312	WT	H
10	BAL	B	*CYB* 2	7	*SOD* 1	5	β-*TUB* 1	WT	WT	I
11	BAL	ND	*CYB* 2 + *CYB* 8	7 + 3	*SOD* 2	ND	β-*TUB* 1 + β-*TUB* 3	*DHFR* 312	WT	Mixed
12	BAL	B2	*CYB* 5	7	*SOD* 1	1	β-*TUB* 1	WT	WT	J
13	BAL	A3	*CYB* 8	8	*SOD* 2	5	β-*TUB* 3	WT	WT	K
14	BAL	A3	*CYB* 2	3	*SOD* 1	5	β-*TUB* 3	WT	WT	L
15	BAL	A4	*CYB* 2	8	*SOD* 1	5	β-*TUB* 3	WT	WT	M
16	BAL	B3	*CYB* 1	2	*SOD* 1	9	β-*TUB* 1	WT	WT	N
17	BAL	A4	*CYB* 6	8	*SOD* 2	10	β-*TUB* 3	WT	WT	O
18	BAL	A3	*CYB* 1 + *CYB* 2	3	*SOD* 1	5	β-*TUB* 1	WT	WT	Mixed
19	TRA	A3	*CYB* 1	3	*SOD* 1	5	β-*TUB* 1	*DHFR* 312	ND	P
20	BAL	A4	*CYB* 6	2	*SOD* 2	ND	β-*TUB* 1 + β-*TUB* 3	ND	WT	Mixed
21	BAL	B1	*CYB* 1	8	*SOD* 5	5	β-*TUB* 3	WT	WT	Q
22	BAL	B1	*CYB* 8	7	*SOD* 1	5	β-*TUB* 1 + β-*TUB* 3	WT	WT	Mixed
23	BAL	B	*CYB* 8	2	*SOD* 2	5	β-*TUB* 1	WT	WT	R
24	BAL	A3	*CYB* 2	3	ND	5	β-*TUB* 3	ND	WT	L
25	BAL	B	*CYB* 2	8	*SOD* 5	6	β-*TUB* 3	WT	WT	S
26	BAL	B	*CYB* 1 + *CYB* 6	2	*SOD* 1	1 + 5	β-*TUB* 1	WT	WT	Mixed
27	BAL	B	*CYB* 8	8	*SOD* 1	5	β-*TUB* 1	ND	WT	T
28	BAL	B	*CYB* 7	7	*SOD* 1	5	β-*TUB* 1	WT	WT	U
29	BAL	ND	*CYB* 1 + *CYB* 7	7	*SOD* 1	1 + 5	β-*TUB* 3	WT	WT	Mixed
30	SPU	ND	*CYB* 1	7 + 8 + 2	*SOD* 2	5	β-*TUB* 1 + β-*TUB* 3	*DHFR* WT + *DHFR*312	WT	Mixed
31	BAL	B6	*CYB* 1	7	*SOD* 2	7	β-*TUB* 1	WT	WT	V
32	BAL	B	*CYB* 3 + *CYB* 1	7 + 8	*SOD* 4 + *SOD* 3	5	β-*TUB* 1	WT	WT	Mixed
33	BAL	ND	*CYB* 8	7 + 3	*SOD* 1	ND	β-*TUB* 1	WT	WT	Mixed

[a] New genotypes are underlined.
[b] BAL, bronchoalveolar lavage fluid; TRA, tracheal aspiration; SPU, sputum.
[c] ND, not determined.
[d] WT, wild type.
[e] Mixed, more than one *P. jirovecii* genotype. For each sample or patient displaying a mixed genotype, the putative combination of genotypes is given.
[f] The ITS1 genotype observed for patient 5 (T/2, TT/8–10, A/11, T/17, T/22, TC/46–47, 10 × T/54–62, GG/70–71, TTA/111–113) that has been previously reported but never designated was therefore named A5.

patients (Table 4). Other loci, such as β-*TUB*, *DHFR*, and *DHPS* displayed only a few allelic polymorphisms, leading to a lower H-index. For example, only two β-*TUB* genotypes (β-*TUB* 1 and β-*TUB* 3) could be identified in this data set (H-index, 0.517). Except for the sample from patient 19, in which *DHPS* could not been amplified, all the remaining patients had the wild-type sequence (i.e., no substitution was observed at residues 55 and 57). Apart from the eight samples and patients displaying the synonymous mutations T201A or T312C, no other mutation was identified at the *DHFR* locus. From a genotyping perspective, taken individually, none of these loci was polymorphic enough to enable a sufficient discrimination of *P. jirovecii* isolates (H-index, <0.95), highlighting the need for a multilocus typing strategy.

