Diversité des gènes de Pneumocystis jirovecii codant pour les cibles de molécules ayant une activité anti-Pneumocystis potentielle ou démontrée

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Résumé

Pneumocystis jirovecii est un champignon transmissible responsable de pneumonies sévères (pneumonies à *Pneumocystis* [PPC]) chez les patients immunodéprimés. Une pression de sélection sur *P. jirovecii*, principalement due aux sulfamides, a été décrite depuis les années 90. Par ailleurs, les échinocandines et l'atovaquone ciblant respectivement la 1,3-bêta-D-glucane synthase et le cytochrome b sont utilisées comme thérapies de deuxième intention de la PPC en France. Le méthotrexate (MTX) est utilisé pour traiter les maladies auto-immunes ou les cancers et cible largement la dihydrofolate réductase (DHFR) des cellules humaines. Cependant, il possède également une activité anti-*Pneumocystis* potentielle puisqu'il pourrait cibler la DHFR de *Pneumocystis*. L'objectif de cette thèse était d'évaluer la pression de sélection potentielle exercée par ces trois médicaments sur *P. jirovecii*. Le gène *gsc1* codant pour la 1,3-bêta-D-glucane synthase, le gène *dhfr* et le gène CYB des isolats de *P. jirovecii* provenant de patients infectés ont été amplifiés et séquencés.

Dans le chapitre 1, deux groupes de patients ont été comparés. Les 27 patients du premier groupe avaient été exposés aux échinocandines et les 24 patients du deuxième groupe n'avaient aucune exposition connue. La diversité génétique du gène *gsc1* était similaire chez les patients avec ou sans exposition préalable aux échinocandines.

Au chapitre 2, deux groupes de patients ont été comparés. Les 21 patients du premier groupe avaient été exposés au MTX et les 22 patients du deuxième groupe n'avaient aucune exposition connue. La diversité du gène *dhfr* était similaire entre les patients avec ou sans exposition préalable au MTX.

Au chapitre 3, deux groupes de patients ont été comparés. Les 14 patients du premier groupe avaient été exposés à l'atovaquone et les 109 patients du deuxième groupe n'avaient aucune exposition connue. La diversité du gène *CYB* était différente selon les patients avec ou sans exposition préalable à l'atovaquone. Plus précisément, les deux mutations faux-sens C431T (Ala144Val) et C823T (Leu275Phe) situées sur le site actif de l'enzyme cytochrome bc1, codé par *CYB*, étaient associées de manière significative à une exposition antérieure à l'atovaquone (test exact de Fisher, $P < 0{,}001$).

Nos résultats suggèrent que la pression de sélection globale exercée par les échinocandines, le MTX et l'atovaquone sur *P. jirovecii* en France reste faible. À l'inverse, la pression de sélection exercée par l'atovaquone apparaît élevée chez les patients directement exposés à ce médicament**.**

Summary

Pneumocystis jirovecii is a transmissible fungus responsible for severe pneumonia (*Pneumocystis* pneumonia [PCP]) in immunocompromised patients. Selective pressure on *P. jirovecii* organisms mostly due to sulfonamides has been described since nineties. In other respects, the echinocandins and atovaquone targeting 1,3-beta-D-glucan synthase and cytochrome b respectively are used as second line therapies of PCP in France. Methotrexate (MTX) is used to treat autoimmune diseases or cancers and it widely targets the dihydrofolate reductase (DHFR) of human cells. However, it also has a putative anti-*Pneumocystis* activity since it may target the DHFR of *Pneumocystis*. The aim of this PhD thesis was to evaluate the putative selective pressure exerted by these three drugs on *P. jirovecii* organisms. The gene *gsc1* encoding the 1,3-beta-D-glucan synthase, the gene *dhfr* and the gene *CYB* of *P. jirovecii* isolates from infected patients were amplified and sequenced.

In chapter 1, two groups of patients were compared. The 27 patients of the first group had been exposed to echinocandins and the 24 patients in the second group had no known exposure. The genetic diversity of the gene *gsc1* was similar in patients with or without prior exposure to echinocandins.

In chapter 2, two groups of patients were compared. The 21 patients of the first group had been exposed to MTX and the 22 patients in the second group had no known exposure. The diversity of the *dhfr* gene was similar between patients with or without prior exposure to MTX. Thus, no selective pressure due to MTX has been pointed out.

In chapter 3, two groups of patients were compared. The 14 patients of the first group had been exposed to atovaquone and the 109 patients in the second group had no known exposure. The diversity of the *CYB* gene was different between patients with or without prior exposure to atovaquone. Specifically, both missense mutations C431T (Ala144Val) and C823T (Leu275Phe) located at the active site of the enzyme cytochrome bc1, encoded by *CYB*, were significantly associated with prior atovaquone exposure (Fisher's exact test, $P < 0.001$).

Our results suggest that the overall selective pressure exerted by echinocandins, MTX, and atovaquone on *P. jirovecii* organisms in France remains low. Conversely, selective pressure exerted by atovaquone appears high in patients directly exposed to this drug.

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Liste des abréviations

ADN : acide désoxyribonucléique

AMM : autorisation de mise sur le marché

ANOFEL : association française des enseignants et praticiens hospitaliers titulaires de parasitologie et mycologie médicales

ARN : acide ribonucléique

CYB : gène codant pour la sous-unité b du cytochrome bc1

gsc1 : gène codant pour la sous-unité Gsc1 de l'enzyme ß-1,3-D-glucane synthase

DHFS : Dihydrofolate Synthase

DHFR : Dihydrofolate Réductase

DHPPP : 6-hydroxyméthyle-7,8-dihydroptérine pyrophosphate

DHPS : Dihydroptéroate Synthase

FDA : Food and Drug Association

IMPDH : inosine 5'-monophosphate déshydrogénase

LAM : Leucémie Aiguë Myéloïde

LBA : lavage broncho-alvéolaire

MLST : Multi-Locus Sequence Typing

MPA : acide mycophénolique

mtLSUrRNA : gène codant pour l'ARN de la grande sous-unité du ribosome de la mitochondrie

MTX : méthotrexate

NGS : Next Generation Sequencing (séquençage à haut debit)

PABA : para-aminobenzoic acid (acide para-aminobenzoïque)

PCR : Polymerase Chain Reaction (réaction de polymérisation en chaîne)

PPC/PCP : Pneumonie à *Pneumocystis* / *Pneumocystis* Pneumonia

RFC : Reduced Folate Carrier

SIDA : Syndrome d'Immunodéficience Acquise

SMX : sulfaméthoxazole

SMX-TMP : sulfaméthoxazole-triméthoprime

SNP : Single Nucleotide Polymorphism (polymorphisme mononucléotidique)

SOD : superoxyde dismutase

TMP : triméthoprime

VIH : Virus de l'Immunodéficience Humaine

Introduction

Le genre *Pneumocystis* désigne des micromycètes se positionnant dans l'embranchement des *Ascomycota*, la classe des *Pneumocystidomycetes*, l'ordre des *Pneumocystidales* et la famille des *Pneumocystidaceae* [1,2]. Cinq espèces du genre *Pneumocystis*, chacune spécifique de son espèce mammifère hôte, ont été décrites. Il s'agit de *P. carinii* et *P. wakefieldiae* chez le rat, *P. murina* chez la souris, *P. oryctolagi* chez le lapin et *P. jirovecii* chez l'Homme [2]. D'autres espèces spécifiques d'autres mammifères hôtes restent à caractériser. *P. jirovecii* se développe au contact des pneumocytes de type I dans les alvéoles pulmonaires humaines, seul réservoir actuellement connu pour cette espèce [3]. *P. jirovecii* est acquis par voie aérienne et transmis par la même voie entre un individu index et un autre individu susceptible [revues *in* 4]. Ce mode de transmission explique la survenue d'infections nosocomiales révélées par des cas groupés d'infections à *P. jirovecii* en milieu hospitalier [revues *in* 4, 5]. Il s'agit d'un champignon opportuniste, agent de la pneumonie à *Pneumocystis* (PPC), infection invasive sévère chez le patient immunodéprimé. La PPC est la plus fréquente des infections opportunistes chez les patients atteints du VIH en France métropolitaine, classant la maladie virale chez le patient au stade clinique 4 [6,7]. La PPC survient chez d'autres patients immunodéprimés non infectés par le VIH. En France, le nombre de cas de PPC rapportés chez les immunodéprimés non infectés par le VIH (69%) est supérieur à celui des patients infectés par le VIH (31%) [8,9]. Ces patients présentent des facteurs de risque de la PPC tels qu'une transplantation d'organe, des hémopathies malignes, des cancers ou des maladies auto-immunes [10]. La PPC est d'évolution plus rapide et sévère chez les patients immunodéprimés non infectés par le VIH, avec un risque d'insuffisance respiratoire aigüe et de mortalité plus élevé [revues *in* 11].

L'infection à *P. jirovecii* est suspectée devant un tableau clinique associant toux sèche, dyspnée et fièvre. L'imagerie révèle un syndrome alvéolo-interstitiel bilatéral diffus se traduisant par des images en verre dépoli dans les deux champs pulmonaires sur le scanner thoracique. Le diagnostic de certitude repose sur l'examen mycologique d'un prélèvement pulmonaire, de préférence un liquide de lavage broncho-alvéolaire (LBA). L'examen mycologique comprend un examen microscopique direct après coloration panoptique au May-Grünwald Giemsa associé à une autre coloration permettant la

visualisation de la paroi des champignons, comme le calcofluor-white. La réalisation d'une amplification génique spécifique par « Polymerase Chain Reaction » (PCR) en complément des techniques microscopiques est effectuée en routine par les laboratoires spécialisés [12,13]. Le dosage du ß-1,3-D-glucane sérique vient compléter l'examen mycologique, il peut notamment être couplé à une PCR sur aspiration naso-pharyngée avec de bonnes performances lorsque la réalisation d'un LBA est impossible [14]. Les champignons du genre *Pneumocystis* restent jusqu'à présent non cultivables dans une démarche de diagnostic de routine [15,16]. La PCR permet de pallier le manque de sensibilité de l'examen microscopique, et ainsi de détecter les faibles charges fongiques [17, revues *in* 18]. En cas de faible charge fongique, de résultats négatifs pour la détection microscopique et de diagnostic alternatif à la PPC, les patients sont généralement considérés comme colonisés par *P. jirovecii* [19].

Une particularité du genre *Pneumocystis* est l'absence d'ergostérol membranaire, un stérol membranaire généralement présent au sein du règne fongique [revues *in* 20, 21]. L'absence d'ergostérol explique sa résistance aux traitements antifongiques ciblant l'ergostérol comme l'amphotéricine B et les azolés largement utilisés en mycologie médicale. En effet, l'amphotéricine B exerce son activité fongicide en se liant à l'ergostérol membranaire, induisant une augmentation de perméabilité et une fuite des composants intracellulaires, tandis que les azolés inhibent l'enzyme lanostérol 14 alpha-déméthylase, responsable de la synthèse de l'ergostérol [revues *in* 22]. Les échinocandines, qui inhibent la synthèse du ß-1,3-D-glucane (Figure 1), composant majeur de la paroi des champignons, dont les asques de *P. jirovecii*, sont employées principalement pour le traitement de première ligne des candidémies [23] et de seconde intention des aspergilloses invasives [24] .

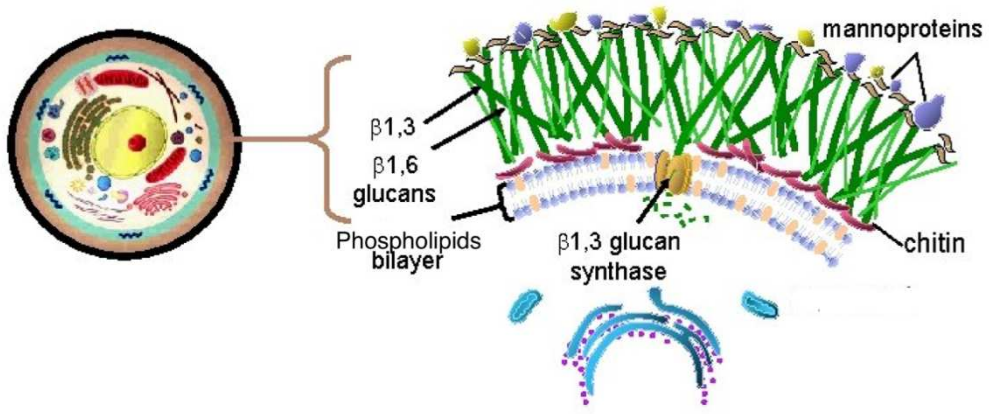

Figure 1. Schéma de la paroi et de la membrane fongiques. Adapté de Maertens et al., 2002 [24].

Bien que le traitement de la PPC n'ait pas fait partie des indications initiales des échinocandines [25], ces molécules représentent des traitements alternatifs de la PPC [26]. L'usage de ces molécules hors des conditions de l'attribution de l'AMM reste néanmoins limité dans ce contexte. Des mutations non synonymes conférant une résistance à la caspofungine, l'échinocandine la plus fréquemment prescrite, ont été identifiées sur le gène *fks1* codant pour la sous-unité Gsc1 de l'enzyme ß-1,3-D-glucane synthase au niveau de deux hot spots HS1 et HS2 chez *Candida albicans, C. krusei, C. tropicalis* et *C. glabrata* [27-29]. En revanche, la résistance acquise aux échinocandines chez *P. jirovecii* reste à explorer puisqu'il existe actuellement très peu de données concernant le gène *gsc1* (homologue du gène *fks1*) de ce champignon. Le gène *gsc1* a pu être caractérisé récemment [30] à la suite de la publication du génome complet de *P. jirovecii* [31,32]. Celui-ci peut donc être analysé chez des isolats de *P. jirovecii* provenant de patients infectés et exposés préalablement aux échinocandines.

Le traitement de la PPC repose en première intention sur l'association sulfaméthoxazole-triméthoprime (SMX-TMP) [26], ces deux molécules agissant sur la voie de synthèse des folates. En effet, *P. jirovecii* ne peut utiliser le folate exogène et doit nécessairement le synthétiser. Deux enzymes sont nécessaires au métabolisme des folates : la dihydroptéroate synthase (DHPS) et la dihydrofolate réductase (DHFR) [revues *in* 33]. Le sulfaméthoxazole (SMX) et le triméthoprime (TMP) agissent respectivement sur la DHPS et la DHFR de façon synergique en tant qu'inhibiteurs compétitifs [34]. La DHFR permet la réduction du dihydrofolate en tétrahydrofolate, nécessaire à la synthèse de l'ADN (Figure 2).

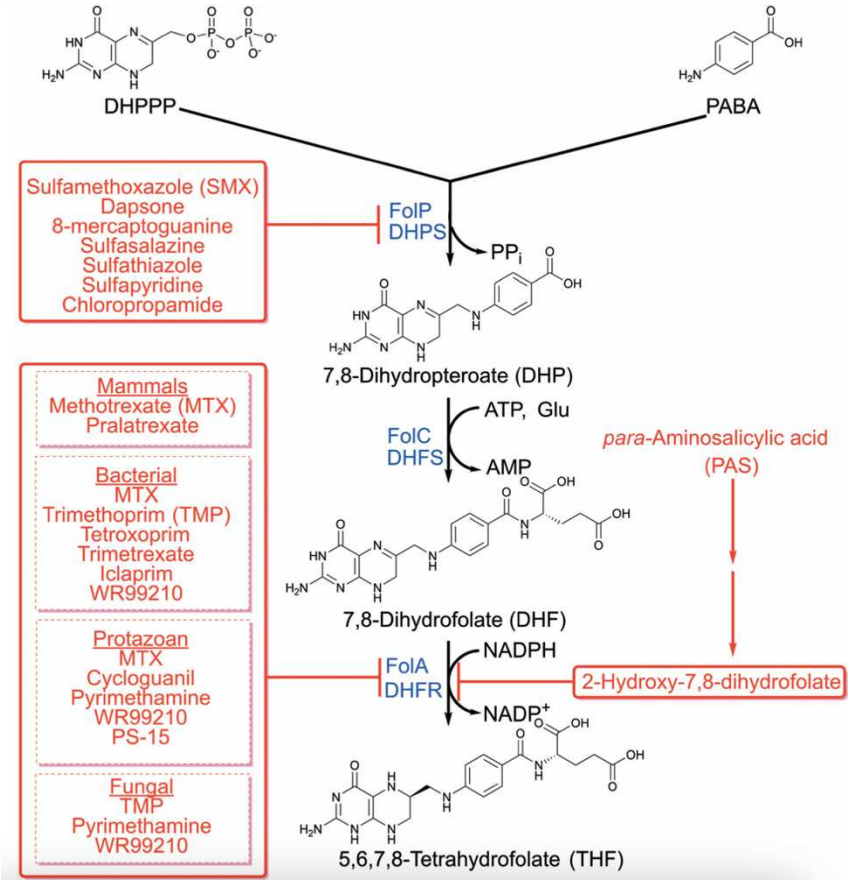

Figure 2. Voie de synthèse des folates et inhibiteurs enzymatiques chez les eucaryotes et les bactéries. DHFR : Dihydrofolate réductase. DHFS : Dihydrofolate synthase. DHPPP : 6-hydroxyméthyle-7,8-dihydroptérine pyrophosphate. DHPS : Dihydroptéroate synthase. MTX : Méthotrexate. PABA : Acide para-aminobenzoïque. TMP : Triméthoprime. D'après Kordus et al., 2019 [33].

Des mutations non synonymes ont été décrites au niveau du gène codant pour la DHFR de *P. jirovecii* [35-39]. La mutation Ser37Thr située au niveau du site actif de la DHFR a été associée à des échecs de prophylaxie par les associations SMX-TMP ou pyriméthamine-sulfadiazine, le TMP et la pyriméthamine étant tous deux inhibiteurs de la DHFR [34]. De plus, des expériences menées *in vitro* ainsi que des modélisations informatiques ont démontré une résistance vis-à-vis du TMP d'enzymes de *P. jirovecii* portant les mutations Ser31Phe, Phe36Cys, Leu65Pro, Ala67Val, Val79Ile, et Ile158Val [40,41]. La mutation Val134Ala sur le gène *dhfr* pourrait être associée à une augmentation de la mortalité chez les enfants atteints de PPC [42].

Le méthotrexate (MTX) est un inhibiteur du cycle cellulaire structurellement proche du TMP. Il s'agit également d'un inhibiteur compétitif de la DHFR à laquelle il se lie de

manière irréversible au niveau du site de liaison du dihydrofolate. Les modes d'action du MTX et du TMP sont identiques, mais la nature de la liaison à la DHFR diffère pour des raisons structurelles. En effet, la structure du MTX est plus proche de celle du dihydrofolate que ne l'est celle du TMP [revues *in* 33] (Figure 3).

