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Highly Conserved *gsc1* Gene of *Pneumocystis jirovecii* in Patients with or without Prior Exposure to Echinocandins

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ABSTRACT Echinocandins are noncompetitive inhibitors of the GSC1 subunit of the enzymatic complex involved in synthesis of 1,3-beta-D-glucan, a cell wall component of most fungi, including *Pneumocystis* spp. Echinocandins are widely used for treating systemic candidiasis and rarely used for treating *Pneumocystis* pneumonia. Consequently, data on *P. jirovecii* *gsc1* gene diversity are still scarce compared to that for the homologous *fks1* gene of *Candida* spp. In this study, we analyzed *P. jirovecii* *gsc1* gene diversity and the putative selection pressure of echinocandins on *P. jirovecii*. *gsc1* gene sequences of *P. jirovecii* specimens from two patient groups were compared. One group of 27 patients had prior exposure to echinocandins, whereas the second group of 24 patients did not, at the time of *P. jirovecii* infection diagnoses. Two portions of the *P. jirovecii* *gsc1* gene, HS1 and HS2, homologous to hot spots described in *Candida* spp., were sequenced. Three single-nucleotide polymorphisms (SNPs) at positions 2204, 2243, and 2303 close to the HS1 region and another SNP at position 4540 more distant from the HS2 region were identified. These SNPs represent synonymous mutations. Three *gsc1* HS1 alleles, A, B, and C, and two *gsc1* HS2 alleles, a and b, and four haplotypes, Ca, Cb, Aa, and Ba, were defined, without significant difference in haplotype distribution in both patient groups ($P = 0.57$). Considering the identical diversity of *P. jirovecii* *gsc1* gene and the detection of synonymous mutations in both patient groups, no selection pressure of echinocandins among *P. jirovecii* microorganisms can be pointed out so far.

KEYWORDS *Pneumocystis jirovecii*, *gsc1*, 1,3-beta-D-glucan, echinocandins, genomic diversity, hot spots

Pneumocystis jirovecii is an opportunistic ascomycete responsible for severe pneumonia in immunosuppressed patients (1). *Pneumocystis* pneumonia (PCP) remains the most frequent AIDS-defining illness in developed countries, including France (2, 3). PCP is also observed with increased frequency in non-HIV-infected immunosuppressed patients, such as solid-organ transplant recipients and patients with hematological malignancies or solid cancers (4). For these reasons, PCP is still a public health issue. The drugs for PCP prophylaxis or treatment target enzymes or co-enzymes that are involved in *Pneumocystis* metabolic pathways. The sulfonamides that target the dihydropteroate synthase (DHPS) are widely used in association with trimethoprim or trimethoprim analogues. *P. jirovecii* organisms with nonsynonymous mutations mostly at

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nucleotide positions 165 (A165G; Thr55Ala) and 171 (C171T; Pro57Ser) on the *dhps* locus have been detected in patients with PCP (reviewed in reference 5). Prior exposure to sulfonamide drugs has been identified as a predictor of mutant genotypes (reviewed in reference 5). Establishing correlations between mutants and resistance is problematic, since *Pneumocystis* spp. are uncultivable. Nonetheless, it was shown that the corresponding mutations in a cultivable yeast *Saccharomyces cerevisiae* model may confer sulfonamide resistance (6–8). Trimethoprim and trimethoprim analogues target the dihydrofolate reductase (DHFR). The diversity of the DHFR gene of *P. jirovecii* has been investigated, and it showed numerous nonsynonymous mutations (9, 10). Although it is usually admitted that monotherapy with trimethoprim or trimethoprim analogues plays a minimal role in PCP treatment, specific mutations may contribute to resistance to this drug (9, 10). Atovaquone is a second-line drug for PCP treatment and prophylaxis. The drug targets the co-enzyme cytochrome *b*. Nonsynonymous mutations in the cytochrome *b* gene that may confer resistance to atovaquone have been identified (11). Specifically, we recently described a previously unreported A144V mutation (C431T; Ala144Val) that potentially diminished the sensitivity of *P. jirovecii* to atovaquone, resulting in spread of the fungus among heart transplant recipients submitted to atovaquone prophylaxis (12). Echinocandins are noncompetitive inhibitors of the GSC1 subunit of the enzymatic complex involved in 1,3-beta-D-glucan synthesis, 1,3-beta-D-glucan being a major cell wall component of most fungi, including *Pneumocystis* species asci (13). However, the use of these drugs for PCP treatment was not initially considered (13). On the other hand, echinocandins are widely used as a treatment for systemic candidiasis (14) or invasive aspergillosis (15). In these circumstances, nonsynonymous mutations that confer resistance to caspofungin, the most widely used echinocandin, have been identified on the *fkp1* gene of the fungal pathogens *Candida* spp. (16), whereas data on the diversity of the *gsc1* gene (homologous to *fkp1* gene) of *P. jirovecii* are still scarce (17). The aforementioned mutations were found in two hot spots of the *fkp1* gene, named HS1 and HS2 (18, 19). The objectives of the present study were to provide original data on the diversity of the *gsc1* gene of *P. jirovecii* and to test the hypothesis that echinocandins exert selective pressure on *P. jirovecii* organisms. Partial sequences of the *gsc1* gene of *P. jirovecii* specimens obtained from two patient groups were compared. One group had prior exposure to echinocandins, whereas the second one did not, at the time of *P. jirovecii* infection diagnoses.