Considering the entire data set, 23 distinct *P. jirovecii* multilocus genotypes (MLGs) were evidenced among the 33 patients (Table 2; A to V and mixed genotypes). Ten patients had mixed *P. jirovecii* MLGs, suggesting coinfection by genetically distinct *P. jirovecii* isolates (10 of 33 [30%]). Only two samples (from patients 14 and 24) could not be differentiated due to amplification failures at some loci. However, interhuman transmission of a single *P. jirovecii* clone between these patients is unlikely, as both subjects were hospitalized during two distinct periods (February and October 2010) and in different intensive care units.

In the subset of patients with a single *P. jirovecii* multilocus genotype ($n = 23$ [70%]), the full eight-locus MLST scheme was highly effective and yielded a high discriminatory index (H-index, 0.996), showing that this typing method is a powerful tool for the investigation of PCP outbreaks. Based on this data set, we evaluated several combinations of loci that were previously reported in the literature to propose a reduced scheme that could be used for

TABLE 3 New alleles and nucleotide polymorphisms identified in this study[a]

Locus	Allele/ genotype	Nucleotide position/identity
ITS1	A4	C/2, TT/8–10, A/11, T/17, T/22, TC/46–47, 10 × T/54–62, GG/70–71, TTA/111–113
	B5	T/2, TT/8–10, A/11, A/17, T/22, TATC/46–47, 10 × T/54–62, GAGG/70–71, TTA/111–113
	B6	T/2, TT/8–10, A/11, A/17, T/22, TC/46–47, 11 × T/54–62, GAGG/70–71, TTA/111–113
CYB	CYB8	T/279, C/299, A/348, C/362, G/369, C/516, C/547, C/566, A/675, C/742, TT/832–833, C/838
	CYB9	C/279, C/299, A/348, C/362, G/369, C/516, C/547, C/566, T/675, C/742, TT/832–833, C/838
SOD	SOD5	C/110, C/191, T/215
26S	6	A/3, A/34, A/78, A/212, T/296, ACTCT/301–305, T/306, A/308.1[b]
	7	A/3, A/34, A/78, A/212, T/248.1[b], T/296, ACTCT/301–305, T/306, G/356.1[b]
	8	A/3, A/34, A/78, A/212, TT/248.1[b], T/296, TACTC/301–305, T/306
	9	A/3, G/34, A/78, A/212, (T)/296[c], ACTCT/301–305, T/306
	10	A/3, A/34, A/78, A/212, T/248.1[b], T/296, ACTCT/301–305, T/306

[a] New mutations are underlined.
[b] Nucleotide insertion.
[c] Nucleotide deletion.

TABLE 4 Discriminatory power by locus[a]

Locus	No. of samples used to calculate Hunter index	Total no. of genotypes	Distribution of genotypes (no. of samples)	Hunter index
ITS1	28	9	B (10) A3 (5) B1 (4) A4 (3) B2 (2) B3 (1) A5 (1) B5 (1) B6 (1)	0.828
CYB	28	7	*CYB* 1 (10) *CYB* 2 (7) *CYB* 8 (5) *CYB* 7 (2) *CYB* 6 (2) *CYB* 5 (1) *CYB* 9 (1)	0.794
mt26S	29	4	8 (10) 7 (9) 2 (5) 3 (5)	0.751
SOD	30	3	*SOD* 1 (16) *SOD* 2 (12) *SOD* 5 (2)	0.570
26S	27	7	5 (18) 1 (4) 6 (1) 7 (1) 8 (1) 9 (1) 10 (1)	0.547
β-*TUB*	29	2	β-*TUB* 1 (15) β-*TUB* 3 (14)	0.517
DHFR	29	3	WT[b] (22) *DHFR* 312 (6) *DHFR* 201 (1)	0.394
DHPS	32	1	WT (32)	0