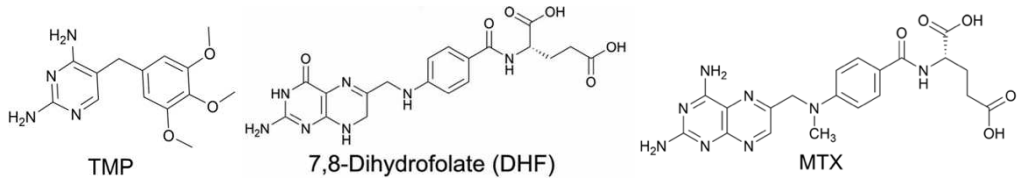

Figure 3. Structures du triméthoprime (TMP), du dihydrofolate (DHF), et du méthotrexate (MTX). D'après Kordus et al., 2019 [33].

Le MTX est fréquemment employé pour le traitement des cancers et des maladies de système telles que la polyarthrite rhumatoïde [revues *in* 43]. Dans ce contexte, le traitement par MTX par son action immunosuppressive représente un facteur de risque d'infections opportunistes dont la PPC [44,45, revues *in* 46]. En raison de l'action potentielle du MTX sur la DHFR de *P. jirovecii*, la PPC dans ce contexte pourrait se rapporter à des souches de *P. jirovecii* sélectionnées par la pression exercée par cette drogue. Cette pression de sélection qui pourrait se révéler par des souches de *P. jirovecii* présentant des mutations sur le gène *dhfr* n'a, à notre connaissance, pas été encore explorée.

L'atovaquone est un traitement de deuxième ligne de la PPC. L'atovaquone est également employée en deuxième ligne pour la prophylaxie de la PPC bien que la prescription dans ce contexte soit hors des conditions de l'attribution de l'AMM [47]. Il s'agit d'un inhibiteur compétitif de la sous-unité b du complexe enzymatique cytochrome bc1. Ce complexe situé au niveau de la membrane mitochondriale participe aux transferts d'électrons nécessaires au fonctionnement de la chaîne respiratoire [revues *in* 48] (Figures 4 et 5).

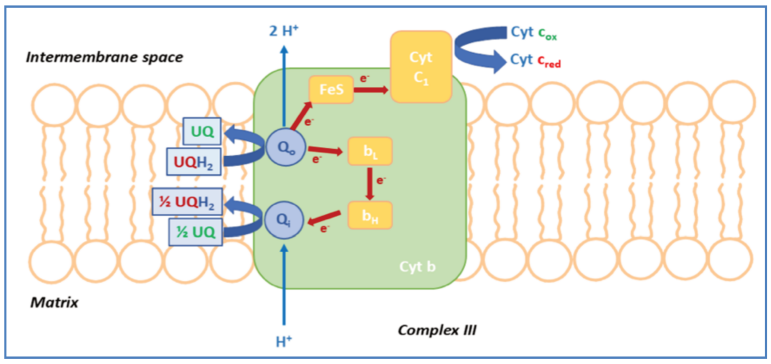

Figure 4. Schéma du complexe enzymatique cytochrome bc1. Ce complexe homodimérique est constitué de trois sous-unités catalytiques : le cytochrome b (Cyt b) avec deux molécules d'hème type b (b_L et b_H), la protéine fer-soufre de Rieske (FeS), et le cytochrome c1 (Cyt c_1) avec une molécule d'hème de type c. Les deux sites de liaison des inhibiteurs et de l'ubiquinone (UQ), Q_i et Q_o, sont également représentés. D'après Musso et al., 2020 [48].

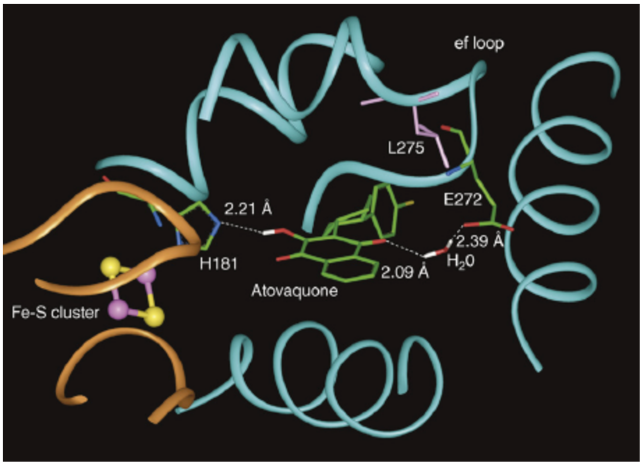

Figure 5. Structure à énergie minimisée de la liaison de l'atovaquone dans la poche d'oxydation de l'ubiquinol au centre P du complexe bc1 d'une levure. Le cytochrome b est représenté en cyan et une partie de la protéine fer-soufre de Rieske est de couleur dorée. L'amas fer-soufre est situé en bas à gauche, avec des atomes de fer et de soufre colorés respectivement en violet et jaune. L'hélice C du cytochrome b s'étend verticalement sur la droite, et l'hélice cd1 est en bas de la poche de liaison. Les résidus Leu_{275} et Glu_{272} situés dans la boucle ef du cytochrome b sont également représentés. L'atovaquone est liée par une liaison hydrogène au résidu His_{181} de la protéine de Rieske et à une molécule d'eau qui forme un pont vers le résidu Glu_{272} via une seconde liaison hydrogène. Le cycle hydroxynaphtoquinone est en avant et le cycle chlorophényle s'étend vers l'arrière. Les atomes de carbone de l'atovaquone, His_{181} et Glu_{272} sont verts, les atomes d'oxygène sont rouges, les atomes d'azote sont bleus et les atomes d'hydrogène sont blancs. Le résidu Leu_{275} est violet. D'après Kessl et al., 2003 [49].

Les mutations non synonymes Thr127Ile, Ile147Val, Thr148Ile, Leu150Phe, Ser152Ala, Ser216Leu, Pro266Leu, Leu275Phe, et Leu307Phe, ont été mises en évidence sur le gène codant pour le cytochrome b de *P. jirovecii* chez des patients sidéens développant une PPC, avec ou sans prophylaxie par atovaquone [50-52]. Ces mutations pourraient entrainer une résistance de *P. jirovecii* à l'atovaquone. À notre connaissance, aucune étude n'a démontré de lien entre la présence de ces mutations et un échec de traitement de la PPC par atovaquone. Cependant, des expériences *in vitro* introduisant les mutations Ile147Val, Thr148Ile, Leu150Phe, Ser152Ala, Pro266Leu et Leu 275Phe chez *Saccharomyces cerevisiae* montrent que leur présence entraine une diminution de sensibilité *in vitro* du cytochrome b à l'atovaquone [53-55]. Plus récemment, Argy et collaborateurs ont rapporté une nouvelle mutation C431T (Ala144Val) chez *P. jirovecii* provenant de patients transplantés cardiaques impliqués dans des cas groupés de PPC à l'hôpital Bichat, à Paris. Cette mutation serait potentiellement impliquée dans la diminution de la sensibilité de *P. jirovecii* à l'atovaquone. Les auteurs ont vérifié l'hypothèse que l'usage de la prophylaxie par atovaquone au lieu de l'association SMX-TMP avait conduit à la sélection d'une souche mutante de *P. jirovecii*. La modélisation du cytochrome b de la souche mutante a plaidé en faveur de la résistance à l'atovaquone (Figure 6). La souche mutante résistante a ensuite été transmise entre patients transplantés au sein de l'hôpital [56].

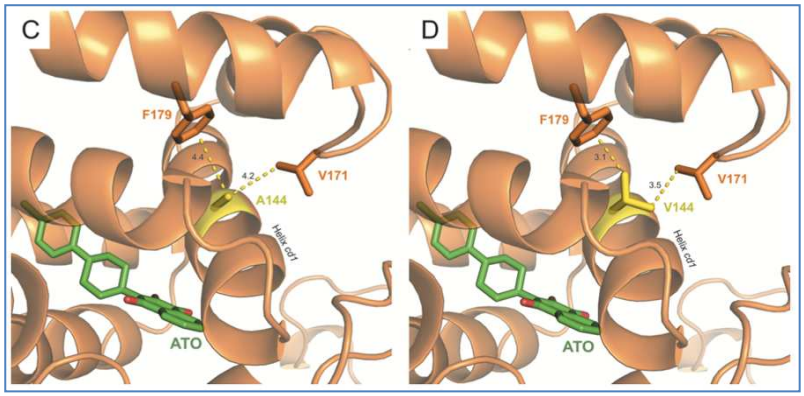

Figure 6. Modélisation du cytochrome b de type sauvage chez *P. jirovecii*. Présence d'une alanine en position 144 (à gauche). Modélisation du cytochrome b d'une souche mutante de *P. jirovecii* associée à une résistance clinique à l'atovaquone. Présence d'une valine en position 144 (à droite). Le résidu valine en position 144 a été généré avec la fonction swapaa (mutagénèse in silico) de l'outil Chimera. D'après Argy et al, 2018 [56].

L'usage de l'atovaquone étant désormais relativement répandu, le niveau de pression de sélection exercé par cette molécule sur *P. jirovecii* en France mériterait d'être exploré.

En résumé, nos objectifs, au cours de ce travail de thèse, ont été de définir le niveau de pression de sélection exercé par les échinocandines en particulier la caspofungine, le méthotrexate et l'atovaquone sur les populations de *P. jirovecii*. Pour cela, nous avons analysé la diversité des gènes de *P. jirovecii* codant pour les enzymes ß-1,3-D-glucane synthase, DHFR et cytochrome b, cibles des échinocandines, du méthotrexate et de l'atovaquone respectivement, ces drogues ayant une activité anti-*Pneumocystis* connue ou potentielle. Les possibles corrélations entre la présence d'éventuelles souches mutantes sur ces gènes et les antécédents de traitement par ces drogues ont été analysées. Nous présentons nos résultats sous la forme de trois chapitres successifs correspondant à trois publications, suivis d'une discussion générale et d'une conclusion. Nous apportons par ce travail des données originales sur l'épidémiologie de *P. jirovecii* valorisées par ces trois publications internationales.

Chapitre 1 : Étude de la sensibilité aux échinocandines de *P. jirovecii* chez les patients pré-exposés

Les échinocandines sont largement utilisées pour traiter les candidoses systémiques [23] mais restent peu utilisées en pratique en deuxième ligne de traitement de la PPC, alors que cette utilisation a été récemment recommandée par un groupe de travail de l'European Conference on Infections in Leukaemia (ECIL) [26]. L'hypothèse d'une pression de sélection exercée par les échinocandines sur *P. jirovecii* chez des patients ayant été traités par ces drogues et ayant ensuite développé une PPC méritait d'être testée. Dans ce but, nous avons analysé la diversité du gène *gsc1* de *P. jirovecii* dans deux groupes de patients présentant une infection à *P. jirovecii*. Un groupe de 27 patients avait été exposé aux échinocandines, tandis que le deuxième groupe de 24 patients ne l'avait pas été. Deux portions du gène *gsc1*, HS1 et HS2, homologues des hot spots décrits chez *Candida* spp., ont été séquencées. Quatre polymorphismes mononucléotidiques (SNP) aux positions 2204, 2243, 2303 et 4540 ont été identifiés. Trois allèles *gsc1* HS1, A, B et C, deux allèles *gsc1* HS2, a et b, et quatre haplotypes, Ca, Cb, Aa et Ba, ont été définis, sans différence significative dans la distribution des haplotypes entre les deux groupes de patients (P = 0,57). Compte tenu de l'absence de mutation non synonyme dans les deux groupes de patients, aucune pression de sélection des échinocandines sur *P. jirovecii* n'a pu être objectivée.

Ces résultats sont présentés dans l'article « **Highly Conserved *gsc1* Gene of *Pneumocystis jirovecii* in Patients with or without Prior Exposure to Echinocandins.** Bonnet PL, Le Gal S, Hoffmann CV, Morio F, Diabira F, de Quélen A, Curral G, Negri S, Cogulet V, Huon JF, Grégoire M, Papon N, Le Pape P, Bouchara JP, Nevez G. Antimicrob Agents Chemother **2022**; 66:e0156321. *Impact Factor*: 5,191».

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EPIDEMIOLOGY AND SURVEILLANCE

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 *fks1* gene of the fungal pathogens *Candida* spp. (16), whereas data on the diversity of the *gsc1* gene (homologous to *fks1* gene) of *P. jirovecii* are still scarce (17). The aforementioned mutations were found in two hot spots of the *fks1* 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

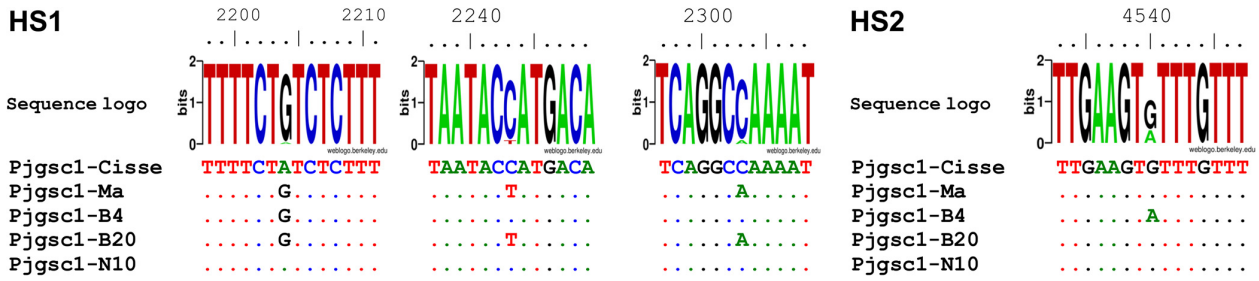

FIG 1 Single-nucleotide polymorphisms in *Pneumocystis jirovecii* hot spot 1 (HS1) and HS2 DNA regions after sequencing. Analysis of 51 *P. jirovecii* specimens led to the identification of three SNPs close to the HS1 region at positions 2204, 2243, and 2303 and one SNP more distant from the HS2 region 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

	HS1 region				HS2 region		
Patient or reference	2204	2243	2303	Allele	4540	Allele	Haplotype
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

[a]ND, not determined.
[b]Haplotype 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.
[c]Mixed 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

	HS1 region				HS2 region		
Patient or reference	**2204**	**2243**	**2303**	**Allele**	**4540**	**Allele**	**Haplotype**
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

[a]Haplotype was not determined because of mixed infections that concerned the two HS1 and HS2 regions simultaneously.
[b]Mixed 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
C. albicans	641	**F**	L	T	L	**S**	L	R	D	**P**	649	1351	N	I	A	P	A	V	D	**W**	1358
C. tropicalis	641	F	L	T	**L**	S	L	R	D	P	649	1351	N	L	S	P	A	V	D	W	1358
C. krusei	655	F	L	I	**L**	S	I	R	D	P	663	1358	N	L	A	P	A	I	D	W	1365
S. cerevisiae	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 *Pneumocystis* infection onset (days)	Duration of echinocandin treatment (days)	Sample	Presentation of *Pneumocystis* infection (alternative diagnosis of PCP)	Treatment of *Pneumocystis* 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[j]
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	Atovaquone	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	Atovaquone	Improvement
N14	M	43	Acute lymphoid leukemia	Micafungin	497	13	BAL	PCP	—[i]	Deterioration
N15	F	47	Lung transplant	Caspofungin	450	8	BAL	PCP	Atovaquone	Deterioration
N16	M	31	Heart-lung transplant	Caspofungin	621	9	BAL	PCP	Atovaquone	Improvement
N17	M	54	Myeloma, COPD[d]	Caspofungin	1,547	14	BAL	PCP	Atovaquone	Improvement

[a]Patients 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).

[b]Patients 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).

[c]M, male, F, female.

[d]Chronic obstructive pulmonary disease.

[e]Bronchoalveolar 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.

[h]Sulfamethoxazole-trimethoprim.

[i]—, limitation of therapy.

[j]Concomitant bacteremia was a potential factor of deterioration.

[k]Patients 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 *fks1* 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'Etoile, 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 (AGGCCTGTTTGGGAATTATTTA)/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 PNEJI1_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 *P. jirovecii* infection (alternative diagnosis of PCP)	Treatment of *P. jirovecii* 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

[a]Patients 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).
[b]M, male, F, female.
[c]Chronic obstructive pulmonary disease.
[d]Bronchoalveolar lavage.
[e]Endotracheal 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*.
[h]Sulfamethoxazole-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 noninterventional 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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Chapitre 2 : Étude de la pression de sélection du méthotrexate sur le gène *dhfr* de *P. jirovecii*

Des mutations non synonymes ont été décrites au niveau du gène *dhfr* de *P. jirovecii* [35-39] et des variants présentant des mutations non synonymes ont été associés à des échecs de prophylaxie par les inhibiteurs de la Dihydrofolate Réductase (DHFR) comme le triméthoprime et les analogues du triméthoprime [36]. Par ailleurs, des cas de PPC chez des patients traités par MTX ont été rapportés [revues *in* 46], et des expériences menées *in vitro* ainsi que des modélisations informatiques ont montré une diminution de la sensibilité au MTX d'enzymes arborant des mutations non synonymes sur le gène *dhfr* [40,41]. Considérant que le MTX est un inhibiteur compétitif de la DHFR très puissant [57], nous avons émis l'hypothèse que ce médicament pourrait exercer une pression de sélection sur *P. jirovecii*. Nous avons donc analysé les séquences du gène *dhfr* de *P. jirovecii* chez deux groupes de patients ayant développé une infection à *P. jirovecii*. Un groupe de 21 patients avait été exposé au MTX tandis qu'un second groupe de 22 patients n'y avait pas été exposé. La diversité du gène *dhfr* était très proche entre les deux groupes de patients. Ces résultats ne sont donc pas en faveur d'une pression de sélection exercée par le MTX sur *P. jirovecii*.

Ce deuxième volet de notre travail de thèse a permis d'apporter des résultats originaux sur la diversité du gène *dhfr* chez des patients exposés ou non exposés au MTX. Les résultats sont présentés dans l'article « **Apparent Absence of Selective Pressure on *Pneumocystis jirovecii* Organisms in Patients with Prior Methotrexate Exposure**. Bonnet PL, Le Gal S, Gautier C, Hoffmann CV, Le Nan N, Happe A, Le Berre R, Saraux A, Nevez G. Antimicrob Agents Chemother **2022**; 1:e0099022. *Impact Factor*: 5,938 ».