RESULTS

The sequencing of the *gsc1* gene was performed on *P. jirovecii* specimens from 51 patients focusing on two regions, HS1 and HS2. However, the HS1 region was not amplified in patient B31 and the HS2 region was not amplified in patients B27 and B29. Three SNPs were observed at nucleotide positions 2204 (A or G), 2243 (C or T), and 2303 (C or A), as previously reported by Luraschi et al. (17), based on sequences referenced by Cissé et al. (20) and Ma et al. (21). A previously undescribed SNP was observed at nucleotide position 4540 with residue A or G (Fig. 1, Tables 1 and 2). The aforementioned SNPs close to the HS1 region and more distant from the HS2 region correspond to synonymous mutations that were not involved in codon changes. However, examination of HS1 and HS2 regions *stricto sensu* did not reveal any SNPs. Alignments of *P. jirovecii* GSC1 proteins with GSC1 proteins of *C. albicans*, *C. tropicalis*, *C. krusei* (18), and *S. cerevisiae* (22) determined that *P. jirovecii* HS1 and HS2 regions were located at amino acids positions 714 to 722 and 1393 to 1400, respectively (Fig. 2).

Three *P. jirovecii* *gsc1* HS1 alleles, named A, B, and C, were determined considering the SNPs found close to the HS1 region. Two *gsc1* HS2 alleles, named a and b, were determined considering the SNP found close to the HS2 region (Tables 1 and 2). Allele C was observed in 24 patients of the first group and in 23 patients of the second group. Allele B was observed in one patient of each group. Allele A was observed in two patients of each group.

Allele a was observed in 17 patients of the first group and in 19 patients of the second group. Allele b was observed in 11 patients of the first group and in seven patients