[a] Samples containing mixed genotypes were not considered. New genotypes are underlined.
[b] WT, wild type.

preliminary investigations of PCP outbreaks. Interestingly, the four-locus-based scheme relying on ITS1, 26S, mt26S, and β-*TUB*, first published by Hauser and coworkers and now used in other studies, displayed a high discriminatory power (H-index, 0.987) (Table 5). Of note, the discriminatory power of this scheme was previously estimated to be 0.93 (30). One explanation for the lower H-index reported by Hauser is that the scheme was first used as a PCR-single-strand conformation polymorphism (PCR-SSCP) rather than an MLST. Importantly, two three-locus MLST schemes also displayed a high H-index, even greater than the scheme described by Hauser: ITS1, mt26S, and *CYB* (H-index, 0.996), and *SOD*, mt26S, and *CYB* (H-index, 0.987). Whereas the former scheme displayed high discriminatory power nearly equal to that of the eight-locus MLST procedure, the lower amplification efficiency noted for ITS1 might limit its use in routine clinical practice. Decreasing the number of loci significantly reduced the performance of the method, with only two combinations displaying an H-index of >0.95: ITS1 with *CYB* (H-index, 0.983) and mt26S with *CYB* (H-index, 0.957) (Table 5).

In all, two distinct MLST schemes, (26S, mt26S, ITS1, and β-*TUB*) and (mt26S, *CYB*, and *SOD*), offered high performance for the molecular typing of *P. jirovecii* from clinical samples, the latter offering the advantages of relying on three loci only and providing high amplification efficiency even without using a nested-PCR strategy.

DISCUSSION

Since the first putative description of a nosocomial cluster of *P. jirovecii*, considerable advances have been made in the understanding of *P. jirovecii* biology and epidemiology (12). It is now clear that the prevalence of *P. jirovecii* in humans, its only host, is high in the general population and that airborne appears to be the main route for interhuman transmission (9, 10). In the past 10 years, increasing numbers of nosocomial outbreaks of PCP have been described worldwide (11, 14–16, 31, 32). In most instances, these cases were described in kidney transplant recipients, and interhuman transmission was confirmed in most reports by molecular typing (13). In France, to the best of our knowledge, at least eight distinct outbreaks have been reported since 1990 (11, 32–38). Epidemiological investigations of a putative nosocomial cluster of PCP usually rely on the study of patient encounters through

TABLE 5 Performance of several previously published schemes for molecular typing of *P. jirovecii*, evaluated by the Hunter index

Molecular typing scheme	Discriminatory power according to our data (H-index)	No. of clinical samples used for determination of H-index[a]	Reference(s) or source
ITS1, β-*TUB*, 26S, mt26S, *CYB*, *SOD*, *DHPS*, *DHFR*	0.996	23	This study
ITS1, mt26S, *CYB*	0.996	23	This study
SOD, mt26S, *CYB*	0.987	22	This study
ITS1, 26S, mt26S, β-*TUB*	0.987[b]	22	14, 15, 17, 30–32
ITS1, *CYB*	0.983	22	This study
mt26S, *CYB*	0.957	23	This study
ITS1, mt26S	0.948	22	24
ITS1	0.828	28	21
mt26S	0.751	29	22, 33

[a] Only samples containing a single *P. jirovecii* genotype were included in the analysis.
[b] The discriminatory power of this method (when used as a PCR-SCCP) was 0.93.

a transmission map (11, 14–16), combined with the molecular typing of *P. jirovecii* performed directly on clinical samples, as this fungal pathogen cannot be cultured *in vitro* (1). Whereas >15 distinct polymorphic DNA regions within the *P. jirovecii* genome have been investigated to date, no consensus MLST scheme for the investigation of PCP outbreaks has been clearly defined and evaluated (18). As a consequence, because most centers use their own strategy, results cannot be compared, thus making population studies unconceivable. In the present study, our aim was to evaluate the performance of an eight-locus MLST scheme on a cohort of 33 epidemiologically unrelated patients who had respiratory samples that were positive for *P. jirovecii*.