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EPIDEMIOLOGY AND SURVEILLANCE

Apparent Absence of Selective Pressure on *Pneumocystis jirovecii* Organisms in Patients with Prior Methotrexate Exposure

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ABSTRACT *Pneumocystis jirovecii* infections occur in patients treated with methotrexate (MTX) because of immunosuppressive effects of this highly potent dihydrofolate reductase (DHFR) inhibitor. Conversely, MTX may act as an anti-*P. jirovecii* drug and consequently may exert a selective pressure on this fungus. In this context, we compared the sequences of the *dhfr* gene of *P. jirovecii* isolates obtained from two groups of patients with *P. jirovecii* infections. The first group, with systemic diseases or malignancies, had prior exposure to MTX (21 patients), whereas the second group (22 patients), the control group, did not. Three single nucleotide polymorphisms (SNPs) were observed at positions 278, 312, and 381. The first one was located in the intronic region and the two others were synonymous. Based on these SNPs, three *P. jirovecii dhfr* alleles, named A, B, and C, were specified. Allele A was the most frequent, as it was observed in 18 patients (85.7%) and in 16 patients (72.7%) of the first and second groups, respectively. No significant difference in *P. jirovecii dhfr* gene diversity in the two patient groups was observed. In conclusion, these original results suggest that MTX does not exert an overt selective pressure on *P. jirovecii* organisms.

KEYWORDS *Pneumocystis jirovecii*, *dhfr*, dihydrofolate reductase, genetic diversity, methotrexate

Pneumocystis jirovecii is an opportunistic and transmissible fungus responsible for severe pneumonia, *Pneumocystis* pneumonia (PCP), in immunocompromised patients. PCP remains the most frequent AIDS-defining illness in human immunodeficiency virus (HIV)-infected patients in metropolitan France (1). PCP also occurs in other immunosuppressed patients not infected with HIV, such as those treated with immunosuppressive and/or cytostatic therapies (2). In this context, cases of PCP in patients treated with methotrexate (MTX) monotherapy or MTX associated with other immunosuppressive drugs have been reported (3). Using analysis of databases on ncbi.nlm.nih.gov (PubMed), we identified about 180 citations comprising *Pneumocystis* and MTX (key words "pneumocystis [Title/Abstract]" and "methotrexate [Title/Abstract]") during the period from 1976 to 2021, mostly related to patients with systemic diseases, specifically rheumatoid arthritis (75 citations from 1983 to 2021).

In other respects, *P. jirovecii* infection prophylaxis and treatment are based on the wide use of sulfamethoxazole and trimethoprim (SMX-TMP), which target dihydropteroate

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synthase (DHPS) and dihydrofolate reductase (DHFR), respectively, two enzymes involved in folate biosynthesis. Two major nonsynonymous mutations have been described at nucleotide positions 165 and 171 of the *dhps* locus of *P. jirovecii* (4). Variants harboring these mutations have been associated with failure of sulfonamide prophylaxis and putative resistance of *P. jirovecii* organisms to these drugs (reviewed in reference 5). Currently, prior exposure to sulfonamides and the city of patient residence have been identified as the two main predictors of *P. jirovecii* DHPS mutants worldwide (6, 7). Nonsynonymous mutations have also been described in the *dhfr* gene of *P. jirovecii* (8–12). Some variants harboring nonsynonymous mutations have been associated with failure of prophylaxis by trimethoprim and trimethoprim analogues (9) and putative resistance of *P. jirovecii* organisms to these drugs (13). *In vitro* experiments have also shown resistance to trimethoprim inhibition in recombinant enzymes harboring nonsynonymous mutations (14, 15). Considering that MTX is a highly potent DHFR inhibitor in mammalian cells and in *P. carinii* organisms (16), we hypothesized that beyond its cytotoxic activity, the drug may exert selective pressure on *P. jirovecii* organisms. To test this hypothesis, genetic diversities of *dhfr* gene of *P. jirovecii* organisms obtained from two patient groups were compared. One group had prior exposure to MTX, whereas the second one did not, at the time of *P. jirovecii* infection diagnosis.

RESULTS

Taken together, no *P. jirovecii dhfr* single nucleotide polymorphisms (SNPs) were observed in 31 patients (17 from the first group and 14 from the control group) in comparison to the reference sequence AF090368 described by Ma and colleagues (17). Three *P. jirovecii dhfr* SNPs were observed among the 12 other patients (four from the first group and eight from the control group). The first SNP, at nucleotide position 312 (T312C), was observed in patients B4, B10 and B18 of the first group and in patients B22, B25, B29, B31, B32, B35, B37, and B43 of the control group. The second SNP, at nucleotide position 381 (C381T), was observed only in patient B37 of the control group. These two SNPs correspond to synonymous mutations previously reported in references 17 and 11, respectively. The third SNP, previously described (10) and located in the unique intronic region of *dhfr* gene, was observed at nucleotide position 278 (C278T) only in patient B15 of the first group. Four cases of mixed infections were detected. Specifically, a C residue and a T residue were identified at nucleotide position 312 in patients B18, B32, and B43. In patient B37, a C residue and a T residue were identified at nucleotide positions 312 and 381 (Table 1 and Table 2).

Three *P. jirovecii dhfr* alleles, named A, B, and C, were determined in relation to the three aforementioned SNPs (Table 1 and Table 2). Allele A was the most frequent allele and was observed in 18 patients of the first group and in 16 patients of the second group. Allele B was observed in three patients of the first group and in seven patients of the control group. Allele C was observed only in one patient of the first group. The allele mix was not determined in patient B37 because of the presence of several different alleles that could not be identified (Table 1 and Table 2). No significant difference in allele distribution was shown between the two groups of patients (Fisher's exact test, $P = 0.28$).

DISCUSSION

In this study, we provided the first data on *P. jirovecii dhfr* gene diversity in patients with prior exposure to MTX, compared to patients without prior exposure to MTX. Three different SNPs whose association made it possible to describe three putative different alleles, A, B, and C, were detected. The results pointed out the diversity of the *P. jirovecii dhfr* gene for all patients taken together. Nonetheless, this genetic diversity does not differ in patients with or without prior exposure to MTX, considering the absence of any significant difference in allele distribution effectively observed in these two patient groups.

Nahimana and colleagues have reported a significant association between nonsynonymous mutations on the *dhfr* gene of *P. jirovecii* and failure of PCP prophylaxis

TABLE 1 Characteristics of 21 patients with prior exposure to methotrexate at the time of *Pneumocystis jirovecii* infection diagnosis and the identified SNPs and alleles of the *P. jirovecii dhfr* gene

Patient code[a]	Underlying condition(s)	Patient still on MTX at time of *Pneumocystis* diagnosis	Duration of MTX treatment before *Pneumocystis* diagnosis (days)	Sample	Presentation of *Pneumocystis* infection (alternative diagnosis of PCP)	Treatment of *P. jirovecii* infection	Outcome	Residue at position:			Allele
								278	312	381	
B1	Horton's disease	Yes	>90	BAL[d]	PCP[e]	SMX-TMP[g]	Improvement	C	T	C	A[j]
B2	Rheumatoid arthritis, glioblastoma	No[c]	>90	Sputum	PCP	SMX-TMP	Improvement	C	T	C	A
B3	Rheumatoid arthritis	Yes	15	BAL	Colonization (bacterial pneumonia)[f]	—[h]	Improvement	C	T	C	A
B4	Still's disease	Yes	>90	BAL	Colonization (autoimmune lung disease)[f]	—	Improvement	C	C	C	B
B5	Rheumatoid arthritis	Yes	>90	BAL	Colonization (iatrogenic lung disease)[f]	—	Improvement	C	T	C	A
B6	Rheumatoid arthritis	Yes	>90	BAL	PCP	SMX-TMP	Deterioration	C	T	C	A
B7	Rheumatoid arthritis	Yes	>90	BAL	PCP	SMX-TMP	Improvement	C	T	C	A
B8	Wegener's granulomatosis	Yes	>90	BAL	PCP	Atovaquone	Improvement	C	T	C	A
B9	Acute myeloid leukemia	Yes	18	BAL	PCP	SMX-TMP	Improvement	C	T	C	A
B10	Polyarteritis nodosa	Yes	44	BAL	PCP	SMX-TMP	Improvement	C	C	C	B
B11	Rheumatoid arthritis, bronchial carcinoma	Yes	68	Sputum	PCP	SMX-TMP	Deterioration	C	T	C	A
B12	Psoriasis	Yes	>90	BAL	PCP	SMX-TMP	Improvement	C	T	C	A
B13	Rheumatoid arthritis	Yes	>90	BAL	PCP	SMX-TMP	Improvement	C	T	C	A
B14	Rheumatoid arthritis	Yes	>90	BAL	PCP	SMX-TMP	Improvement	C	T	C	A
B15	Psoriasis	No[c]	>90	Sputum	Colonization (autoimmune lung disease)[f]	—	Improvement	T	T	C	C
B16	Rheumatoid arthritis	Yes	>90	BAL	Colonization (autoimmune lung disease)[f]	—	Improvement	C	T	C	A
B17	Rheumatoid arthritis	Yes	>90	BAL	PCP	SMX-TMP	Deterioration	C	T	C	A
B18[b]	Breast carcinoma	Yes	>90	BAL	PCP	SMX-TMP	Deterioration	C	C/T[i]	C	A and B
B19	Polymyalgia rheumatica	Yes	>90	Sputum	Colonization (iatrogenic lung disease)[f]	—	Improvement	C	T	C	A
B20	Polymyalgia rheumatica	No[c]	>90	BAL	Colonization (bacterial pneumonia)[f]	—	Improvement	C	T	C	A
B21	Horton's disease	Yes	41	Sputum	PCP	SMX-TMP, atovaquone	Improvement	C	T	C	A

[a]Patients numbered from B1 to B21 were monitored at Brest University Hospital, Brest, France. Age, sex, and dates were not indicated *in extenso*, since they could be identifiable patient information.
[b]Mixed infection in patient B18.
[c]For patients B2, B15, and B20, methotrexate treatment was stopped 97, 4, and 17 days, respectively, before *P. jirovecii* infection diagnoses.
[d]BAL, bronchoalveolar lavage fluid.
[e]PCP, *Pneumocystis* pneumonia.
[f]*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*.
[g]SMX-TMP, sulfamethoxazole-trimethoprim.
[h]—, patients B3, B4, B5, B15, B16, B19, B20 did not receive any specific treatment against *P. jirovecii* infection.
[i]Two nucleotides were identified at this position on sequence electrophoregrams.
[j]Allele A corresponds to the reference sequence (GenBank accession no. AF090368) (17). It harbors C, T, and C residues at nucleotide positions 278, 312, and 381, respectively.

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TABLE 2 Characteristics of 21 patients without known prior exposure to methotrexate at the time of *Pneumocystis jirovecii* infection diagnoses and the identified SNPs and alleles of the *P. jirovecii dhfr* gene

Patient code[a]	Underlying condition(s)	Sample	Presentation of *P. jirovecii* infection (alternative diagnosis of PCP)	Treatment of *P. jirovecii* infection	Outcome	Residue at position: 278	312	381	Allele
B22	HIV infection	BAL[d]	PCP[f]	SMX-TMP[h]	Improvement	C	C	C	B
B23	HIV infection	BAL	PCP	SMX-TMP	Improvement	C	T	C	A[k]
B24	Esophageal carcinoma, COPD[c]	BAL	Colonization (iatrogenic lung disease)[g]	—[i]	Improvement	C	T	C	A
B25	Kidney transplant	BAL	PCP	SMX-TMP, atovaquone	Deterioration	C	C	C	B
B26	Myeloma	BAL	PCP	SMX-TMP	Deterioration	C	T	C	A
B27	Cholangiocarcinoma	BAL	PCP	SMX-TMP	Deterioration	C	T	C	A
B28	Prostatic carcinoma	BAL	PCP	SMX-TMP	Deterioration	C	T	C	A
B29	Kidney transplant	BAL	PCP	SMX-TMP	Improvement	C	C	C	B
B30	HIV infection	BAL	Colonization (bacterial pneumonia)[g]	—[i]	Improvement	C	T	C	A
B31	Esophageal carcinoma	BAL	PCP	SMX-TMP	Deterioration	C	C	C	B
B32[b]	Lymphoma	BAL	PCP	SMX-TMP	Deterioration	C	C/T[j]	C	A and B
B33	HIV infection	Sputum	PCP	Atovaquone	Deterioration	C	T	C	A
B34	Glioblastoma	Sputum	PCP	SMX-TMP	Deterioration	C	T	C	A
B35	Kidney transplant	BAL	PCP	SMX-TMP	Deterioration	C	C	C	B
B36	HIV infection	BAL	PCP	SMX-TMP	Improvement	C	T	C	A
B37[b]	Lung carcinoma	Sputum	PCP	SMX-TMP	Deterioration	C	C/T[j]	C/T[j]	Mixed[l]
B38	Kidney-liver transplant	Sputum	PCP	SMX-TMP	Deterioration	C	T	C	A
B39	Acute myeloid leukemia	BAL	PCP	SMX-TMP, atovaquone	Deterioration	C	T	C	A
B40	Lymphoma	ETA[e]	PCP	SMX-TMP	Improvement	C	T	C	A
B41	Lung carcinoma	BAL	PCP	SMX-TMP	Improvement	C	T	C	A
B42	Lymphoma	Sputum	PCP	Atovaquone, caspofungin, pentamidine	Deterioration	C	T	C	A
B43[b]	HIV infection	BAL	PCP	SMX-TMP	Improvement	C	C/T[j]	C	A and B

[a]Patients numbered from B22 to B43 were monitored at Brest University Hospital, Brest, France. Age, sex, and dates were not indicated *in extenso*, since it could be identifiable patient information.
[b]Mixed infections in patients B32, B37, and B43.
[c]COPD, chronic obstructive pulmonary disease.
[d]BAL, bronchoalveolar lavage fluid.
[e]ETA, endotracheal aspiration.
[f]PCP, *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*.
[h]SMX-TMP, sulfamethoxazole-trimethoprim.
[i]—, patients B24 and B30 did not receive any specific treatment against *P. jirovecii* infection.
[j]Two nucleotides were identified at this position on sequence electrophoregrams.
[k]Allele A corresponds to reference sequence (GenBank accession no. AF090368) (17). It harbors C, T, and C residues at nucleotide positions 278, 312, and 381, respectively.
[l]Allele mix was not determined because of the presence of several different alleles that could not be identified. Considering previous description of SNP C381T by Costa et al. (11), the association of allele B with another putative allele harboring 278C, 312T, and 381T is the most likely hypothesis.

based on DHFR inhibitors (9). These inhibitors were mainly pyrimethamine, rarely trimethoprim, and not MTX. Obviously, MTX is not used for PCP prophylaxis or treatment because of its cytotoxic activity, while it is mainly used as a long-term treatment for systemic diseases or malignancies. This cytotoxic activity is partly due to the fact that mammalian cells harbor a high-affinity transmembrane transport system, the reduced folate carrier (RFC), which is the major route for MTX uptake (18).

In the present study, 18 out of 21 patients with prior exposure to MTX were still treated with MTX when *P. jirovecii* infections were diagnosed. Occurrence of these infections in this context suggested ineffectiveness of the drug against the fungus and consequently in PCP prevention. Conversely, MTX is known to have a strong *in vitro* competitive inhibitor effect on *Pneumocystis* DHFR (16). These discrepant data between *in vitro* and *in vivo* observations can be explained by the fact that *Pneumocystis* organisms may not have the aforementioned transmembrane transport system, the absence of which reduces MTX uptake (16). Indeed, no significant similarity was found in genomes and proteomes of *P. jirovecii* (BioProject no. PRJEA68827 from Cisse and colleagues [19]; BioProject no. PRJNA223510 from Ma and colleagues [20]), *Pneumocystis carinii* (BioProject no. PRJNA223511 [20]), *Pneumocystis murina* (BioProject no. PRJNA70803 [20]), and other closely related fungi, i.e., *Saccharomyces cerevisiae* (BioProject no. PRJNA393501 [21]) and *Schizosaccharomyces pombe* (BioProject no. PRJNA527756 [22]), using the RFC protein of *Homo sapiens*, *Pan troglodytes* (chimpanzee), *Bos taurus* (cattle), *Rattus norvegicus* (Norway rat), and *Mus musculus* (mouse) (GenBank accession no. AAC50180, XP_001157360, NP_001069921, NP_001030309, and NP_112473, respectively) as query sequences in tblastn and blastp searches (https://blast.ncbi.nlm.nih.gov/Blast.cgi). This decreased MTX uptake may explain the apparent low selective pressure exerted by MTX. Moreover, the absence of nonsynonymous mutation in the *dhfr* gene of *P. jirovecii* in the 16 patients with long-term MTX exposure (more than 90 days) is consistent with a low selective pressure exerted by MTX. Our approach was essentially based on detection and identification of nonsynonymous mutations as recently reviewed and published by Leidner and colleagues (15). However, it has been suggested that synonymous mutations may affect protein translational kinetics and drug-target interactions (23–25). This may represent a new field of investigation for studies on *P. jirovecii*. Be that as it may, in the present study, no significant difference in SNP distributions was shown between patients with and without prior MTX exposure.

The average/low number of patients enrolled in this retrospective monocentric study may explain the absence of any significant difference of *P. jirovecii dhfr* gene diversity in patients with or without prior exposure to MTX. Indeed, despite the 14-year period of inclusion, only 21 patients met the criteria for being enrolled in the first patient group of this study. In conclusion, these original results show that MTX does not exert an overt selective pressure on *P. jirovecii* organisms so far.

MATERIALS AND METHODS

A request was made with the collaboration of the Clinical Data Center regarding patients who tested positive for *P. jirovecii* infection at Brest University Hospital during the period from 31 March 2008 to 30 March 2022 on the basis of the patients' prior exposure to MTX during the year before *P. jirovecii* infection diagnosis, whatever the MTX dosage and its duration. Among the 43 patients initially selected, only 21 were retained on the basis of the availability and quality of patients' archival *P. jirovecii* DNA specimens. In this first group of 21 patients, the median age was 74 years (range, 42 to 89 years); the male/female ratio was 10/11. Underlying conditions were autoimmune diseases (17 patients), glioblastoma and rheumatoid arthritis (1 patient), bronchial carcinoma and rheumatoid arthritis (1 patient), acute myeloid leukemia (1 patient), and breast carcinoma (1 patient) (Table 1). A second group of patients contemporaneously diagnosed with *P. jirovecii* infection served as a control group, consisting of 22 patients without known prior exposure to MTX at the time of *P. jirovecii* infection diagnosis. The median age was 64 years (range, 30 to 81 years); the male/female ratio was 18/4. Underlying conditions included cancer (6 patients), HIV infection (6 patients), hematological malignancy (5 patients), organ transplantation (4 patients), and esophageal carcinoma with chronic obstructive pulmonary disease (COPD) (1 patient) (Table 2). None of the patients of the first and second groups had sulfonamide PCP prophylaxis. Only one patient of the second group (patient B33) had prior exposure to sulfonamides related to one PCP treatment 2 years earlier.