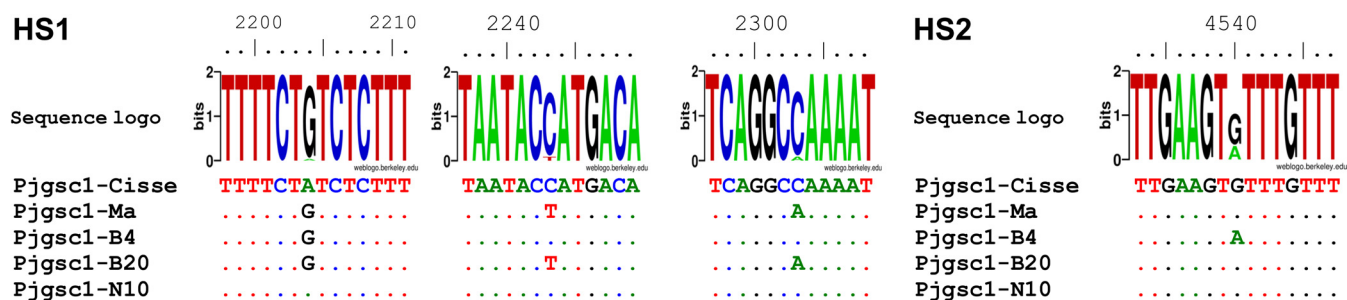


FIG 1 Single-nucleotide polymorphisms in *Pneumocystis jirovecii* hot spot 1 (HS1) and HS2 DNA regions after sequencing. Analysis of 51 *P. jirovecii* specimens led to the identification of three SNPs close to the HS1 region at positions 2204, 2243, and 2303 and one SNP more distant from the HS2 region at position 4540. Only the surrounding regions of polymorphic positions are shown. Sequence logos were created using the WebLogo application (<https://weblogo.berkeley.edu/logo.cgi>). The height of each amino acid symbol indicates the relative frequency of each amino acid at that position. Dots indicate identical nucleotides to the reference sequence. Pjgsc1-Cisse, *gsc1* gene sequence of *P. jirovecii* retrieved from Cissé and colleagues' genome (20); Pjgsc1-Ma, *gsc1* gene sequence of *P. jirovecii* retrieved from Ma and colleagues' genome (21); Pjgsc1-B4, *gsc1* gene sequence of *P. jirovecii* from patient B4; Pjgsc1-B20, *gsc1* gene sequence of *P. jirovecii* from patient B20; Pjgsc1-N10, *gsc1* gene sequence of *P. jirovecii* from patient N10.

of the second group. Considering the aforementioned SNPs, four haplotypes combining HS1 and HS2 alleles, named Ca, Cb, Aa, and Ba, were identified. Mixed infections that corresponded to more than one putative haplotype in the same specimen were detected in three patients, N2, N12, and N15, of the first group and in three patients, B2, B21, and B23, of the second group. Haplotypes were not determined when mixed

TABLE 1 SNPs, alleles, and haplotypes of *Pneumocystis jirovecii* *gsc1* gene identified in patients with prior exposure to echinocandins at the time of diagnosis of *P. jirovecii* infections

Patient or reference	HS1 region				HS2 region		Haplotype
	2204	2243	2303	Allele	4540	Allele	
Cissé et al. (20)	A	C	C	B	G	a	Ba
Ma et al. (21)	G	T	A	A	G	a	Aa
B25	G	C	C	C	A	b	Cb
B26	G	C	C	C	G	a	Ca
B27	G	C	C	C	ND	ND	— ^b
B28	G	C	C	C	G	a	Ca
B29	G	C	C	C	ND	ND	— ^b
B30	G	C	C	C	G	a	Ca
B31	ND ^a	ND	ND	ND	A	b	— ^b
B32	G	C	C	C	A	b	Cb
B33	G	C	C	C	G	a	Ca
B34	G	C	C	C	G	a	Ca
N1	G	C	C	C	G	a	Ca
N2 ^c	G	C	C	C	A/G	a and b	Ca and Cb
N3	G	C	C	C	G	a	Ca
N4	G	C	C	C	G	a	Ca
N5	G	C	C	C	A	b	Cb
N6	G	C	C	C	G	a	Ca
N7	G	C	C	C	G	a	Ca
N8	G	C	C	C	G	a	Ca
N9	G	C	C	C	A	b	Cb
N10	A	C	C	B	G	a	Ba
N11	G	C	C	C	A	b	Cb
N12 ^c	G	C/T	C/A	A and C	A/G	a and b	— ^b
N13	G	C	C	C	A	b	Cb
N14	G	C	C	C	G	a	Ca
N15 ^c	G	C	C	C	A/G	a and b	Ca and Cb
N16	G	C	C	C	A	b	Cb
N17	G	T	A	A	G	a	Aa

^aND, not determined.

^bHaplotype was not determined because of mixed infections that concerned the two HS1 and HS2 regions or because of undetermined alleles of HS1 or HS2 regions.

^cMixed infections in patients N2, N12, and N15.

TABLE 2 SNPs, alleles, and haplotypes of *Pneumocystis jirovecii* *gsc1* gene identified in patients without prior exposure to echinocandins at the time of diagnosis of *P. jirovecii* infections

Patient or reference	HS1 region				HS2 region		Haplotype
	2204	2243	2303	Allele	4540	Allele	
Cissé et al. (20)	A	C	C	B	G	a	Ba
Ma et al. (21)	G	T	A	A	G	a	Aa
B1	G	C	C	C	G	a	Ca
B2	A/G	C	C	B and C	A/G	a and b	— ^{a,b}
B3	G	C	C	C	G	a	Ca
B4	G	C	C	C	A	b	Cb
B5	G	C	C	C	G	a	Ca
B6	G	C	C	C	G	a	Ca
B7	G	C	C	C	G	a	Ca
B8	G	C	C	C	A	b	Cb
B9	G	C	C	C	G	a	Ca
B10	G	C	C	C	G	a	Ca
B11	G	C	C	C	G	a	Ca
B12	G	C	C	C	G	a	Ca
B13	G	C	C	C	G	a	Ca
B14	G	C	C	C	G	a	Ca
B15	G	C	C	C	G	a	Ca
B16	G	C	C	C	A	b	Cb
B17	G	C	C	C	G	a	Ca
B18	G	C	C	C	A	b	Cb
B19	G	C	C	C	G	a	Ca
B20	G	T	A	A	G	a	Aa
B21	G	C	C	C	A/G	a and b	Ca and Cb ^b
B22	G	C	C	C	G	a	Ca
B23	G	C/T	C/A	A and C	G	a	Ca and Aa ^b
B24	G	C	C	C	A	b	Cb

^aHaplotype was not determined because of mixed infections that concerned the two HS1 and HS2 regions simultaneously.