As expected from previous studies, variable amplification rates were observed at each individual locus. Amplification failures were mainly observed for ITS1, making this locus unavailable for study in some patient samples. These findings, which have been also reported by others, might be explained by (i) the number copies of each locus within the *P. jirovecii* genome, (ii) the low fungal burden observed in some patients, such as those being colonized by *P. jirovecii*, (iii) and/or the use of noninvasive methods for collecting respiratory samples (24, 25, 39–42). Several authors have overcome this problem by using a nested-PCR approach (11, 16, 42). Here, we decided not to use nested-PCR due to the potential risk of carryover contamination. Importantly, this single-round PCR strategy allowed for the amplification and sequencing of nearly all analyzed loci for each of the 33 patients included in this study. However, this might be considered a limitation of our study, making difficult the investigation of patients who are colonized by *P. jirovecii*.

Infection of a single patient by two (or more) *P. jirovecii* isolates seems to be a common event and has been reported by several authors (17, 28, 41, 43). Such infections can be easily detected by MLST, as infection by genetically distinct strains must theoretically lead to one (or more) heterozygous positions. In the present study, mixed infections have been identified in 10 of the 33 patients (30%). However, we cannot exclude the possibility that the real prevalence of mixed infections might be higher in our data set, as PCR amplification and direct sequencing could theoretically have failed to detect a minority genotype. Several new genotypes resulting from new allelic combinations, and new single-nucleotide polymorphisms were identified and highlight the considerable number of genetic polymorphisms of the *P. jirovecii* genome. According to Tsolaki and coworkers (44), the number of T's at positions 54 to 62 might vary within a single sample when resequencing is performed. However, in agreement with the approach in other studies, this poly(T) tract was not considered in this study, as we never observed this phenomenon in our data set (14, 15). The design of our study in relying on the analysis of clinical samples obtained from epidemiologically unrelated patients, meaning that these patients had probably acquired PCP from independent sources of infection (as they were hospitalized at distinct time periods and in different medical units), is of overall importance. Indeed, it represents a perfect situation for investigating the performance of a molecular typing method, since all *P. jirovecii* isolates could be assumed to be genetically distinct (also confirmed by our data). In the present article, we underline that the choice of loci used for the molecular typing of *P. jirovecii* from clinical specimens is crucial and may affect discriminatory power. One of our important findings is the high performance of the eight-locus MLST scheme as demonstrated by the H-index value (H-index, 0.996). However, as this procedure can be time-consuming, a second aim of this project was to examine a reduced scheme displaying sufficient discriminatory power to be used for preliminary investigations of PCP outbreaks, as well as to examine the performance of several MLST schemes that were previously published by others (17, 20, 22, 24, 33). Unfortunately, the recently proposed MLST scheme relying on five loci (mt26S, ITS1, ITS2, β-*TUB*, and *DHPS*) could not be evaluated, as *ITS2* was not included in our study (http://mlst.mycologylab.org). Our data suggest that at least three to four loci are required for sufficient discrimination of *P. jirovecii* isolates. Indeed, some previously published schemes relying on one or two loci displayed insufficient performance (H-index, <0.95) and may lead to underestimating the genetic diversity of *P. jirovecii*, increasing the risk that the isolates will be identified, by default, as a clonal cluster (20, 24, 33). In our study, two MLST schemes appeared to provide suitable discriminatory power to be used for primary investigations of PCP outbreaks: the four-locus scheme first described by Hauser (ITS1, 26S, mt26S, and β-*TUB*) and the new scheme evaluated in the present study (*SOD*, mt26S, and *CYB*). The latter strategy offers several advantages over the former, in that it is easy to use (three loci only) and has high amplification efficiency while maintaining a similar discriminatory power.

In conclusion, this study highlights the overall importance of the choice and number of loci of the MLST scheme for the molecular investigation of nosocomial outbreaks of *P. jirovecii*. Based on our findings, the full MLST scheme relying on eight loci appears to be a powerful method for the molecular typing of *P. jirovecii*. In the clinical setting, using a simplified procedure, such as *SOD*, mt26S, and *CYB* or ITS1, 26S, mt26S, and β-*TUB* is proposed to be an efficient alternative strategy for preliminary investigations. Together with an analysis of patient encounters, these methods would allow for a rapid conclusion to be made about possible interhuman transmission of *P. jirovecii* in a medical unit.

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