The diagnosis of PCP was initially made in the parasitology and mycology laboratory using direct

microscopy on bronchoalveolar lavage (BAL) specimens (calcofluor white and Wright-Giemsa stains) and a real-time PCR assay targeting the *P. jirovecii* mitochondrial large subunit rRNA gene (*mtLSUrRNA*) (26, 27) on BAL, endotracheal aspirate, or induced sputum specimens. The PCR assays were performed after a DNA extraction procedure using NucliSens easyMag (bioMérieux, Marcy l'Etoile, France). Seven patients of the first group and two patients of the second group were considered to be colonized by *P. jirovecii*, since they presented alternative diagnoses of PCP and/or did not receive specific treatment against *P. jirovecii*. *P. jirovecii* was only detected by the PCR assay because of the low fungal burdens in these patients. Thirty-four patients, including 14 patients of the first group and 20 of the second group, had overt PCP, with diagnoses based on clinical and radiological criteria combined with *P. jirovecii* detection (Table 1 and Table 2). The corresponding 43 *P. jirovecii* isolates had been stored at −80°C for further examination.

The primer pair FR208 and FR1018 was used to amplify the full length of the *dhfr* gene as described elsewhere (17). Primer specificity was checked using the Basic Local Alignment Search Tool (BLAST) (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 $MgCl_2$ at a final concentration of 3 mM. Amplification was conducted under the following conditions: an 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 and extension at 72°C for 1 min, and then 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 of 832 bp. 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. To monitor for possible contamination, negative controls were included. PCR products were purified using ExoSAP-IT (Affymetrix, Inc., USA) 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 ABI Prism 3130xl DNA sequencer (Applied Biosystems, Courtaboeuf, France).

DNA sequences of the *dhfr* gene were edited using Pregap and Gap software (Staden package version 2.0.0b11-2016; Staden Group) (28). Consensus sequences were aligned with the reference sequence (GenBank accession no. AF090368 [17]) using the BioEdit software with the ClustalW program.

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 noninterventional and did not require informed consent and ethical approval according to French laws and regulations (CSP Art L1121e1.1).

Accession number(s). The nucleotide sequences of the *dhfr* gene obtained in the present study have been deposited in GenBank under accession no. ON932600 to ON932642.

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Chapitre 3 : Étude de la pression de sélection de l'atovaquone sur le gène du cytochrome b de *P. jirovecii* en France et revue de la littérature

Les effets indésirables potentiels du SMX-TMP peuvent conduire à l'usage de l'atovaquone en seconde ligne de prophylaxie ou traitement de la PPC. Il s'agit d'un inhibiteur compétitif de la sous-unité « b » du complexe enzymatique cytochrome bc1. Des échecs de prophylaxie par atovaquone chez des patients à risque de PPC ont été documentés [50,51,56,58,59]. L'objectif de cette troisième partie du travail de thèse était de caractériser la pression de sélection exercée par l'atovaquone sur *P. jirovecii* en France. Dans ce contexte, nous avons analysé rétrospectivement le gène *CYB* d'isolats de *P. jirovecii* issus de 14 patients exposés et de 109 patients non exposés à l'atovaquone au moment du diagnostic de l'infection par *Pneumocystis*. Ce travail, associé à une revue de la littérature sur les données disponibles sur le gène *CYB* de *P. jirovecii*, suggère que la pression de sélection globale exercée par l'atovaquone sur *P. jirovecii* en France reste faible actuellement. En revanche, cette pression de sélection est élevée chez les patients pré-exposés à l'atovaquone.

Ce troisième volet de notre travail a permis d'apporter des résultats originaux sur la diversité du gène du cytochrome b chez des patients exposés ou non-exposés à l'atovaquone. Les résultats sont présentés dans l'article « **Atovaquone exposure and *Pneumocystis jirovecii* cytochrome b mutations: French data and review of the literature**. Bonnet PL, Hoffmann CV, Le Nan N, Bellamy L, Hoarau G, Flori P, Demar M, Argy N, Morio F, Le Gal S, Nevez G. Medical Mycology **2023**; 61:myad095. *Impact Factor*: 3,747».

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Atovaquone exposure and *Pneumocystis jirovecii* cytochrome b mutations: French data and review of the literature

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Abstract

Pneumocystis jirovecii is a transmissible fungus responsible for severe pneumonia (*Pneumocystis* pneumonia [PCP]) in immunocompromised patients. Missense mutations due to atovaquone selective pressure have been identified on cytochrome b (*CYB*) gene of *P. jirovecii*. It was recently shown that atovaquone prophylaxis can lead to the selection of specific *P. jirovecii CYB* mutants potentially resistant to atovaquone among organ transplant recipients. In this context, our objectives were to provide data on *P. jirovecii CYB* mutants and the putative selective pressure exerted by atovaquone on *P. jirovecii* organisms in France. A total of 123 patients (124 *P. jirovecii* specimens) from four metropolitan hospitals and two overseas hospitals were retrospectively enrolled. Fourteen patients had prior exposure to atovaquone, whereas 109 patients did not at the time of *P. jirovecii* detection. A 638 base-pair fragment of the *CYB* gene of *P. jirovecii* was amplified and sequenced. A total of 10 single nucleotide polymorphisms (SNPs) were identified. Both missense mutations C431T (Ala144Val) and C823T (Leu275Phe), located at the Q_o active site of the enzyme, were significantly associated with prior atovaquone exposure, these mutations being conversely incidental in the absence of prior atovaquone exposure ($P < 0.001$). Considering that the aforementioned hospitals may be representative of the national territory, these findings suggest that the overall presence of *P. jirovecii CYB* mutants remains low in France.

Lay summary

The mutations C431T (Ala144Val) and C823T (Leu275Phe) at the cytochrome b (*CYB*) active site of *Pneumocystis jirovecii* are associated with patient prior exposure to atovaquone. Conversely, these mutations are incidental in the absence of exposure. Overall, the presence of *P. jirovecii CYB* mutants remains low in France.

Key words: *Pneumocystis jirovecii*, cytochrome b, *CYB*, atovaquone, selective pressure.

Introduction

Pneumocystis jirovecii is a non-cultivable, opportunistic, and transmissible fungus responsible for severe pneumonia (*Pneumocystis* pneumonia [PCP]) in immunocompromised patients. PCP remains the most frequent acquired immunodeficiency syndrome (AIDS) defining infection in patients infected with the human immunodeficiency virus (HIV) in developed countries. In France, at present, PCP cases in HIV-infected patients are less frequent than cases in other immunosuppressed patients not infected with HIV.[1,2] The first-line treatment and prophylaxis of PCP are based on the combination of sulfamethoxazole with trimethoprim (SMX-TMP), which targets the dihydropteroate synthase (DHPS) and the dihydrofolate reductase (DHFR), respectively, two enzymes involved in folate biosynthesis. Two major missense mutations that occur at nucleotide positions 165 and 171 have been described at the *dhps* locus of *P. jirovecii*.[3] Variants harboring these mutations have been associated with sulfonamide exposure and the putative resistance of *P. jirovecii* organisms to these drugs.[4] Currently, prior exposure to sulfonamides and the city of patient residence have been identified as the two main predictors of *P. jirovecii* DHPS mutants worldwide.[5,6] Moreover, occasional severe adverse effects of SMX-TMP lead to the use of second-line treatments or prophylaxis. Atovaquone is an alternative used for both PCP treatment and prophylaxis in the USA, mainly in patients with

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Table 1. Characteristics of 123 patients with or without prior atovaquone exposure at the time of *P. jirovecii* infection diagnoses.

	Patients with prior atovaquone exposure at the time of *P. jirovecii* infection diagnoses	Patients without prior atovaquone exposure at the time of *P. jirovecii* infection diagnoses
No. of patients	14	109
Median age [range]	53 [35-81]	61 [0.1-89]
Sex ratio (M/F)	9/5	74/35
Samples	BAL ($n = 9$); Sputum ($n = 3$); NS ($n = 2$)	BAL ($n = 77$); Sputum ($n = 10$); NS ($n = 1$); ETA ($n = 1$); NPA ($n = 21$)
Presentation of *Pneumocystis* infection	PCP ($n = 12$); colonization[a] ($n = 2$)	PCP ($n = 82$) ; colonization[a] ($n = 6$); primary infection ($n = 21$)
Underlying conditions (No. of patients)	SOT ($n = 8$); SOT and HIV ($n = 1$); H ($n = 2$); AI ($n = 2$); Cancer ($n = 1$)	SOT ($n = 26$); HIV ($n = 22$); H ($n = 14$); Cancer ($n = 11$); AI ($n = 7$); Premature birth ($n = 4$); Cancer and COPD ($n = 1$); Others[b] ($n = 14$); None[c] ($n = 10$)

BAL, bronchoalveolar lavage; NS, nasopharyngeal swab; ETA, endotracheal aspiration; NPA, nasopharyngeal aspiration; PCP, *Pneumocystis* pneumonia; M, male; F, female; SOT, solid organ transplant; H, hemopathy; AI, autoimmune disease; HIV, human immunodeficiency virus; COPD, Chronic Obstructive Pulmonary Disease

[a] *Pneumocystis jirovecii* was not detected by microscopic examination, it was only detected by PCR assay. The patients presented alternative diagnoses of PCP.

[b] Interstitial lung disease ($n = 3$); Lymphopenia ($n = 2$); Congenital heart disease ($n = 2$); Hypersensitivity pneumonitis ($n = 1$); Non-X histiocytosis ($n = 1$); West syndrome ($n = 1$); Tetralogy of Fallot ($n = 1$); Primary immunodeficiency ($n = 1$); Laryngomalacy ($n = 1$); Down's syndrome ($n = 1$)

[c] Immunocompetent neonates who developed *Pneumocystis* primary infection.

hematological malignancies or solid organ transplants. It is only an approved second-line drug for PCP treatment in France, but despite this restriction, it is also used as PCP prophylaxis in patients that cannot be given first-line therapy. This drug is a competitive inhibitor of the b subunit of the cytochrome bc1 enzyme complex. This complex, located in the mitochondrial membrane, participates in the electron transfers necessary for the functioning of the respiratory chain.[7] Missense mutations in the cytochrome b gene (*CYB*) consisting of the amino acid changes Ile147Val, Thr148Ile, Leu150Phe, Ser152Ala, Pro266Leu, and Leu275Phe have been observed in *P. jirovecii* strains, and others were found originally in *Saccharomyces cerevisiae* (Leu275Phe/Ser/Thr), *Plasmodium yoelii* (Tyr279Cys), and *P. falciparum* (Tyr279Ser).[8–10] The aforementioned mutations in *P. jirovecii* and *Plasmodium* sp. confer reduced sensitivity to atovaquone in enzymatic models from *S. cerevisiae*.[8–10] Moreover, failure of prophylaxis based on the use of atovaquone in patients at risk for PCP has been documented.[11–15] Specifically, Argy and colleagues recently described a previously unreported C431T mutation (Ala144Val) that diminished the sensitivity of *P. jirovecii* to atovaquone, which may explain the spread of the fungus among heart transplant recipients receiving atovaquone prophylaxis.[14]

The objectives of the present study were to provide data on *P. jirovecii CYB* mutants and information on the putative selective pressure exerted by atovaquone on *P. jirovecii* organisms in France. In this context, we retrospectively analyzed the *CYB* gene of a collection of *P. jirovecii* specimens in patients from six French hospitals, exposed or not exposed to atovaquone, at the time of *P. jirovecii* detection. We also provided a review of the literature on *P. jirovecii CYB* diversity and the description of new alleles.

Materials and Methods

A total of 123 patients (124 *P. jirovecii* specimens) who tested positive for *P. jirovecii* infection were retrospectively enrolled in the study (Table 1). They were monitored from February 2009 to March 2022. Seventy-eight *P. jirovecii* specimens were from patients monitored at Brest University Hospital. The remaining 46 *P. jirovecii* specimens were from patients monitored in five other hospitals: three metropolitan hospitals: Nantes (six patients), Paris Bichat Hospital (19 patients), Saint-Étienne (one patient), and two overseas hospitals: Cayenne, French Guiana (six patients), and Réunion Island (14 patients). *Pneumocystis jirovecii* was initially detected by microscopic examination of pulmonary specimens and/or PCR assays. Ninety-four patients were considered to develop overt PCP; eight patients were considered to be colonized by the fungus; and 21 infants were considered to develop *Pneumocystis* primary infection. The colonized patients presented with alternative diagnoses of PCP. *Pneumocystis jirovecii* in colonized patients as well as in primary infected infants was not detected by microscopy, while it was detected by PCR.

Fourteen patients had prior atovaquone exposure (the first group). Atovaquone exposure was defined on the basis of the patients' prior exposure during the year before *P. jirovecii* detection, whatever the atovaquone dosage and its duration. The median age in this group was 53 years (range, 35–81 years); the male-female ratio was 9/5. Underlying conditions were solid-organ transplantation (eight patients), hematological malignancy (two patients), solid-organ transplantation and HIV (one patient), giant cell arteritis (one patient), epidermoid carcinoma (one patient), and myasthenia gravis (one patient) (Tables 1 and 2). The second group comprised 109 patients who had no known prior exposure to atovaquone at the time of *P. jirovecii* detection (second group). This group included patients without a history of atovaquone treatment or prophylaxis within the year before *P. jirovecii* detection. The median age was 61 years (range, 0.1–89 years); the male-female ratio was 74/35. Underlying conditions included organ transplantation (26 patients), HIV infection (22 patients), hematological malignancy (14 patients), cancer (11 patients), autoimmune disease (seven patients), premature birth (four patients), interstitial lung disease (three patients), lymphopenia (two patients), congenital heart disease (two patients), hypersensitivity pneumonitis (one patient), non-X histiocytosis (one patient), West syndrome (one patient), tetralogy of Fallot (one patient), primary immunodeficiency (one patient), laryngomalacy (one patient), Down's syndrome (one patient), esophageal carcinoma and COPD (Chronic Obstructive Pulmonary Dis-

Table 2. Characteristics of 14 patients with prior exposure to atovaquone at the time of *P. jirovecii* infection diagnoses and *P. jirovecii* alleles at the *CYB* gene.

Patient code[a]	Underlying condition(s)	Patient still under atovaquone at the time of *Pneumocystis* infection diagnoses	Sample	Presentation of *Pneumocystis* infection	Treatment of *Pneumocystis* infection	Outcome	CYB allele[j]	Missense mutation(s) at the Q_o active site
P1	Kidney and heart transplant, HIV[b]	Yes	BAL[c]	PCP[e]	SMX-TMP[g]	Improvement	CYB12[k]	C431T[m]
P2	Heart transplant	Yes	Sputum	PCP	SMX-TMP	Improvement	CYB12	C431T
P3	Heart transplant	Yes	BAL	PCP	SMX-TMP	Improvement	CYB12	C431T
P4	Heart transplant	Yes	BAL	PCP	SMX-TMP, atovaquone and pentamidine	Improvement	CYB12	C431T
P5	Heart transplant	Yes	BAL	PCP	SMX-TMP	Improvement	CYB12	C431T
P6	Heart transplant	Yes	NS[d]	PCP	SMX-TMP	Improvement	CYB12	C431T
P7	Heart transplant	Yes	NS	Colonization[f]	- [h]	Improvement	CYB12	C431T
P8	Heart transplant	Yes	BAL	Colonization	-	Deterioration	CYB1	-
P9	Epidermoid carcinoma	Yes	Sputum	PCP	SMX-TMP	Deterioration	CYB6	-
LR1	Myeloma	Yes	BAL	PCP	Atovaquone, pentamidine	NA[i]	CYB11	C823T[m]
LR2	Acute lymphoblastic leukemia	Yes	BAL	PCP	SMX-TMP	NA	CYB11	C823T
B1	Giant cell arteritis	Yes	Sputum	PCP	SMX-TMP, Atovaquone	Improvement	CYB1	-
B2	Kidney transplant	Yes	BAL	PCP	Pentamidine	Deterioration	CYB2	-
S1	Myasthenia gravis	Yes	BAL	PCP	Atovaquone, SMX-TMP	Improvement	Mixed[l]	C431T and C823T

[a]Patients P1 to P9 were monitored at Bichat–Claude Bernard hospital, Paris, France, and correspond to the same cluster during a *P. jirovecii* infection outbreak. Patients LR1 and LR2 were monitored at South Réunion Island University Hospital, Réunion Island, France. Patients B1 and B2 were monitored at Brest University Hospital, Brest, France. Patient S1 was monitored at Saint-Étienne University Hospital, Saint-Étienne, France. All *P. jirovecii* specimens were examined at the *CYB* gene.
[b]HIV, Human immunodeficiency virus.
[c]BAL, Bronchoalveolar lavage.
[d]NS, Nasopharyngeal swab.
[e]PCP, *Pneumocystis* pneumonia.
[f]*Pneumocystis jirovecii* was not detected by microscopic examination, it was only detected by PCR assay. The patients presented alternative diagnoses of PCP.
[g]SMX-TMP, Sulfamethoxazole-trimethoprim.
[h]Patients P7 and P8 did not receive any specific treatment against *P. jirovecii* infection since they were considered to be colonized.
[i]NA, Not available.
[j]CYB, cytochrome b. Alleles were named using scoring nucleotide positions 597, 823, and 919, as previously described.[15–17]
[k]Initially named CYB2 considering nucleotide position 919,[16] and renamed CYB12 in this study considering additional SNP at nucleotide position 431.
[l]Allele was not determined because of putative mixed infections revealed by more than one SNP at more than one nucleotide position simultaneously.
[m]SNPs C431T and C823T from the CYB whole gene corresponded respectively to nucleotide positions C350T and C742T using the partial reference sequence (GenBank accession number AF074871).[11]

ease) (one patient), and unknown underlying condition (10 patients) (Table 1 and Supplementary Table S1). Some patients may have been previously enrolled in other studies, and their data described in part elsewhere.[13–15,18–21] *Pneumocystis jirovecii* DNA specimens were stored at -20°C until further examination.

The pair of the primers CytbFw and CytbRw was used to amplify a section of the *CYB* gene, as described elsewhere.[16] PCR reactions were carried out as previously described.[13] The expected size of the amplicons was 638 bp. 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. To monitor for possible contamination, negative controls were included. PCR products were purified using ExoSAP-IT® (Affymetrix Inc., USA) 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, USA) on the ABI Prism 3130xl DNA sequencer (Applied Biosystems, Courtaboeuf, France). DNA sequences of *CYB* were edited using Pregap and Gap software (Staden package version 2.0.0b11-2016; Staden Group).[22] Consensus sequences were aligned with the reference sequence (GenBank accession number AF074871)[11] using the BioEdit software

with the Clustal W program. The putative nucleotide positions were not those initially numbered by Walker and colleagues to describe the SNPs.[11] We renumbered them considering the whole *CYB* gene included in the *P. jirovecii* mitochondrial genome assembly from Ma and colleagues (GenBank accession number JX855938) (Table 3).[23] Alleles were named using the nomenclature based on scoring nucleotide positions 360, 380, 429, 443, 450, 597, 628, 647, 756, 823, 913, 914, and 919.[15–17]

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. Ethical review and approval were waived for this study, in accordance with the French public health law (CSP Art L1121e1.1), because this study was non-interventional, focused on *P. jirovecii* specimens, and used samplings and data obtained from the patients' standard routine care.