^bMixed infections in patients B2, B21, and B23.

infections concerned the two hot spots simultaneously, e.g., in patients B2 and N12, or when HS1 or HS2 region amplification failed, e.g., in patients B27, B29, and B31 (Tables 1 and 2). Ca was the most frequent haplotype and was detected in 14 out of 23 patients of the first group for whom positive results of haplotype identification were obtained and in 17 out of 23 patients of the second group (Table 2). No significant difference in haplotype distribution was shown between the two groups of patients (Fisher's exact test, $P = 0.57$). Thus, both groups of patients appear to be infected with similar strains of *P. jirovecii*.

DISCUSSION

In this study, we obtained the first results of diversity of the *gsc1* gene among *P. jirovecii* specimens from 51 patients routinely diagnosed with *P. jirovecii* infections,

	HS1	HS2
Pj_Ma	714 F L S L S L R D P 722	1393 S L A P V L D W 1400
Pj_Cisse	714 F L S L S L R D P 722	1393 S L A P V L D W 1400
<i>C. albicans</i>	641 F L T L S L R D P 649	1351 N I A P A V D W 1358
<i>C. tropicalis</i>	641 F L T L S L R D P 649	1351 N L S P A V D W 1358
<i>C. krusei</i>	655 F L I L S I R D P 663	1358 N L A P A I D W 1365
<i>S. cerevisiae</i>	639 F L V L S L R D P 647	1347 N F Q P A V D W 1354

FIG 2 Position of HS1 and HS2 of wild GSC1 protein types of *C. albicans*, *C. tropicalis*, *C. krusei* (Desnos-Olivier et al. [18]), and *Saccharomyces cerevisiae* (Luraschi et al. [22]) aligned with the GSC1 protein type of *P. jirovecii* (Cissé et al. [20], Ma et al. [21]). Boldfaced amino acids indicate known positions of mutations at HS1 and HS2 of GSC1 protein of *Candida* species. HS1 and HS2 GSC1 protein sequences of *P. jirovecii* in the present study were identical to those described by Cissé et al. (20) and Ma et al. (21).

whereas Cissé et al. (20) and Ma et al. (21) examined only one specimen each when they sequenced the whole genome of *P. jirovecii* and Luraschi et al. (17) did not examine clinical specimens when they characterized the *gsc1* gene of *P. jirovecii* or investigated *P. jirovecii* sensitivity to caspofungin (22). We chose to examine HS1 and HS2, since nonsynonymous mutations that confer resistance to caspofungin have been identified on homologous sequences of *Candida* spp. (16, 18, 19, 23). The amplification of HS1 failed in patient B31, and the amplification of HS2 failed in patients B27 and B29. These results could be explained by technical issues due to low burdens of *P. jirovecii* DNA in pulmonary specimens combined with putative DNA degradation in relationship with successive freezing-thawing cycles contemporaneous to specimen reexamination. Indeed, patients B31 and B27 did not develop overt PCP, were colonized by the fungus, and consistently harbored low *P. jirovecii* pulmonary burdens. Patient B29 developed PCP but only a sputum sample was examined, this kind of sample being characterized by poor cellular quality and low *P. jirovecii* burdens.