Results

Pneumocystis jirovecii CYB nucleotide sequences of the 123 patients (124 specimens) were obtained and compared with the wild-type reference sequence (GenBank accession number AF074871).[11] Overall, 10 SNPs were observed at nucleotide positions 360 (C or T), 363 (C or T), 429 (A or G), 431 (C or T), 597 (C or T), 628 (C or T), 647 (C or T), 823 (C or T), 846 (G or A), and 919 (C or T) (Table 2 and Supplementary Table S1). SNPs C360T, A429G, C597T, and C628T, correspond to previously described synonymous mutations that were not involved in amino acid changes (Tables 2 and 3, and Supplementary Tables S1, S2, and S3).[11,12,24–27] They were observed in 17, 4, 1, and 26 patients of the second patient group, respectively. Two previously undescribed SNPs, C363T and G846A, corresponding to synonymous mutations were found in one and two patients of the second patient group, respectively (GenBank accession numbers OR192130 and OR192131, respectively). The SNP at position 363 was detected in only one patient. It was confirmed with a second PCR-sequencing experiment. Moreover, one SNP at position 597 was also detected in one patient of the first group.

Four SNPs that corresponded to four previously described missense mutations [C431T (Ala144Val), C647T (Ser216Leu), C823T (Leu275Phe), and C919T (Leu307Phe)] were identified (Tables 2 and 3, and Supplementary Tables S2 and S3). Both C431T (Ala144Val) and C823T (Leu275Phe), located at the Q_o site of the mitochondrial cytochrome bc1, the active site of the enzyme, were identified in eight and three patients of the first patient group, respectively (one patient harbored both mutations). C431T (Ala144Val) was also evidenced in two patients from the second group. C647T (Ser216Leu) was only found in the second patient group (four patients). C919T (Leu307Phe) was found in both patient groups (in eight and 43 patients, respectively). These latter mutations were not located at the Q_o site of the enzyme. Mixed infections were observed in 23 patients, one of the first group and 22 of the second group. Mixed infections are defined by the presence of more than one SNP at more than one nucleotide position simultaneously in the same *P. jirovecii* specimen.

Overall, 10 *CYB* alleles named CYB1 to CYB8, CYB10, and CYB11 were identified (Table 2 and Supplementary Tables S1, S2, and S3). CYB1 and CYB2 were the most frequent alleles in

Table 3. Scoring positions and corresponding *CYB* alleles found in this study in patients with or without prior atovaquone exposure at the time of *P. jirovecii* infection diagnoses.

Nucleotide positions															Prior atovaquone exposure at the time of *P. jirovecii* infection (%)	No prior atovaquone exposure at the time of *P. jirovecii* infection (%)	Total (%)
360[a]	363	380	429	431	443	450	597	628	647	756	823	846	913	914	919	Allele			
C	C	C	A	C	C	G	C	C	C	A	C	G	T	T	C	CYB1	2 (14.3)	46 (36.5)	48 (34.3)
C	C	C	A	C	C	G	C	C	C	A	C	G	T	T	**T**[c]	CYB2	1 (7.1)	37 (29.4)	38 (27.1)
C	C	C	**G**	C	C	G	**T**	C	C	A	C	G	T	T	C	CYB3	0	2 (1.6)	2 (1.4)
C	C	C	A	C	C	G	C	**T**	C	A	C	G	T	T	C	CYB4	0	1 (0.8)	1 (0.7)
T	C	C	A	C	C	G	**T**	C	C	A	C	G	T	T	C	CYB5	0	5 (4)	5 (3.6)
C	C	C	A	C	C	G	**T**	C	C	A	C	G	T	T	C	CYB6	1 (7.1)	11 (8.6)	12 (8.6)
C	C	C	A	C	C	G	C	C	**T**	A	C	G	T	T	C	CYB7	0	4 (3.2)	4 (2.9)
T	C	C	A	C	C	G	C	C	C	A	C	G	T	T	C	CYB8	0	8 (6.3)	8 (5.7)
C	C	C	A	C	C	G	C	C	C	**T**	C	G	T	T	C	CYB10	0	2 (1.6)	2 (1.4)
C	C	C	A	C	C	G	C	C	C	A	**T**	G	T	T	C	CYB11	2 (14.3)	0	2 (1.4)
C	C	C	A	**T**	C	G	C	C	C	A	C	G	T	T	**T**	CYB12	7 (50)	2 (1.6)	9 (6.4)
C	**T**	C	A	C	C	G	C	C	C	A	C	G	T	T	C	CYB13	0	1 (0.8)	1 (0.7)
C	C	C	A	C	C	G	C	C	C	A	C	**A**	T	T	C	CYB14	0	1 (0.8)	1 (0.7)
																Undetermined[b]	1 (7.1)	6 (4.8)	7 (5)

[a]Alleles were named using scoring nucleotide positions 360, 380, 429, 443, 450, 597, 628, 647, 756, 823, 913, 914, and 919, as previously described.[15–17] Nucleotide positions 363 and 846 were used for the first time in this study for the description of alleles CYB13 and CYB14, respectively.
[b]Allele was not determined because of putative mixed infections revealed by more than one SNP at more than one nucleotide position simultaneously.
[c]Bold letters correspond to the SNPs.

the second group of patients, as they represented 36.5% and 29.4% of alleles in this patient group, respectively. Seven *P. jirovecii* strains from the first group harbored the CYB2 allele with an additional C431T (Ala144Val) missense mutation. This allele, CYB2, could be renamed as a new allele, CYB12, which was the most frequent allele in the first group. Alleles corresponding to the previously undescribed SNPs C363T and G846A could be renamed as new alleles CYB13 and CYB14, respectively. The allele was not determined in patients B4, B26, B27, B37, B56, S1, and LR12 because of putative mixed infections. A significant difference was observed between the two patient groups, atovaquone exposed vs. atovaquone non-exposed, at the time of *P. jirovecii* detection when comparing the repartition of alleles including synonymous mutations (CYB1, CYB3 to CYB6, CYB8, CYB13, and CYB14), other alleles including missense mutations far from the active site (CYB2, CYB7, and CYB10), and other alleles including missense mutations at the Q_o site of the enzyme (CYB11 and CYB12) (Fisher's exact test, $P < 0.001$). These findings suggest that atovaquone exposure leads to allele selection, specifically those harboring missense mutations at the Q_o site. Nonetheless, if we consider only the patients effectively cured with atovaquone for PCP treatment, none of the aforementioned allele groups was significantly associated with improvement or deterioration (Fisher's exact test, $P = 0.19$).

Discussion

Mutations in *P. jirovecii* strains potentially resulting from atovaquone prior exposure have been already reported.[11–15] Here, we assessed the selective pressure exerted by atovaquone on *P. jirovecii* through a French multicenter study. We chose an approach that compared specifically patients with prior atovaquone exposure and patients without prior atovaquone exposure at the time of PCP, *P. jirovecii* colonization, or primary infection diagnoses. The association of the two previously described missense mutations C431T (Ala144Val) and C823T (Leu275Phe) located at the Q_o active site of the mitochondrial cytochrome bc1,[12–15] with prior atovaquone exposure was strengthened. Indeed, none of the patients without atovaquone exposure were infected with *P. jirovecii* harboring the aforementioned mutations, except the two patients who contracted the fungus in a context of nosocomial acquisition during an outbreak.[13,14]

If the following missense mutations Ile147Val, Thr148Ile, Leu150Phe, Ser152Ala, Pro266Leu, and Leu275Phe in the CYB gene that confer *in vitro* resistance to atovaquone have already been described,[8–10] to the best of our knowledge, no study highlighting clinical failure of PCP atovaquone treatment related to *P. jirovecii CYB* mutant strains is presently available. Our study did not bring any additional data of this topic.

Two previously undescribed SNPs were found in this study in patients without prior atovaquone exposure at nucleotide positions 363 (C or T) and 846 (G or A), both located far from the active site of the *CYB* subunit. They may reflect genomic diversity and are less likely to be involved in atovaquone selective pressure. Nonetheless, it has been suggested elsewhere that synonymous mutations may affect protein translational kinetics and drug-target interactions.[28–30] We did not consider this field of investigation in this study.

A significant difference in allele distribution between the two patient groups, atovaquone exposed vs. atovaquone non-exposed at the time of *P. jirovecii* detection, was observed. There was a different distribution between alleles related to synonymous mutations, alleles related to missense mutations out of the active site, and alleles related to missense mutations at the active site of the enzyme (Fisher's exact test, $P < 0.001$). These results suggest that atovaquone exposure exerts a selective pressure on *P. jirovecii* organisms revealed by missense mutations at the Q_o site of its target gene. Nonetheless, overstatement of the presence of C431T (Ala144Val) from CYB12 in the first patient group and consequently of this putative selective pressure of atovaquone is possible since nine of the patients monitored in Paris Bichat hospital were involved in an outbreak in the course of which a *P. jirovecii* strain harboring this mutation was transmitted.

The low number of patients enrolled in this retrospective study may represent its limitation. Indeed, despite the enrollment of patients from six French centers, only 14 patients met the criteria for being enrolled in the first patient group, i.e., atovaquone-exposed patients. This is explained by the fact that atovaquone remains a second-line therapy or prophylaxis for PCP. Despite $P < 0.001$ as shown by Fisher's exact test, our analysis may lack power. Moreover, the low frequency of *CYB* mutants observed in the patient population not exposed to atovaquone could be due to the lack of sensitivity of Sanger sequencing. Indeed, next-generation sequencing was found to be more effective in detecting minor haplotypes.[25] Nonetheless, we detected mixed infections in 45/123 patients using Sanger sequencing, which then may seem efficient enough to detect potential minor haplotypes.

In this study, atovaquone exposure was defined on the basis of the patients' prior exposure during the year before *P. jirovecii* detection, whatever the atovaquone dosage and its duration. We assumed that the regimens consisted of 750 mg twice a day. However, the duration of the treatment, plasma/serum atovaquone levels, and treatment compliance were unavailable data. This may represent another limitation of this study.

One patient from the first group was infected with *P. jirovecii* organisms harboring the SNP C431T (Ala144Val) but was not concerned by the cluster that occurred in Paris Bichat Hospital. The patient was monitored in another French hospital, Saint-Étienne University Hospital, 550 km from Paris. *P. jirovecii* organisms in this patient harbored also the SNP C823T (Leu275Phe). The two other patients infected with *P. jirovecii* organisms that harbored C823T (Leu275Phe) were monitored at Réunion University Hospital, 9400 km from metropolitan France. These three patients belong to the first patient group and had prior atovaquone exposure at the time of the *P. jirovecii* detection. It is noteworthy that the nucleotide change C823T was previously observed by Kazanjian and colleagues in a *P. jirovecii* specimen from a patient who presented with prior atovaquone exposure.[12] Be that as it may, these observations are in favor of the implication of atovaquone exposure in the selection of mutants harboring C431T (Ala144Val) and C823T (Leu275Phe) mutations, located at the active site of the *CYB* subunit.

These results brought additional data to that of Walker et al.[11] and Kazanjian et al.,[12] published in 1998 and 2001, respectively. Both studies described the first SNPs in the *CYB* potentially related to atovaquone prophylaxis failure: C380T (Thr127Ile), A439G (Ile147Val), C443T (Thr148Ile), G450T (Leu150Phe), T454G (Ser152Ala), C797T (Pro266Leu), and C823T (Leu275Phe). They did not name the correspond-

ing *CYB* alleles. The present study describes again atovaquone PCP prophylaxis failure in patients with prior atovaquone exposure, potentially due to missense mutations C823T (Leu275Phe) and C431T (Ala144Val) located at the active site of the *CYB* enzyme, the target of atovaquone. Esteves et al.,[16] Maitte et al.,[17] and Le Gal et al.[15] named for the first time *CYB* alleles from CYB1 to CYB11 in years 2010, 2013, and 2020, respectively. In the present study, we propose to name new alleles, from CYB12 to CYB14.

In conclusion, the results suggest that the direct selective pressure exerted by atovaquone among *P. jirovecii* microorganisms in patients with prior exposure to the drug is high. Conversely, the indirect selective pressure of atovaquone, which could have been revealed by the circulation, and the presence of *P. jirovecii CYB* mutants in patients without prior exposure to the drug remains incidental and does not represent a public health issue in France so far.

Supplementary material

Supplementary material is available at *Medical Mycology* online.

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Author contributions

Pierre L. Bonnet (Data curation, Formal analysis, Investigation, Methodology, Resources, Writing—original draft), Claire V. Hoffmann (Data curation, Resources, Writing—review & editing), Nathan Le Nan (Data curation, Resources), Lorenn Bellamy (Data curation, Resources), Gautier Hoarau (Resources, Writing—review & editing), Pierre Flori (Resources, Writing—review & editing), Magalie Demar (Resources), Nicolas Argy (Resources), Florent Morio (Data curation, Resources, Writing—review & editing), Solène Le Gal (Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Project administration, Resources, Software, Supervision, Validation, Visualization, Writing—original draft, Writing—review & editing), Gilles Nevez (Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Project administration, Resources, Supervision, Validation, Visualization, Writing—original draft, Writing—review & editing)

Conflicts of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and the writing of the paper.

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Discussion générale

Au cours de ce travail nous avons étudié trois gènes de *P. jirovecii*, le gène *gsc1*, le gène *dhfr* et le gène du cytochrome b (*CYB).* Ces trois gènes codent pour des enzymes cibles de molécules thérapeutiques pouvant exercer une pression de sélection sur *P. jirovecii* et ainsi favoriser l'émergence de mutants potentiellement résistants. Les gènes *gsc1* et *CYB* codent pour la sous-unité Gsc1 de l'enzyme ß-1,3-D-glucane synthase et l'enzyme cytochrome b qui sont les cibles respectives des échinocandines et de l'atovaquone, molécules ayant une activité anti-*Pneumocystis*. Le gène *dhfr* code pour l'enzyme DHFR cible du méthotrexate, molécule employée à visée immunosuppressive. Cette molécule possède également une action antifongique potentielle car sa cible, l'enzyme DHFR, est par ailleurs la cible du triméthoprime qui associé au sulfaméthoxazole représente le traitement et la prophylaxie de première ligne de la PPC. Le gène *fas* codant pour la dihydroptéroate synthase (DHPS) de *P. jirovecii*, la cible du sulfaméthoxazole, aurait pu être analysé d'autant que les données brestoises et rennaises remontent à des travaux datant de 2012 et 2013 [60,61]. Néanmoins, Il nous a semblé plus pertinent de nous focaliser en première intention sur des gènes peu ou pas encore étudiés à Brest et en France.

Nous avons obtenu les premiers résultats en matière d'analyse de la diversité du gène *gsc1* de *P. jirovecii* chez 51 patients. Avant ces travaux, les données sur la diversité du gène *gsc1* étaient des plus limitées car basées sur l'analyse d'un seul échantillon clinique ayant servi au séquençage du génome entier de *P. jirovecii* [31,32] ou sur une analyse fonctionnelle du gène par transfection dans *S. cerevisiae* [62].
Nous avons choisi d'amplifier deux portions du gène *gsc1,* c'est-à-dire deux hot spots dénommés HS1 et HS2 homologues de ceux connus sur le gène *fks1* de *Candida albicans.* En effet, les mutations non synonymes associées à une diminution de la sensibilité de *C. albicans* aux échinocandines qui ont été précédemment rapportées [27-29,63] se situaient au niveau de ces deux hot spots.
La collecte d'échantillons de *P. jirovecii* chez les patients exposés aux échinocandines et développant une PPC a été difficile. En effet, les échinocandines ne représentent qu'un traitement de seconde ligne de la PPC. Celui-ci est réservé aux patients atteints d'hémopathies ou transplantés d'organes solides non infectés par le VIH, en association avec le TMP-SMX [26]. Les échinocandines ne sont en revanche pas

proposées pour la prophylaxie de la PPC. Notre approche a été d'analyser des échantillons de *P. jirovecii* provenant de patients ayant développé une PPC mais ayant été exposés aux échinocandines en raison d'antécédents d'infections fongiques invasives effectivement traitées par ces antifongiques. Cette approche a permis d'inclure 27 patients infectés par *P. jirovecii* et ayant été exposés aux échinocandines. Nous avons également inclus 24 autres patients infectés par *P. jirovecii* mais non exposés aux échinocandines, ceux-ci représentant un groupe contrôle.

Trois polymorphismes mononucléotidiques (SNP) aux positions 2204, 2243 et 2303 ont été identifiés sur HS1, et un SNP à la position 4540 a été identifié sur HS2. Trois allèles *gsc1* HS1, A, B et C, deux allèles *gsc1* HS2, a et b, et quatre haplotypes, Ca, Cb, Aa et Ba, ont été définis. Nous n'avons pas mis en évidence de différence significative dans la distribution des haplotypes entre les deux groupes de patients (*P* = 0,57). Cependant, 25 patients n'étaient plus traités par échinocandines au moment du diagnostic de l''infection par *P. jirovecii*. La possibilité que les échinocandines aient exercé une pression de sélection sur *P. jirovecii* doit être discutée. Le délai médian entre la fin du traitement par échinocandines et le diagnostic de l'infection par *P. jirovecii* était de 562 jours (152 - 1533 jours). Ce délai rend peu compatible cette pression de sélection sauf si les patients ayant développé l'infection par *P. jirovecii* étaient « porteurs » du champignon avant l'arrêt du traitement par échinocandines. Cela est possible car il a été établi que la PPC peut faire suite à une période de colonisation pulmonaire pouvant représenter une étape d'incubation dont la durée n'est pas bien connue. En revanche, si l'on prend en compte la durée d'incubation de sept à 90 jours qui a été rapportée dans la littérature [64-67] ou le fait que la PPC résulte de l'acquisition de novo de *P. jirovecii* avec une période d'incubation courte, ce délai reste incompatible avec une pression de sélection exercée par les échinocandines. De plus, deux patients étaient traités par échinocandines au moment du diagnostic de l'infection par *P. jirovecii*. Les conditions étaient donc réunies pour observer les effets d'une pression de sélection sur le gène *gsc1* de *P. jirovecii*. Toutefois, ces deux patients portaient l'allèle Ca de *P. jirovecii*. Cet allèle était le plus fréquemment observé quels que soient les patients inclus dans l'étude (i.e. exposés ou non exposés aux échinocandines). Il n'était pas caractérisé par des mutations non-synonymes et ne représentait donc pas la signature de l'exposition aux échinocandines.

L'analyse du gène *gsc1* de *P. jirovecii* a permis l'identification de trois allèles sur HS1 et deux allèles sur HS2, formant quatre haplotypes différents, Ca, Cb, Aa et Ba. En termes de génotypage, il a été possible d'établir un index (H) de Hunter [68] à 0,21 pour HS1 et 0,44 pour HS2 pris séparément. En cas de combinaison d'HS1 et HS2, l'index H s'élève à 0,67. Malgré cela, cette valeur reste insuffisante au regard des recommandations sur le pouvoir de discrimination d'une méthode de génotypage qui requiert un index H supérieur à 0,95 [69,70]. Ces réserves pourraient être aisément levées par l'adjonction de l'analyse du gène *gsc1* à la méthode de génotypage MLST comprenant les gènes mtLSUrRNA, cytochrome b (CYB), et superoxyde dismutase (SOD) qui est utilisée par notre équipe [58,71].

Malgré d'obtention de données originales sur la diversité du gène *gsc1* de *P. jirovecii*, la principale limite de notre travail reste le faible nombre de patients effectivement exposés aux échinocandines ainsi qu'une définition rigoureuse de l'exposition aux échinocandines. Le prolongement de cette étude monocentrique par une étude multicentrique nationale permettrait d'inclure un plus grand nombre de patients et d'obtenir un plus grand nombre d'échantillons de *P. jirovecii*. Une large diffusion des recommandations de l'ECIL proposant l'usage des échinocandines comme traitement de seconde ligne de la PPC [26] en cancérologie, pourrait conduire à terme à une augmentation de l'exposition des patients à ces drogues et jeter les bases de cette étude multicentrique. Par ailleurs, une période maximale d'exposition de trois mois précédant le diagnostic de l'infection par *P. jirovecii* pourrait être définie comme critère d'inclusion comme cela a été retenu lors d'études sur la DHPS et l'exposition aux sulfamides [65,66]. Cette période correspond à la durée maximale d'incubation admise.

Chez les rongeurs, les échinocandines apparaissent efficaces pour la prophylaxie et le traitement de la PPC [revues *in* 72]. L'usage des échinocandines en monothérapie pour le traitement de la PPC en pratique clinique reste discuté [revues *in* 72, revues *in* 73, revues *in* 74]. Ces molécules semblent actives sur les asques et non les formes trophiques de *P. jirovecii* [75]. En revanche, l'association des échinocandines et du triméthoprime-sulfaméthoxazole (TMP-SMX), pouvant couvrir tout le cycle biologique du champignon, a été présentée comme une alternative à la monothérapie [75]. La bithérapie pourrait également permettre de diminuer les doses de TMP-SMX et donc les effets indésirables potentiels.

De nouvelles molécules ciblant la ß-1,3-D-glucane synthase sont en cours de développement. La rezafungine est une nouvelle génération d'échinocandine dont l'administration est hebdomadaire. Son efficacité en matière de prophylaxie et de traitement a été démontrée dans le modèle murin sur les asques et à long terme sur les formes trophiques de *P. murina* [76,77]. Des études de phase III sont en cours concernant l'usage de la rezafungine dans le cadre des infections fongiques invasives dont les candidoses invasives, les aspergilloses invasives et la PPC [78]. L'ibrexafungerp est un triterpénoïde qui cible la ß-1,3-D-glucane synthase, mais dont la zone cible diffère légèrement de la zone cible des échinocandines. C'est ainsi que la résistance aux échinocandines n'est pas systématiquement accompagnée d'une résistance à l'ibrexafungerp [revues *in* 79]. Son efficacité a été démontrée sur les asques de *P. murina* en prophylaxie et traitement de la PPC [revues *in* 79, 80]. Bien que nous n'ayons pas observé de pression de sélection sur le gène *gsc1* au cours de notre première approche, la diffusion de ces nouvelles molécules en pratique clinique dans les années futures va contribuer à cette pression sur les espèces fongiques impliquées en médecine dont *P. jirovecii*. Dans ce contexte, la poursuite d'une surveillance se justifie.

Nous avons obtenu dans le deuxième volet du travail de thèse les premières données sur la diversité du gène *dhfr* de *P. jirovecii* chez 21 patients exposés au méthotrexate (MTX). Nahimana et coll. ont rapporté l'existence d'une association significative entre des mutations non synonymes sur le gène *dhfr* et l'échec de la prophylaxie de la PPC par les inhibiteurs de l'enzyme DHFR, principalement la pyriméthamine et éventuellement le triméthoprime (TMP) [36]. Le MTX est communément employé pour le traitement de maladies inflammatoires systémiques ou de cancers tels que la leucémie aiguë lymphoblastique et le lymphome de Burkitt [43]. En revanche, Il ne peut être utilisé comme traitement ou prophylaxie de la PPC en raison de son action cytotoxique. Quoiqu'il en soit, il cible bien la DHFR humaine et potentiellement celle de *P. jirovecii* [57]. Des patients traités par MTX peuvent développer une PPC en raison de leur statut d'immunodépression [revues *in* 46] et pourraient être infectés par des souches de *P. jirovecii* mutantes sur le gène *dhfr*.
L'analyse des séquences du gène *dhfr* de *P. jirovecii* provenant de ces 21 patients et de 22 patients contrôles non exposés au MTX a permis d'identifier trois SNPs. L'association de ces différents SNPs a permis de définir trois allèles qui ont été

dénommés A, B et C. Aucune différence n'a été mise en évidence en termes de distribution des allèles de *P . jirovecii* dans les deux groupes de patients (test exact de Fisher, *P* = 0,28). Plus précisément, la diversité du gène *dhfr* de *P. jirovecii* apparait ainsi identique qu'il s'agisse de patients exposés ou non exposés au MTX. Le MTX bien que ciblant théoriquement la DHFR de *P. jirovecii* ne semble pas exercer de pression de sélection.

Nous n'avons retrouvé que 21 patients présentant les critères d'inclusion du groupe traités par MTX malgré une requête sur notre système informatique général du laboratoire portant sur 14 ans. Ce faible nombre pourrait expliquer en partie l'absence de différence significative observée en matière de diversité génétique de *dhfr* entre les patients exposés et non exposés au MTX.

Dix-huit patients sur les 21 inclus dans l'étude étaient encore traités par MTX au moment du diagnostic d'infection par *P. jirovecii*. Les conditions étaient donc bien réunies pour que le MTX puisse théoriquement exercer une pression de sélection sur *P. jirovecii*. De plus, les patients traités par MTX ont bien développé une PPC qui pourrait, en conséquence comme nous l'avons écrit plus haut, résulter de souches mutantes sur le gène *dhfr*. Notre démarche était pertinente mais notre hypothèse n'a pas été confirmée.

La survenue de l'infection à *P. jirovecii* chez ces patients pourrait s'expliquer par l'inefficacité *in vivo* du MTX sur le champignon alors que la drogue présente bien une forte activité inhibitrice *in vitro* sur la DHFR de *P. jirovecii* [57]. Cette discordance entre l'activité *in vitro* et l'activité *in vivo,* c'est-à-dire sur *P. jirovecii* en situation pathogène chez le patient, pourrait s'expliquer par le fait que le MTX n'atteint pas la DHFR intracellulaire du champignon. Les cellules des mammifères possèdent un transporteur membranaire de haute affinité, le RFC (Reduced Folate Carrier), qui est la voie majoritaire de captation du MTX [revues *in* 81]. Ce transporteur n'a pas été identifié chez *P. jirovecii*. En effet, nous n'avons pas retrouvé de séquences homologues du RFC dans les génomes et protéomes de *P. jirovecii* (BioProject PRJEA68827 de Cissé et coll. [31] ; BioProject PRJNA223510 de Ma et coll. [32]), *P. carinii* (BioProject PRJNA223511 [32]), ou de *P. murina* (BioProject PRJNA70803 [32]) et d'autres champignons apparentés à *Pneumocystis* sp*. tels que Saccharomyces cerevisiae* (BioProject PRJNA393501 [82]) ou *Schizosaccharomyces pombe* (BioProject PRJNA527756 [83]), en utilisant comme requête les séquences de la protéine RFC de mammifères tels que *Homo sapiens*, *Pan troglodytes* (chimpanzé), *Bos taurus* (bovin),

Rattus norvegicus (rat brun), et *Mus musculus* (souris) (numéros d'accession GenBank AAC50180, XP_001157360, NP_001069921, NP_001030309 et NP_112473, respectivement) dans tblastn et blastp (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Cette protéine serait donc absente du protéome des champignons évoqués ci-dessus, dont *P. jirovecii*. Une captation cellulaire réduite ou l'absence de captation du MTX chez *P. jirovecii* pourrait ainsi expliquer l'absence d'activité et en conséquence l'absence de pression de sélection du MTX sur *P. jirovecii*.

Des mutations chez *P. jirovecii* résultant potentiellement d'une exposition antérieure à l'atovaquone ont déjà été rapportées [50,51,56,58,59]. En 1998, Walker et coll. ont décrit chez des patients pris en charge aux États-Unis et au Royaume-Uni les mutations faux-sens C443T (Thr148Ile) et G450T (Leu150Phe) [50]. En 2001, les mutations faux-sens C380T (Thr127Ile), A439G (Ile147Val), T454G (Ser152Ala), C797T (Pro266Leu), C823T (Leu275Phe) et C919T (Leu307Phe) ont été décrites par Kazanjian et coll. chez des patients pris en charge aux États-Unis [51]. Nous avons évalué ici la pression de sélection exercée par l'atovaquone sur *P. jirovecii* à travers une étude multicentrique française. Nous avons choisi une approche comparant spécifiquement 14 patients ayant déjà été exposés à l'atovaquone et 109 patients sans exposition préalable à l'atovaquone, au moment du diagnostic de PPC, de colonisation par *P. jirovecii* ou de primo-infection.
L'association probable des deux mutations faux-sens précédemment décrites C431T (Ala144Val) et C823T (Leu275Phe) situées au niveau du site actif Q_o du cytochrome b mitochondrial [51,56,58,59] avec une exposition préalable à l'atovaquone a à nouveau été suggérée par nos résultats. En effet, les échantillons de *P. jirovecii* caractérisés par les mutations C431T (Ala144Val), et/ou C823T (Leu275Phe) concernaient 74,1% des patients ayant déjà été exposés à l'atovaquone. Par ailleurs, aucun des patients non exposés à l'atovaquone n'a été infecté par une souche de *P. jirovecii* porteuse des mutations précitées, à l'exception des deux patients ayant été infectés par le champignon dans un contexte d'infection nosocomiale lors d'une épidémie à l'hôpital Paris Bichat au cours de laquelle une souche de *P. jirovecii* caractérisée par la mutation C431T (Ala144Val) a été transmise [56].
Si les mutations Ile147Val, Thr148Ile, Leu150Phe, Ser152Ala, Pro266Leu, Leu275Phe ont entraîné l'apparition d'une résistance à l'atovaquone dans un modèle enzymatique

in vitro [revues *in* 84], à notre connaissance aucune étude n'a démontré l'existence d'échecs thérapeutiques lié à des souches mutantes de *P. jirovecii* au niveau du gène *CYB*. Notre étude n'a pas apporté de données supplémentaires à ce sujet étant donné que seuls deux patients infectés par *P. jirovecii* ayant des mutations non synonymes sur le site actif du cytochrome b ont été traités par atovaquone pour leur PPC. Cependant, les mutations Tyr268Ser/Cys identifiées chez *Plasmodium falciparum*, correspondant aux mutations Tyr279Cys/Ser chez *S. cerevisiae*, ont été mises en évidence chez des patients en échec thérapeutique suite à un traitement par atovaquone ou atovaquone-proguanil [85,86]. Elles entrainent la perte d'un résidu aromatique au sein du site de liaison de l'atovaquone, entraînant un défaut dans la stabilisation par interaction hydrophobe entre l'atovaquone et les résidus du site actif Q_o du cytochrome b et potentiellement une déformation structurelle de ce même site [revues *in* 84]. Il est donc probable que les mutations au sein du site Q_o de *P. jirovecii* mènent également à des échecs thérapeutiques.

Deux SNP non décrits auparavant ont été observés au cours de notre travail de thèse chez des patients sans exposition préalable à l'atovaquone aux positions nucléotidiques 363 (C ou T) et 846 (G ou A), toutes deux situées loin du site actif du cytochrome b. Ces mutations synonymes peuvent refléter une diversité génomique et sont en conséquence moins susceptibles d'être impliquées dans la pression de sélection exercée par l'atovaquone.

Une différence significative dans la distribution des allèles entre les deux groupes de patients, exposés ou non exposés à l'atovaquone au moment de la détection de *P. jirovecii*, a été observée. Les allèles caractérisés par des mutations synonymes étaient CYB1, CYB3 à CYB6, CYB8, CYB13 et CYB14. Les allèles caractérisés par des mutations faux-sens situées loin du site actif étaient CYB2, CYB7 et CYB10, et les allèles caractérisés par des mutations faux-sens au niveau du site Q_o de l'enzyme étaient CYB11 et CYB12. La distribution de ces trois groupes d'allèles diffère entre les deux groupes de patients, les allèles caractérisés par des mutations faux-sens situées au niveau du site Q_o étant plus fréquents chez les patients exposés à l'atovaquone (test exact de Fisher, $P < 0.001$). Ces résultats suggèrent que l'exposition à l'atovaquone exerce une pression de sélection sur *P. jirovecii* révélée par des mutations faux-sens au niveau du site Q_o du cytochrome. Néanmoins, une surestimation de la présence du SNP C431T (Ala144Val) caractérisant l'allèle CYB12 dans le premier groupe de patients, et par conséquent de cette potentielle pression de

sélection exercée par l'atovaquone, est possible. En effet, neuf des patients suivis à l'hôpital Paris Bichat ont été impliqués dans une épidémie au cours de laquelle les mutants de *P. jirovecii* portant cette mutation ont été transmis.

Il est important de considérer que *P. jirovecii* est un microorganisme transmissible qui circule au sein des populations humaines. En prenant en compte cette circulation de *P. jirovecii*, il est probable que des mutants en lien avec une pression de sélection soient détectés chez des patients non exposés à l'atovaquone. Dans ce cas, l'analyse du gène du cytochrome b et la détection de mutants au niveau du gène *CYB* peuvent représenter un marqueur de circulation de *P. jirovecii* au sein des populations humaines. Ce concept a été étudié par des chercheurs s'intéressant à la présence de mutants au niveau de l'enzyme DHPS chez *Pneumocystis* malgré l'absence d'exposition aux sulfamides [60,87,88]. La présence de ces mutants a été expliquée par la circulation de *P. jirovecii* au sein de la population humaine, à l'hôpital ou en communauté. Pour cette raison, le groupe de 109 patients non exposés de notre étude est utilisé en tant qu'élément comparatif avec le groupe de 14 patients exposés, et permet de documenter la circulation des mutants dans les populations.

Un patient du groupe exposé à l'atovaquone était infecté par une souche de *P. jirovecii* caractérisée par le SNP C431T (Ala144Val) mais n'était pas concerné par le cluster survenu à l'hôpital Bichat à Paris que nous avons évoqué plus haut. Le patient était suivi dans un autre hôpital français, le CHU de Saint-Étienne, à 550 km de Paris. *P. jirovecii* chez ce patient portait en plus le SNP C823T (Leu275Phe). Les deux patients infectés par *P. jirovecii* ayant le SNP C823T (Leu275Phe) étaient suivis au CHU de La Réunion, à 9 400 km de la France métropolitaine et appartenaient également au groupe exposé à l'atovaquone. Il convient de noter que le changement nucléotidique C823T a déjà été observé par Kazanjian et coll. chez *P. jirovecii* provenant d'un patient également exposé à l'atovaquone [51]. Ces mutations ont donc été observées chez *P. jirovecii* provenant de patients a priori non liés sur le plan épidémiologique. C'est un argument supplémentaire en faveur de l'implication de l'exposition à l'atovaquone dans la sélection de mutants porteurs des mutations C431T (Ala144Val) et C823T (Leu275Phe), localisées au niveau du site actif du cytochrome b.

Walker et coll. [50] et Kazanjian et coll. [51] ont décrit les premiers SNPs sur le gène *CYB* potentiellement liés à l'échec de la prophylaxie par l'atovaquone mais ces deux équipes n'ont pas nommé les allèles pouvant être caractérisé par ces SNPs. Esteves et coll., Maitte et coll., et Le Gal et coll. ont nommé les allèles CYB de CYB1 à CYB11

[52,59,89]. Nous avons proposé de nommer de nouveaux allèles de CYB12 à CYB14 caractérisés par les mutations C431T (Ala144Val) et C919T (Leu307Phe) pour CYB12, C363T pour CYB13 et G846A pour CYB14.

Le faible nombre de patients inclus dans ce troisième volet de travail de thèse représente une de ses limites. En effet, malgré le recrutement rétrospectif de patients provenant de six centres français, seulement 14 patients répondaient aux critères d'inclusion dans le premier groupe de patients, à savoir les patients exposés à l'atovaquone. Ceci s'explique par le fait que l'atovaquone reste un traitement ou une prophylaxie de deuxième intention de la PPC.

L'atovaquone pourrait à l'avenir être employée à la fois en tant que thérapie anti-cancéreuse et prophylaxie de la PPC au cours des leucémies aiguës myéloïdes (LAM) en pédiatrie [90]. En effet, l'atovaquone entrave la phosphorylation oxydative au sein des cellules cancéreuses et inhibe ainsi ce mécanisme respiratoire alternatif qui permet à ces cellules de résister à la chimiothérapie standard par anthracyclines et aracytine [91]. L'emploi de cette molécule pourrait en conséquence devenir plus fréquent. Les essais de phase III sont en faveur de cette option thérapeutique pour traiter les LAM en pédiatrie. La pression de sélection de l'atovaquone sur *P. jirovecii* pourrait augmenter dans ces circonstances. Il s'agit d'un argument supplémentaire pour poursuivre la surveillance de l'émergence de *P. jirovecii* mutants potentiellement résistants à l'atovaquone.

Au final, quels que soient les volets de notre travail de thèse, les limites étaient le faible nombre de patient inclus, notamment ceux exposés aux échinocandines, au MTX ou à l'atovaquone. Ces limites seront aisément contournables pour des travaux futurs en faisant un appel national au sein de la collégiale ANOFEL (association française des enseignants et praticiens hospitaliers titulaires de parasitologie et mycologie médicales) pour des PHRC nationaux et des enquêtes multicentriques éventuellement dans une démarche prospective selon des règles d'inclusion rigoureuses en termes de nombre de patients à inclure.