Since echinocandins were not initially considered for PCP treatment (13) and were only secondarily proposed as a second line of PCP treatment in leukemic patients (24), no archival *P. jirovecii* specimens from patients who were effectively treated with echinocandins for one or recurrent episodes of PCP were available in our laboratory. For these reasons, our approach consisted of examining *P. jirovecii* specimens from patients who developed a PCP episode after being treated with echinocandins for another invasive fungal infection. This method made possible the collection of 27 *P. jirovecii* specimens in this context. It was noteworthy that two patients, B34 and B30, were treated with echinocandins when PCP was diagnosed. Echinocandin treatment was maintained after PCP diagnosis during 5 days and 15 days in the first and second patient, respectively. These two patients were treated with caspofungin because of allergic bronchopulmonary aspergillosis and invasive pulmonary aspergillosis, respectively. Both harbored allele Ca, which was the major allele for all patients enrolled in this study, whatever their characteristics. The other 25 patients were no longer treated with echinocandins when PCP was diagnosed, the median duration of echinocandin treatment being 15 days (range, 1 to 29 days) and the median time between the end of echinocandin treatment and PCP diagnosis was 562 days (range, 152 to 1,533 days) (Table 3). Considering the hypothesis that PCP results from reactivation of long-term pulmonary colonization with *P. jirovecii*, the patients could have still been under selection pressure, whatever the duration of echinocandin treatment and the time between the end of echinocandin treatment and PCP diagnosis. The second hypothesis is that PCP results from *de novo* acquisition of the fungus. In this case, the duration of PCP incubation is considered highly variable, from 7 to 90 days (25–28), but a duration of less than 3 months is generally assumed. In 25 patients who were no longer treated with caspofungin during the 3 months preceding PCP diagnoses, there would no longer be any selection pressure at the time of the acquisition of *P. jirovecii*, while for the two patients who had been treated with caspofungin during the three preceding months, caspofungin might have exerted selection pressure at the time of the acquisition of *P. jirovecii*. This small number of two patients could represent the main limit of the study. It could be interesting to enroll a number of patients effectively treated with echinocandins or having been treated with echinocandins within the 3 months preceding PCP diagnosis. This will be possible in future years, given the new ECIL recommendations that retain echinocandins as a second line of PCP treatment (24). Recurrences or relapses of PCP in patients initially treated with echinocandins will provide *P. jirovecii* specimens that may be examined for this purpose.

The analysis of the *gsc1* gene focusing on the two hot spots permitted the identification of three *P. jirovecii gsc1* HS1 alleles, two *P. jirovecii gsc1* HS2 alleles, and four haplotypes combining the HS1 and HS2 alleles. The Hunter (H) index (29) of the putative genotyping method, based on examination of the HS1 and HS2 regions, was evaluated at 0.21 and 0.44, respectively. The H index based on the two hot spots taken together was evaluated at 0.67. These values appeared insufficient according to recommendations

TABLE 3 Characteristics of 27 patients with prior exposure to echinocandins at the time of *Pneumocystis jirovecii* infection diagnoses

Patient code ^{a,b}	Sex ^c	Age (yr)	Underlying disease	Prior echinocandin treatment	Time between echinocandin treatment start and <i>Pneumocystis</i> infection onset (days)	Duration of echinocandin treatment (days)	Sample	Presentation of <i>Pneumocystis</i> infection (alternative diagnosis of PCP)	Treatment of <i>Pneumocystis</i> infection	Outcome
B25	F	58	Chronic myelogenous leukemia	Caspofungin	181	29	BAL ^e	Colonization ^f		Improvement
B26	F	46	Acute myeloid leukemia	Micafungin, caspofungin	447	29	BAL	Colonization (bacterial pneumonia)		Improvement
B27	M	63	Bronchial carcinoma	Caspofungin	1,134	18	BAL	Colonization (bacterial pneumonia)		Improvement
B28	M	57	Acute myeloid leukemia	Caspofungin	822	23	Sputum	PCP ^g	SMX-TMP ^h	Improvement
B29	M	65	Acute myeloid leukemia	Caspofungin	308	3	Sputum	PCP	SMX-TMP	Deterioration/Improvement
B30	M	35	Biphenotypic acute leukemia	Caspofungin	56	71 ^k	BAL	PCP	SMX-TMP	Improvement
B31	F	82	Rheumatoid arthritis, methotrexate	Caspofungin	197	14	BAL	Colonization (bacterial pneumonia)		Improvement
B32	F	50	Acute myeloid leukemia	Caspofungin	522	1	BAL	PCP	SMX-TMP	Improvement
B33	F	34	Acute myeloid leukemia	Caspofungin	936	16	BAL	PCP	SMX-TMP	Improvement
B34	M	60	Asthma	Caspofungin	1	6 ^k	BAL	Colonization (hypersensitivity pneumonitis)		Improvement
N1	F	37	Lung transplant	Caspofungin	405	15	BAL	PCP	SMX-TMP	Improvement
N2	F	21	Lung transplant	Caspofungin, micafungin	454	18	BAL	PCP	SMX-TMP	Improvement
N3	M	25	Lung transplant	Caspofungin	956	4	BAL	PCP	SMX-TMP	Improvement
N4	F	35	Heart-lung transplant	Caspofungin, micafungin	724	20	BAL	PCP	SMX-TMP	Improvement
N5	M	34	Acute myeloid leukemia	Micafungin	1,000	28	BAL	PCP	SMX-TMP	Improvement
N6	F	47	Myelodysplasia	Caspofungin	365	8	BAL	PCP	SMX-TMP	Improvement
N7	M	23	Lung transplant	Caspofungin	580	18	BAL	PCP	SMX-TMP	Improvement
N8	F	53	Acute lymphoid leukemia	Micafungin	721	29	BAL	PCP	SMX-TMP	Improvement
N9	F	58	Kidney-pancreas transplant	Micafungin	432	9	BAL	PCP	SMX-TMP	Improvement
N10	M	64	Acute myeloid leukemia	Micafungin	1,114	20	BAL	PCP	SMX-TMP	Improvement
N11	F	47	Lung transplant	Micafungin	833	14	BAL	PCP	SMX-TMP	Improvement
N12	M	58	Lung transplant	Caspofungin	706	15	BAL	PCP	SMX-TMP	Improvement
N13	F	56	Lung transplant	Caspofungin	535	29	BAL	PCP	SMX-TMP	Improvement
N14	M	43	Acute lymphoid leukemia	Micafungin	497	13	BAL	PCP	SMX-TMP	Improvement
N15	F	47	Lung transplant	Caspofungin	450	8	BAL	PCP	SMX-TMP	Improvement
N16	M	31	Heart-lung transplant	Caspofungin	621	9	BAL	PCP	SMX-TMP	Deterioration
N17	M	54	Myeloma, COPD ^l	Caspofungin	1,547	14	BAL	PCP	SMX-TMP	Deterioration
									Atovaquone	Improvement
									Atovaquone	Improvement