Nous avons retenu le gène *gsc1* car peu de données sur sa diversité étaient disponibles. Le gène *dhfr* avait été plus fréquemment étudié, mais l'activité antifongique secondaire potentielle du MTX sur *P. jirovecii* n'avait pas encore été explorée. L'étude du cytochrome b de *P. jirovecii* pouvait apparaitre peu innovante,

mais les données françaises sur le sujet étaient parcellaires. Ces arguments justifient notre choix de ces trois gènes codant pour des cibles thérapeutiques.

Une autre thèse effectuée parallèlement au sein de notre équipe de recherche porte sur l'étude du gène *impdh*, codant pour l'enzyme IMPDH (inosine 5'-monophosphate déshydrogénase), cible de l'acide mycophénolique (MPA) employé en tant que traitement anti-rejet chez les transplantés d'organes solides [92]. Les premiers résultats obtenus démontrent la pression de sélection du MPA sur *P. jirovecii*. Une étude multicentrique au niveau national viendra compléter cette première approche. Celle-ci est en cours de publication. Les données nationales et bretonnes sur la diversité du locus de la DHPS de *P. jirovecii* cible des sulfamides remontant à 2012 et 2013 respectivement [60,61], il sera nécessaire d'effectuer une actualisation .

Notre approche a été basée sur la détection et l'identification de mutations non synonymes. Cependant, il a été suggéré que des mutations synonymes pourraient affecter la cinétique de la traduction ainsi que les interactions entre les drogues et leur cible [93-95]. Ceci représente une autre approche pertinente que nous n'avons pas développée dans ce travail de thèse. Il s'agit d'un nouveau champ d'investigation.

Quelles que soient les cibles génomiques analysées, la faible fréquence de mutants observée pourrait être due au manque de sensibilité du séquençage selon la méthode de Sanger. En effet, le séquençage de nouvelle génération (NGS) s'est révélé plus efficace pour détecter les haplotypes mineurs [96-98]. Alanio et coll., Charpentier et coll., et Gits-Muselli et coll. ont pu détecter entre 66% et 92% d'infections mixtes en couplant une technique de MLST au NGS [96-98]. A contrario, Maitte et coll. n'ont pu détecter que 30% d'infections mixtes à l'aide d'une technique de MLST avec séquençage selon la méthode Sanger [89]. En prenant en compte uniquement le locus CYB, Charpentier et coll. ont mis en évidence 43,75% d'infections mixtes à l'aide du NGS et jusqu'à neuf haplotypes distincts par échantillon avec un seuil de détection de 1% [98]. Au cours du travail sur le gène CYB, nous avons détecté des infections mixtes avec un pourcentage de 36,6% à l'aide du séquençage selon la méthode de Sanger. Cette technique garde tout son intérêt dans sur démarche de génotypage mais le NGS apparait comme une alternative lorsque que le but du séquençage est de détecter précisément les génotypes mineurs.

Conclusion et perspectives

Notre objectif était de caractériser la pression de sélection exercée par les échinocandines, le méthotrexate et l'atovaquone sur les populations de *P. jirovecii*. Chacun des trois volets de ce travail de thèse a été bâti de manière à comparer un groupe de patients exposés et un groupe de patients non exposés à l'une de ces trois drogues. L'amplification et le séquençage des gènes codant pour les cibles de ces trois drogues ont été effectués dans le but de détecter des mutations potentiellement en lien avec une pression de sélection.

Au cours du premier volet, nous avons obtenu les premiers résultats en matière d'analyse de la diversité des hot spots HS1 et HS2 du gène *gsc1* codant pour l'enzyme ß-1,3-D-glucane synthase de *P. jirovecii*, cible des échinocandines. La diversité génétique similaire chez *P. jirovecii* issu de patients exposés ou non exposés aux échinocandines est en faveur de l'absence de pression de sélection. Le deuxième volet a permis d'apporter les premières données sur la diversité du gène *dhfr* codant pour l'enzyme DHFR chez les patients exposés ou non exposés au méthotrexate (MTX). Ce traitement employé à visée immunosuppressive chez l'Homme possède une activité antifongique potentielle qui méritait d'être explorée. Nos résultats suggèrent l'absence de pression de sélection exercée par le MTX sur *P. jirovecii*. Le troisième volet a permis de compléter les données françaises très parcellaires concernant la pression de sélection exercée par l'atovaquone sur l'enzyme cytochrome b de *P. jirovecii*. La pression de sélection de l'atovaquone semble forte sur les patients directement exposés, alors que la pression de sélection indirecte potentielle chez les patients non exposés à l'atovaquone apparait faible.

Notre travail aura été limité par le faible nombre de patients exposés à l'une ou l'autre de ces trois drogues et ayant pu être inclus dans les trois études menées successivement. Bien que la pression de sélection globale exercée par ces trois drogues en France semble faible et ne représente pas actuellement une problématique de santé publique, la surveillance de l'émergence de *P. jirovecii* mutants potentiels reste justifiée.

En termes de perspectives, les travaux futurs pourront porter sur l'actualisation de la pression de sélection exercée par les sulfamides à Brest et en Bretagne. En effet, les dernières données sur la diversité du locus *dhps* du gène *fas* sont anciennes puisqu'elles remontent aux années 2012 et 2013 [60,61].

Par ailleurs, l'équipe de Brest poursuit ses travaux sur le rôle des immunosuppresseurs anti-rejet, précisément l'acide mycophénolique dans la sélection de mutants IMPDH de *P. jirovecii* chez les transplantés d'organes solides [92] et plus largement sur la sélection de souches spécifiques chez ces patients. L'étude d'autres immunosuppresseurs antirejet et leur impact sur *P. jirovecii* comme les inhibiteurs de la calcineurine a été également initiée. Ces travaux font l'objet d'une autre thèse d'université à l'UBO qui se positionnera dans une suite logique à mon propre travail de thèse.

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Annexe : participation à des publications sur le thème de la pneumocystose en parallèle des travaux de thèse

frontiers in Cellular and Infection Microbiology

MINI REVIEW
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Pneumocystis jirovecii in Patients With Cystic Fibrosis: A Review

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Pneumocystis pneumonia (PCP) remains the most frequent AIDS-defining illness in developed countries. This infection also occurs in non-AIDS immunosuppressed patients, e.g., those who have undergone an organ transplantation. Moreover, mild *Pneumocystis jirovecii* infections related to low pulmonary fungal burden, frequently designated as pulmonary colonization, occurs in patients with chronic pulmonary diseases, e.g., cystic fibrosis (CF). Indeed, this autosomal recessive disorder alters mucociliary clearance leading to bacterial and fungal colonization of the airways. This mini-review compiles and discusses available information on *P. jirovecii* and CF. It highlights significant differences in the prevalence of *P. jirovecii* pulmonary colonization in European and Brazilian CF patients. It also describes the microbiota associated with *P. jirovecii* in CF patients colonized by *P. jirovecii*. Furthermore, we have described *P. jirovecii* genomic diversity in colonized CF patients. In addition of pulmonary colonization, it appears that PCP can occur in CF patients specifically after lung transplantation, thus requiring preventive strategies. In other respects, *Pneumocystis* primary infection is a worldwide phenomenon occurring in non-immunosuppressed infants within their first months. The primary infection is mostly asymptomatic but it can also present as a benign self-limiting infection. It probably occurs in the same manner in CF infants. Nonetheless, two cases of severe *Pneumocystis* primary infection mimicking PCP in CF infants have been reported, the genetic disease appearing in these circumstances as a risk factor of PCP while the host-pathogen interaction in older children and adults with pulmonary colonization remains to be clarified.

Keywords: ***Pneumocystis jirovecii*, *Pneumocystis* pneumonia, cystic fibrosis, pulmonary colonization, genomic diversity, microbiota, *Pneumocystis* primary infection, lung transplantation**

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INTRODUCTION

Pneumocystis jirovecii is a transmissible fungus that causes severe pneumonia in immunocompromised patients (Walzer and Cushion, 2005). However, *Pneumocystis* infections cover a spectrum of presentations, in which PCP represents only a small proportion whereas most *Pneumocystis* infections correspond to mild diseases due to low levels of parasitism

(Morris and Norris, 2012). Indeed, polymerase chain reaction (PCR) assays have revealed that patients without PCP can be infected with a few organisms (Nevez et al., 1999). In these circumstances, the term "pulmonary colonization with *P. jirovecii*" is frequently used (Morris and Norris, 2012). Pulmonary colonization arises in immunocompromised patients as well as immunocompetent patients with underlying lung diseases. The potential role of the fungus as a morbidity cofactor has been suggested (Morris and Norris, 2012). Moreover, these colonized patients may act as infectious sources (Le Gal et al., 2012, 2015a). For these reasons, the characterization of colonized populations as well as that of *P. jirovecii* organisms in colonized populations are required. For instance, it has been established that patients with cystic fibrosis (CF) can be colonized by *P. jirovecii* (Calderón et al., 2010). CF is due to mutations of the cystic fibrosis transmembrane conductance regulator (CFTR) gene that encodes a chloride channel involved in electrolytic exchanges and is associated with impairment of the mucociliary clearance (Ferec and Cutting, 2012). The resulting thickening of mucus favors pulmonary colonization or infections due to bacteria and fungi (Lipuma, 2010). The first data on pulmonary colonization with *P. jirovecii* in CF patients were obtained in Munich, Germany (Sing et al., 2001). Further studies provided additional data in this field (Respaldiza et al., 2005; Le Gal et al., 2010; Hernandez-Hernandez et al., 2012; Pederiva et al., 2012; Green et al., 2016; Nevez et al., 2018). Moreover, PCP can also occur in CF patients (Royce and Blumberg, 2000; Quattrucci et al., 2005; Solé et al., 2006; Kaur et al., 2016; Wojarski et al., 2018). The objective of this review is to compile available information on *P. jirovecii* and CF worldwide.

PULMONARY COLONIZATION WITH *P. JIROVECII* IN CF PATIENTS

Differences of *P. jirovecii* Prevalence in European and Brazilian CF Patients

In Munich, Germany, sputum specimens from 95 patients were prospectively assayed using a nested-PCR amplifying the mitochondrial large subunit rRNA (mtLSUrRNA) gene (Sing et al., 2001). *P. jirovecii* was detected in seven patients (7.4%). Two patients who were submitted to recurrent sampling remained positive for *P. jirovecii* detection over a 4–6-week period.

In Seville, Spain, sputum or oropharyngeal wash specimens from 88 patients were assayed using a nested-PCR amplifying the mtLSUrRNA gene. *P. jirovecii* was detected in 19 patients (21.6%) (Respaldiza et al., 2005).

In Brest, Brittany, France, sputum specimens from 76 patients were retrospectively assayed using both qPCR and nested-PCR amplifying the mtLSUrRNA gene (Le Gal et al., 2010). *P. jirovecii* was detected in one patient (1.3%). A second study was performed in Rennes, the largest city in Brittany, in the course of which sputum specimens from 86 patients were retrospectively assayed using the same qPCR (Nevez et al., 2018). The fungus was detected in three patients (3.5%).

In a French multicenter study, sputum specimens from 104 patients who lived in the following four cities, Angers, Bordeaux, Dunkirk and Lille, were prospectively analyzed using a conventional PCR combined with a qPCR amplifying the mtLSUrRNA gene (Hernandez-Hernandez et al., 2012). The fungus was detected in 13 patients (12.5%).

In Manchester, United Kingdom, sputum specimens from 111 patients were prospectively assayed using a qPCR amplifying the mtLSUrRNA gene (Green et al., 2016). The fungus was detected in nine patients (8.1%) with a higher frequency in specimens from patients presenting with pulmonary exacerbation than in those from patients without exacerbation (9.2 vs. 2%, $p = 0.03$). One patient, submitted to recurrent sampling, remained positive for *P. jirovecii* detection over a 3-month period.

In Porto Alegre, Brazil, BAL specimens from 34 patients were retrospectively assayed using a nested-PCR amplifying the mtLSUrRNA gene. *P. jirovecii* was detected in 13 patients (38.2%) (Pederiva et al., 2012).

The frequencies of *P. jirovecii* colonization in patients enrolled in these seven studies differ significantly. Specifically, the frequency observed in Brittany, Western France, differed from that was observed in Seville, Porto Alegre, and other French regions, whereas it did not differ from that observed in Munich and in Manchester (Sing et al., 2001; Respaldiza et al., 2005; Le Gal et al., 2010; Hernandez-Hernandez et al., 2012; Pederiva et al., 2012; Green et al., 2016; Nevez et al., 2018) (**Table 1**).

Pulmonary Microbiota and *P. jirovecii* Colonization in CF Patients

Pulmonary microbiota characteristics in CF patients with or without *P. jirovecii* colonization were partially described in four studies. In the Brest study, 74 out of 76 patients were colonized by bacteria and/or fungi (Le Gal et al., 2010). The low incidence of *P. jirovecii* (1.3%) was not compatible with any comparison of microbiota in patients colonized by *P. jirovecii* or not. It is noteworthy, that the patient colonized by *P. jirovecii* was also colonized by *Pseudomonas aeruginosa*. In the Rennes study, 86 patients were colonized by bacteria and/or fungi (Nevez et al., 2018). Likewise, the low incidence of *P. jirovecii* (3.5%), rendered any comparison difficult. The three patients colonized by *P. jirovecii* were also colonized by *Staphyloccus aureus*, *Stenotrophomonas maltophilia, Candida glabrata* (first patient), by *Saccharomyces* sp. and *Haemophilus influenzae* (second patient), by *Aspergillus fumigatus, Candida albicans, P. aeruginosa* (third patient). In the French multicenter study (Hernandez-Hernandez et al., 2012), the presence of *P. aeruginosa* mucoid-strains showed a negative association with *P. jirovecii* presence. In the Manchester study, no difference of microbiota in CF patients with exacerbation and with or without *P. jirovecii* colonization was observed (Green et al., 2016). These studies were based on the results of microbial cultures combined with those of *P. jirovecii* DNA amplification since this fungus is uncultivable. Recently, next generation sequencing (NGS) studies showed that the pulmonary microbiota of CF patients was more complex than initially supposed, considering the results of microbial cultures (Caverly et al., 2019; Cuthbertson et al., 2020;

TABLE 1 | Comparison of characteristics of cystic fibrosis (CF) patients who underwent *Pneumocystis jirovecii* detection in pulmonary specimens [partly reproduced from Nevez et al. (2018)].

	Germany (Munich)	**Spain (Seville)**	**Brittany, France (Rennes and Brest taken together)**	**Other French regions and cities (Pays de la Loire, Angers; South West, Bordeaux; North, Dunkirk and Lille)**	**Brazil (Porto Alegre)**	**United Kingdom (Manchester)**
Number of patients	95	88	162	104	34	111
Median age, years [range]	23.2	15.8 [1–35]	19.0 [3 months-41]	24.0 [15–32]	11.0 [1–35]	31+/– 10 (mean; sd)
Sex ratio M/F	52/43	41/47	81/81	50/54	17/17	59/52
Inclusion period	NA	May 2001-July 2002	February 2005-August 2007	October 2006-March 2009	March 2006 – August 2009	October 2013 – May 2014
Number of specimens	137	88	324	146	NA	226
Pulmonary samples examined for *Pneumocystis jirovecii* detection	Sputa	Sputa (54) and oropharyngeal washes (34)	Sputa	Sputa	BAL	Sputa
Technique of *Pneumocystis jirovecii* detection in pulmonary specimens	Nested PCR targeting mtLSUrRNA gene	Nested PCR targeting mtLSUrRNA gene	Real-time PCR targeting mtLSUrRNA gene	Conventional PCR followed by a real-time PCR targeting mtLSUrRNA gene	Nested PCR targeting mtLSUrRNA gene	Real-time PCR targeting mtLSUrRNA gene
Number of patients with a prior treatment with cotrimoxazole	0	2	NA (Rennes) 30 out of 76 (Brest)	NA	5 (cotrimoxazole or azithromycin in the 6 months preceding sampling)	102
Number of patients colonized with *Pneumocystis jirovecii*	7 (7.4%; CI 95: 2.1–12.7%)	19 (21.6%; CI 95: 12.9–30.1%)	4 (2.5%; CI 95: 0.1–4.9%)	13 (12.5%; CI 95: 6.1–18.9%)	13 (38.2%; CI 95%:21.9–54.5%)	9 (8.1%; CI 95%: 3–13.2%)

BAL, bronchoalveolar lavage; CI, confidence interval; F, female; M, male; mtLSUrRNA, mitochondrial large subunit ribosomal RNA; NA, not available.

Enaud et al., 2020; Soret et al., 2020). Nonetheless, none of these recent studies has specifically focused on the relationship between microbiota and *P. jirovecii* colonization.

P. jirovecii Genomic Diversity in CF Patients

The pioneer study on *P. jirovecii* genotyping in Seville identified three different mtLSUrRNA genotypes in 11 patients colonized by the fungus (Respaldiza et al., 2005). Genotype 1 (85C/248C) was the most prevalent (5/11, 45.5%) followed by genotype 3 (85T/248C) (3/11, 27.3%) and genotype 2 (85A/248C) (2/11, 18.2%). The genotype distribution was similar to that previously described in a Spanish population presenting PCP and chronic pulmonary diseases, but excluding CF (Montes-Cano et al., 2004).

In the multicenter French study, genotype 1 predominated in the overall population (∼60%), consistent with the results of the Spanish study (Respaldiza et al., 2005; Hernandez-Hernandez et al., 2012). However, geographic variations emerged with higher prevalence of genotype 2 in Dunkirk in the absence of genotype 3, whereas genotype 1 predominated in Angers in the absence of genotype 2 (Hernandez-Hernandez et al., 2012). In the other French study performed in Brittany, genotyping was successful in two out of the four patients colonized by *P. jirovecii* (Nevez et al., 2018). A 13-week old infant had a mixed infection with the combination of genotype 2 and genotype 3, associated with internal transcribed spacer (ITS) haplotype Jf. The second patient harbored genotype 2 in the absence of ITS haplotype identification. Both patients harbored a dihydropteroate synthase (DHPS) wild type (threonine55/ proline57). In the Brazilian study, mtLSUrRNA genotyping was successful in 12 patients and also showed a predominance of genotype 1 (5/12, 41.6%) and lower frequencies of genotype 3 (3/12, 25%) and genotype 2 (2/12, 16.6%). In addition, all patients harbored a DHPS wild type. A Spanish study has analyzed the longitudinal distribution of mtLSUrRNA genotypes in CF patients. During a 1-year follow-up, a continuous infection-and-clearance cycle of genotypes was observed with a switch from genotype 1 to genotype 3 (Montes-Cano et al., 2007). The results of another Spanish study were consistent, since a predominance of genotype 3 in CF adult patients was observed (Montes-Cano et al., 2006).