^aPatients numbered B25 to B34 were monitored in Brest University Hospital, Brest, France. *P. jirovecii* detection was performed using microscopy (Calcofluor white and Wright-Giemsa stains) and a PCR assay amplifying the mitochondrial large subunit RNA gene after DNA extraction using NucliSens easyMag (bioMérieux, Marcy l'Etoile, France).
^bPatients numbered N1 to N17 were monitored in Nantes University Hospital, Nantes, France. *P. jirovecii* detection was performed using microscopy (Gomori-Grocott stain) and a PCR assay amplifying the mitochondrial large subunit RNA gene after DNA extraction using EZ1 DSP virus kit (Qiagen, Courtaboeuf, France).

^cM, male; F, female.

^dChronic obstructive pulmonary disease.

^eBronchoalveolar lavage.

^f*P. jirovecii* was not detected by microscopic examination; it was only detected by PCR assay, the patients presented alternative diagnoses of PCP, and they improved despite the absence of specific treatment against *P. jirovecii*.

^g*Pneumocystis* pneumonia.

^hSulfamethoxazole-trimethoprim.

ⁱ—, limitation of therapy.

^jConcomitant bacteremia was a potential factor of deterioration.

^kPatients B30 and B34 were treated with echinocandins when *Pneumocystis* infections were diagnosed. Echinocandin treatment was maintained after PCP diagnosis during 15 days and 5 days in the first and second patient, respectively.

(30, 31). The discriminatory power of a genotyping method requires an H index of ≥ 0.95 (30). Nonetheless, analysis of HS1 and HS2 can be added to our multilocus sequence type genotyping approach, consisting of mtLSUrRNA, cytochrome *b* (CYB), and superoxide dismutase (SOD) examination, which we have used in the past (32, 33).

In conclusion, our results show similar diversity of the *P. jirovecii gsc1* gene in patients with or without prior exposure to echinocandins at the time of PCP onset. The SNPs that were identified correspond to synonymous mutations that are not involved in codon changes. Despite the limitation of the present study, the results showed that no specific pressure of echinocandins among *P. jirovecii* microorganisms can be pointed out so far. However, given the resistance of *Candida* species *fk1* mutants to echinocandins as previously published (16, 18, 19, 23), the monitoring of the potential emergence of *P. jirovecii gsc1* mutants remains justified.

MATERIALS AND METHODS

A total of 51 patients who tested positive for *P. jirovecii* infection were retrospectively enrolled in the study. They were monitored at Brest University Hospital (34 patients) and Nantes University Hospital (17 patients) from 2 July 2008 to 31 August 2020. These two hospitals are located in the same region of France, 300 km apart.

Twenty-seven patients were selected from the databases of the two pharmacy units. This patient group was defined on the basis of (i) the patients' prior exposure to echinocandins at the time of *P. jirovecii* infection diagnoses, whatever the duration of this exposure, and the time between the end of echinocandin treatment and *P. jirovecii* infection diagnoses, and (ii) the availability of patients' archival *P. jirovecii* DNA specimens. The median age was 47 years (range, 21 to 82 years); the male-female ratio was 13:14. Underlying diseases were solid-organ transplantation (11 patients), hematological malignancy (13 patients), bronchial carcinoma (one patient), rheumatoid arthritis (one patient), and asthma (one patient) (Table 3).

Another group served as a control group and consisted of 24 patients who had no prior exposure to echinocandins at the time of *P. jirovecii* infection diagnoses. The median age was 65 years (range, 30 to 89 years); the male-female ratio was 17:7. Underlying diseases were cancer (seven patients), HIV infection (six patients), hematological malignancy (four patients), solid-organ transplantation (five patients), esophageal carcinoma and chronic obstructive pulmonary disease (one patient), and Horton disease (one patient) (Table 4).

The diagnoses of *P. jirovecii* infections had initially been performed in the laboratories of parasitology and mycology at Brest University Hospital or Nantes University Hospital using direct microscopy on bronchoalveolar lavage (BAL) specimens (Calcofluor-white and Wright-Giemsa stainings or Grocott-Gomori staining, respectively) and a real-time PCR assay targeting the *P. jirovecii* mitochondrial large subunit rRNA gene (*mtLSUrRNA*) (34, 35) on BAL, endotracheal aspirate (ETA), or induced sputum (IS) specimens. The PCR assays were performed after DNA extraction procedure using NucliSens easyMag (bioMérieux, Marcy l'Étoile, France) in Brest and EZ1 DSP virus kit (Qiagen, Courtaboeuf, France) in Nantes. Seven patients were considered colonized by *P. jirovecii*, since they presented alternative diagnoses of PCP and/or they did not receive specific treatment against *P. jirovecii*, whereas 44 patients had overt PCP, diagnosis of which was based on clinical and radiological signs combined with positive results of *P. jirovecii* detection (Tables 3 and 4). The corresponding 51 *P. jirovecii* specimens were stored at -20°C for further experiments.

P. jirovecii sequence analysis was performed at Brest University Hospital. Two primer pairs, HS1-2042F (AGGCTGTTTGGGAATTATTA)/HS1-2440R (CAAGGCGTCCATATAGAAACTC) and HS2-4207F (TGCTCATCC TGGGTTTCA)/HS2-4623R (AATCCACGACCAGTACCAATA), were designed using PrimerQuest Tools software (Integrated DNA Technology) to amplify and sequence the HS1 and HS2 regions of the *gsc1* gene. Primer specificity was checked using Basic Local Alignment Search Tool (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>). PCRs were carried out in a final volume of 50 μl , containing 200 μM each deoxynucleoside triphosphate (deoxynucleoside triphosphate set; Eurogentec, Seraing, Belgium), 0.5 μM forward and reverse primers, 2 U of DNA polymerase (HGS Diamond Taq DNA polymerase, Eurogentec), and MgCl_2 under final concentrations of 3 mM (first primer pair) and 1.5 mM (second primer pair). Amplification was conducted under the following conditions: activation step at 95°C for 10 min followed by 45 cycles including denaturation at 94°C for 30 s, annealing at 60°C for 45 s, extension at 72°C for 1 min, and a final extension step at 72°C for 10 min. The PCR products were electrophoresed on a 1.5% agarose gel containing ethidium bromide to visualize the expected bands for HS1 and HS2 (399 and 417 bp, respectively). To avoid contamination, each step was performed in different areas with different sets of micropipettes. The reagents used in the PCR mixtures were prepared in a laminar-flow cabinet. Negative controls were included to monitor for possible contamination. PCR products were purified using ExoSAP-IT (Affymetrix Inc., US) and sequenced from the two strands using the dideoxy chain termination method (BigDye Terminator version 1.1 cycle sequencing kit; Applied Biosystems, Foster City, CA) on the 3130XL Genetic Analyzer (Applied Biosystems, Courtaboeuf, France).

DNA sequences of the HS1 and HS2 regions were edited using Pregap and Gap software (Staden package version 2.0.0b11-2016; Staden Group) (36). Consensus sequences were aligned using BioEdit software with the Clustal W program. The *gsc1* genomic sequences and open reading frames (ORFs) used for the alignment with consensus sequences corresponded to the PNEJ11_001061 locus in the *P.*

TABLE 4 Characteristics of 24 patients without prior exposure to echinocandins at the time of *Pneumocystis jirovecii* infection diagnoses

Patient code ^a	Sex ^b	Age (yr)		Underlying condition	Sample	Presentation of <i>P. jirovecii</i> infection (alternative diagnosis of PCP)	Treatment of <i>P. jirovecii</i> Infection		Outcome
B1	M	65		HIV infection	BAL ^d	PCP ^f	SMX-TMP ^h		Improvement
B2	M	33		HIV infection	BAL	PCP	SMX-TMP		Improvement
B3	M	60		Esophageal carcinoma, COPD ^c	BAL	Colonization ^g (iatrogenic lung disease)			Improvement
B4	M	74		Kidney transplant	BAL	PCP	SMX-TMP, atovaquone		Deterioration
B5	M	70		Myeloma	BAL	PCP	SMX-TMP		Deterioration
B6	M	81		Cholangiocarcinoma	BAL	PCP	SMX-TMP		Deterioration
B7	M	76		Prostatic carcinoma	BAL	PCP	SMX-TMP		Deterioration
B8	F	78		Kidney transplant	BAL	PCP	SMX-TMP		Improvement
B9	F	55		HIV infection	BAL	PCP	SMX-TMP		Improvement
B10	M	42		HIV infection	BAL	Colonization ^g (bacterial pneumonia)			Improvement
B11	M	73		Esophageal carcinoma	BAL	PCP	SMX-TMP		Deterioration
B12	M	58		Lymphoma	BAL	PCP	SMX-TMP		Deterioration
B13	M	54		HIV infection	Sputum	PCP	Atovaquone		Deterioration
B14	M	77		Glioblastoma	Sputum	PCP	SMX-TMP		Deterioration
B15	F	69		Glioblastoma	Sputum	PCP	SMX-TMP		Improvement
B16	M	64		Kidney transplant	BAL	PCP	SMX-TMP		Deterioration
B17	M	30		HIV infection	BAL	PCP	SMX-TMP		Improvement
B18	M	51		Lung carcinoma	Sputum	PCP	SMX-TMP		Deterioration
B19	F	64		Kidney-Liver transplant	Sputum	PCP	SMX-TMP		Deterioration
B20	F	74		Acute myeloid leukemia	BAL	PCP	SMX-TMP, atovaquone		Deterioration
B21	F	70		Lymphoma	ETA ^e	PCP	SMX-TMP		Improvement
B22	F	89		Horton disease	BAL	PCP	SMX-TMP		Improvement
B23	M	63		Bronchial carcinoma	BAL	PCP	SMX-TMP		Improvement
B24	M	58		Lymphoma	Sputum	PCP	Atovaquone, caspofungin, pentamidine		Deterioration

^aPatients numbered from B1 to B24 were monitored at Brest University Hospital, Brest, France. *P. jirovecii* detection was performed using microscopy (Calcofluor white and Wright-Giemsa stains) and a PCR assay amplifying the mitochondrial large subunit RNA gene after DNA extraction using NucliSens easyMag (bioMérieux, Marcy l'Etoile, France).

^bM, male, F, female.

^cChronic obstructive pulmonary disease.

^dBronchoalveolar lavage.

^eEndotracheal aspiration.

^f*Pneumocystis pneumonia*.

^g*P. jirovecii* was not detected by microscopic examination; the fungus was only detected by PCR assay, the patients presented alternative diagnoses of PCP, and they improved despite the absence of specific treatment against *P. jirovecii*.

^hSulfamethoxazole-trimethoprim.

jirovecii genome assembly version ASM33397v2 (20) and to the T551_02309 locus in the *P. jirovecii* genome assembly version Pneu_jiro_RU7_V2 (21).

Statistical analyses were performed using Prism software (version 7.0; GraphPad Software, San Diego, CA, USA). Qualitative variables were compared using Fisher's exact test. A two-sided *P* value of <0.05 was considered significant. The study was noninterventive and did not require informed consent and ethical approval according to French laws and regulations (CSP Art L1121e1.1).

Nucleotide sequence accession numbers. The nucleotide sequences of the *gsc1* gene obtained in the present study have been deposited in GenBank under accession numbers [MZ670330](#) to [MZ670428](#).

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