Pneumocystis Primary Infection in CF Infants

Pneumocystis primary infection is a common phenomenon in non-immunosuppressed infants without underlying diseases, with a peak of occurrence between the third and fifth months (Vargas et al., 2001; Larsen et al., 2007; Nevez et al., 2020). It occurs contemporaneously to a self-limiting infection in the course of which the pulmonary tract is colonized by the fungus (Vargas et al., 2001; Larsen et al., 2007; Nevez et al., 2020). Primary infection occurs probably in the same manner in CF infants. Consistently, in the course of the study performed in Rennes, *P. jirovecii* was detected in a 13-week old infant who recovered without specific treatment (Nevez et al., 2018). Nonetheless, two PCP cases in CF infants have been recorded in the literature. The first one concerned a 15-week old HIV-seronegative infant newly diagnosed with CF who underwent a BAL to determine pneumonia etiology. BAL examination revealed *P. jirovecii* asci and pathogens usually associated with CF, such as *S. aureus* and *P. aeruginosa*. The patient recovered after cotrimoxazole treatment (Royce and Blumberg, 2000). The second one concerned a 16-week old HIV-seronegative infant newly diagnosed with CF who underwent a BAL to investigate chronic cough etiology. BAL examination revealed *P. jirovecii* asci and *P. aeruginosa*. The patient recovered after cotrimoxazole treatment (Kaur et al., 2016). The young age of the two infants strongly suggested that they effectively developed *Pneumocystis* primary infection. These two case-reports showed that PCP contemporaneous to severe primary infection occurs in CF infants in the apparent absence of risk factors other than the genetic disease itself.

PNEUMOCYSTIS PNEUMONIA IN CF PATIENTS AFTER LUNG TRANSPLANTATION

Beyond pulmonary colonization, overt PCP occurs in CF patients after lung transplantation due to immunosuppressive therapy. In Spain, in the course of a 14-year follow-up, 57 CF patients underwent lung transplantation. Surprisingly none developed PCP (Solé et al., 2006). In Italy, in the course of a 6-year follow-up, 55 CF patients underwent lung transplantation. Three patients developed PCP (5.4%), two of whom died (Quattrucci et al., 2005). In Poland, in the course of a 7-year follow-up, 21 CF patients underwent lung transplantation. Eight patients (38%) developed PCP (Wojarski et al., 2018). Thus, the risk of PCP in CF patients after lung transplantation appears highly variable.

GENERAL DISCUSSION

There are several hypotheses to explain differences in prevalence of pulmonary colonization with *P. jirovecii* in European and Brazilian CF patients.

Differences may result from technical issues. In the European studies, available specimens were sputa whereas in the Brazilian study it was BALs which provided high-quality cells from the alveolus and consequently a higher sensitivity to *P. jirovecii* detection (Pederiva et al., 2012). A qPCR assay was used in the Brittany study (Nevez et al., 2018) instead of two-step PCR assays which were performed in the other studies (Sing et al., 2001; Respaldiza et al., 2005; Hernandez-Hernandez et al., 2012; Pederiva et al., 2012; Green et al., 2016). Sputum examination or the qPCR assay might have been less sensitive than BAL examination and the two-step-PCR assays.

Climatic parameters may affect *P. jirovecii* presence in CF patient populations and may explain geographical variations of prevalence. The factor of a Mediterranean climate in Seville and a temperate, maritime climate in Brittany has already been discussed (Le Gal et al., 2010; Nevez et al., 2018). Unfortunately, information was mainly obtained from Europe and Brazil, which limited the discussion on putative relationship between geographical location and *P. jirovecii* incidence in CF patients. Nonetheless, geographical variations could be non-independent

factors (Miller et al., 2007, 2018). Indeed, population densities in European regions that differ from each other may affect inter-individual encounters and consequently *P. jirovecii* transmission and acquisition.

A history of cotrimoxazole treatment for bacterial infections may play a role in *P. jirovecii* clearance from the lungs, thus influencing the results of *P. jirovecii* detection. For instance, two out of 88 patients in Seville compared to 30 out of 76 patients in Brest had a history of cotrimoxazole treatment (2.2 vs. 39.4% $p < 0.001$) (Respaldiza et al., 2005; Le Gal et al., 2010). Furthermore, in Manchester, *P. jirovecii* was more frequent in patients not treated with cotrimoxazole (29.7 vs. 0%, $p = 0.03$) (Green, 2015).

Attention should be paid to other patients' characteristics, such as CFTR gene mutations. Two of the three patients in the Rennes study who tested positive for *P. jirovecii* were heterozygous for the F508Del mutation (Nevez et al., 2018). Nonetheless, considering the lack of information in the other studies a putative relationship between the presence of *P. jirovecii* and CFTR mutations remains speculative, requiring further evaluation.

The presence of *P. jirovecii,* its relationship with patients' age, pulmonary microbiota and the severity of CF must be debated. Bacterial infections arise earlier than fungal infections during the disease's progression, both acting as aggravating factors in respiratory dysfunction (Caverly et al., 2019; Delhaes et al., 2019; Cuthbertson et al., 2020). CF patients are initially infected with *H. influenzae* and *S. aureus*, and further develop infections with *P. aeruginosa* or *Burkholderia cepacia* complex. The use of antibiotics to prevent or treat infections by these bacteria, has resulted in improving the clinical outcome and increasing the life expectancy of CF patients (Castellani et al., 2018). Consequently, they are at risk for mycoses (Schwarz et al., 2018) but not for *P. jirovecii* infections, according to our analysis. The median ages of the patients enrolled in the Spanish (15.8 years) and Brazilian (11 years) studies (Respaldiza et al., 2005; Pederiva et al., 2012) were lower than those of the patients enrolled in the French multicenter study (23.5 years) (Hernandez-Hernandez et al., 2012), the Brittany study (19 years) (Nevez et al., 2018), and the British study (mean age, 31 years) (Green et al., 2016). The colonization with *P. jirovecii* may be transient and may occur earlier during CF progression, which is consistent with the presence of *P. jirovecii* in younger Spanish and Brazilian patients and the scarcity of *P. jirovecii* detection in older Breton and British patients.

In vivo competitive inhibition between species and networks between fungal and bacterial kingdoms in CF may exist (Soret et al., 2020). For instance, *P. aeruginosa* may limit the spread of *P. jirovecii* within the lungs. Indeed, in the French multicenter study, the patients colonized by *P. aeruginosa* mucoid strains were less likely to be colonized by *P. jirovecii* (Hernandez-Hernandez et al., 2012). Moreover, *P. jirovecii* colonization was associated with less severe lung disease according to FEV1 values, which appeared consistent with the negative correlation between *P. jirovecii* and *P. aeruginosa* mucoid strains. On the other hand, *P. jirovecii* colonization was associated with *P. aeruginosa* colonization in Brazilian patients (Pederiva et al., 2012). However, it remains unknown whether these patients were specifically infected with mucoid strains. and the relationship between *P. jirovecii* and mucoid strains is only one of the possible hypotheses.

P. jirovecii's role as an aggravating factor on its own is unclear in CF patients, while it has been previously suggested in patients with COPD (Morris and Norris, 2012). The Manchester study may be consistent with this role in CF patients since the presence of *P. jirovecii* was significantly associated with exacerbation (Green et al., 2016), in contrast to the French multicenter study (Hernandez-Hernandez et al., 2012), in which the presence of *P. jirovecii* was associated with less severe lung diseases. We can hypothesize that *P. jirovecii* provokes a local inflammation facilitating subsequent infections such as by *P. aeruginosa*, in the course of which the fungus could be detectable or not (Ulrich et al., 2010; Döring et al., 2011; Hernandez-Hernandez et al., 2012; Pederiva et al., 2012). Finally, whether the fungus's presence is the cause or the result of exacerbation remains an open question.

Most data on microbiota resulted from routine culture and identification. Today, NGS methods allow identifying a higher number of microbial species, and thus to analyze cooperative, competitive and/or adaptive interactions between microorganisms that play a role in CF disease progression (Quinn et al., 2014; Layeghifard et al., 2019; Soret et al., 2020). However, these methods are mainly based on amplification of ITS sequences to analyze mycobiota, *i.e.,* fungal microorganisms. These sequences are present in multicopies in most fungal genomes but are unfortunately present in a single copy in the *P. jirovecii* genome (Nahimana et al., 2000). Thus, despite their potential efficiency, NGS methods may not be sensitive enough to capture *P. jirovecii* sequences present in relative average/low abundance within the mycobiota profiles (Delhaes et al., 2012).

Analyses of the results of *P. jirovecii* genotyping from cross-sectional studies must be made cautiously. Indeed, a continuous colonization/clearance cycle with changes of mtLSUrRNA genotype prevalence, e.g., with a switch of genotype 1 to genotype 3 has been identified (Montes-Cano et al., 2007). Other studies not focusing on CF patients suggest that single-nucleotide polymorphism (SNP) at position 85 is somehow correlated to fungal burden levels and virulence. A putative link between SNP 85T or 85A, high fungal burdens and infection severity has been suggested whereas SNP 85C may be related to low/average burdens and less severe infections (Esteves et al., 2010a,b, 2016). The switch from genotype 1 (85C/248C) to genotype 3 (85T/248C) in CF patients who, nevertheless, do not develop severe infections remains unclear. Genotype 3 may be the most adapted in the course of CF disease progression. It is unfortunate that, in the Manchester study which highlighted significant association of *P. jirovecii* and exacerbation, genotyping was not performed. No DHPS mutant-types were identified in patients from Brittany or Brazil, consistent with the general low prevalence of mutant types previously reported in these two locations (Wissmann et al., 2006; Le Gal et al., 2013a). In the Brittany study, a 13-week old infant at risk for *Pneumocystis* primary infection harbored a combination of mtLSUrRNA genotype 2 and genotype 3, associated with ITS

haplotype Jf and a DHPS wild type (Nevez et al., 2018). No additional data on genotypes in a context of primary infection in CF infants are available. Indeed, the two case reports on severe primary infection that we mentioned above did not provide any information about genotypes (Royce and Blumberg, 2000; Kaur et al., 2016). Conversely, data exist on genotypes in non-CF infants developing primary infection (Totet et al., 2003a,b; Nevez et al., 2020). In Brittany, *P. jirovecii* genotyping was performed in non-immunosuppressed infants developing symptomatic primary infection. MtLSUrRNA genotype 2 was the most frequent (13/21, 61, 9%) whereas, none harbored genotype 1. Thus, the presence of genotype 2 in the aforementioned CF infant may reflect particular geographical characteristics of the fungus in infants living in this region. ITS haplotype Jf has rarely been described in France, whatever the patient population (range, 0–7.5%), whereas Eg is the most frequent haplotype (range, 42.8–64.5%) (Nevez, 2003; Le Gal et al., 2013b, 2015b). The presence of type Jf in this infant could simply be a random event. Finally, all these genotypic characteristics were obtained using a unilocus approach and Sanger's method of sequencing, while more informative assays such as multilocus sequence typing (MLST) combined with NGS are available today (Alanio et al., 2016; Charpentier et al., 2017). Moreover, shotgun metagenomics may represent an alternative method to identify pulmonary mycobiota including *P. jirovecii* organisms (Irinyi et al., 2020).

The detection of *P. jirovecii* does not seem to be predictive of subsequent PCP occurrence since no colonized patients enrolled in the above-mentioned studies developed PCP. In the absence of profound immunodeficiency, the shift from colonization to PCP did not occur. In lung transplant recipients with iatrogenic immunodeficiency, attack rates of PCP have been assessed at 5–5.8% or 6.5–43% in patients with or without chemoprophylaxis, respectively (review in reference Iriart et al., 2015). However, precise data on attack rates of PCP among transplanted CF patients were not provided in this review. The differences in PCP occurrence in CF patients from Spain, Italy, and Poland (Quattrucci et al., 2005; Solé et al., 2006; Wojarski et al., 2018) may be explained by different uses of PCP chemoprophylaxis.

Although rare, PCP can also occur contemporaneously to *Pneumocystis* primary infection in CF infants (Royce and Blumberg, 2000; Kaur et al., 2016) and the medical community should be aware of this. Early impairment of pulmonary function due to the genetic disease, in *P. jirovecii* immune-naïve infants, in the apparent absence of immunosuppression, could be the cause of PCP occurrence. Indeed, this early impairment is objectivized by the presence of *S. aureus* and *P. aeruginosa* in infants a few weeks old, while these bacteria are usually detected in older patients with pulmonary dysfunction and more advanced disease. Conversely, this hypothesis is not consistent with the negative association between mucoid *P. aeruginosa* and *P. jirovecii* suggested in the French multicenter study (Hernandez-Hernandez et al., 2012). Nevertheless, microorganism interactions in CF patients including infants are certainly complex. Moreover, whether PCP prophylaxis in CF infants is required remains an open question.

Finally, data on *P. jirovecii* and CF are fragmented. This topic remains an open field of investigation. A multicenter prospective study comprising identical methods of *P. jirovecii* screening in CF patients including those who have undergone lung-transplantation, with patient-age stratification, CFTR mutation analysis, and MLST/NGS analysis of the *P. jirovecii* genome combined with microbiome/mycobiome examination is warranted.

AUTHOR CONTRIBUTIONS

PB and GN: wrote, corrected, and submitted the manuscript. SL: wrote, corrected, and provided biological data from CF patients in Brest. EC: wrote the paragraphs on genomic diversity. LD: wrote paragraphs on microbiota. DQ: performed biological diagnoses and provided biological data from CF patients in Brest. FR-G: read and corrected the manuscript, wrote the reference section, and provided biological data from Rennes. SR: provided clinical data from CF patients and represents the Réseau Muco-Ouest. All authors contributed to the article and approved the submitted version.

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Brief Report

Brief Report

The shift from pulmonary colonization to *Pneumocystis* pneumonia

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Summary

Pulmonary specimen pairs from five patients who presented with pulmonary colonization and later developed *Pneumocystis* Pneumonia (PcP) were retrospectively examined for *P. jirovecii* genotyping. A match of genotypes in pulmonary specimen pairs of three patients was observed, whereas a partial match and a mismatch were observed in the fourth and fifth patients, respectively. The genotyping results suggest that the colonization state can differ from PcP but can also represent the incubation period of PcP. Clinicians should not systematically rule out the treatment of putative colonized patients and should at least discuss the initiation of prophylaxis on a case-by-case basis.

Lay Summary

The results suggest that clinicians should not systematically rule out the treatment of putative patients colonized by Pneumocystis jirovecii and should at least discuss prophylaxis initiation on a case-by-case basis.

Key words: *Pneumocystis jirovecii*, PcP, pulmonary colonization, incubation, genotypes.

Over the past years, the use of PCR assays for the diagnosis of *Pneumocystis* infections has led to the detection of low burdens of *P. jirovecii* organisms in pulmonary samples from patients, who can present with an alternative diagnosis of *Pneumocystis* pneumonia (PcP).[1] These low levels of parasitism have frequently been deemed to reflect torpid infection related to pulmonary colonization by *P. jirovecii*.[1] In a previous study, we followed-up 14 patients for a mean period of 8.6 months (range, 0.2-16 months) after the dates of pulmonary colonization diagnoses.[2] The patients did not develop PcP over this period, despite the absence of specific treatment or prophylaxis.[2] This observation showed that colonization and PcP may be two different clinical entities. Nevertheless, colonization may also evolve to acute pneumonia, the colonization in this case being the PcP incubation period with a putative time continuum between the two clinical presentations. The objective of this study was to test this hypothesis using a longitudinal follow-up of *P. jirovecii* genotypes identified in patients initially diagnosed with pulmonary colonization who later developed PcP. Identifying identical genotypes involved in these two successive steps of infection would be compatible with this hypothesis.

Five patients were retrospectively enrolled. They were initially diagnosed with pulmonary colonization by *P. jirovecii* and later developed PcP over a median period of 41 days (range,

Table 1. Characteristics of patients colonized by *P. jirovecii* who later developed *Pneumocystis* pneumonia and results of *P. jirovecii* genotyping in pulmonary specimen pairs from these patients

				Pulmonary colonization with *Pneumocystis jirovecii*			*Pneumocystis* pneumonia (PcP)			
Patient n°	Sex	Age	Underlying diseases	Date of colonization diagnosis d/m/y	Alternative diagnosis	Results of *P. jirovecii* genotyping	Date of PcP Diagnosis d/m/y	Results of *P. jirovecii* genotyping	Treatment	Outcome
1	F	72	Wegener disease	30/12/y	Bacterial pneumonia	mtLSU3-CYB1-SOD2	06/03/y + 1*[1]	mtLSU3-CYB1-SOD2	Atovaquone	Recovery
2	M	77	RTR	11/03/y	Drug induced pneumonia	mtLSU4-CYB2	21/04/y*[2]	mtLSU4-CYB2-SOD1	Cotrimoxazole	Death[b]
3	M	71	Pulmonary fibrosis Corticosteroids	15/06/y	Hypersensitivity pneumonitis	mtLSU2-CYB1-SOD2	22/06/y*[2]	mtLSU2-CYB1-SOD2	Cotrimoxazole	Recovery
4	F	70	Idiopathic interstitial pneumonia treated with azathioprine	19/04/y	NSIP	mtLSU3-CYB2-mix of SOD allele[a]	15/05/y*[2]	mtLSU3-CYB2&4-mix SOD allele[a]	Cotrimoxazole	Recovery
5	M	57	T lymphoma	04/02/y	Bacterial pneumonia	mtLSU2-CYB1-SOD2	12/09/y + 1*[1]	mtLSU2-CYB2-mix of SOD allele[a]	Cotrimoxazole	Death[c]

Foot notes. *[1] y + 1, next year, *[2] y, same year, years were not indicated since it could be identifiable patient information; RTR, renal transplant recipient; NSIP, nonspecific interstitial pneumonia; mtLSU, mitochondrial large subunit ribosomal RNA gene; CYB, cytochrome b gene; SOD, superoxide dismutase gene. The alleles were identified considering changes at nucleotide positions described elsewhere by Vindrios and colleagues[5] and Maitte and colleagues.[9] A multilocus genotype consisted of the combination of mtLSU, CYB and SOD alleles. [a] Allele mix, presence of several different alleles that were not identified; [b] PCP contributed to death; [c] PCP did not directly contribute to death, patient death was due to cerebral location of lymphoma.

NB. Bronchoalveolar lavage specimens were retrieved in all patients except in patient P1 in whom a bronchial aspirate was sampled at the date of colonization diagnosis.

7–586). The criteria for colonization diagnosis were based on negative results of *P. jirovecii* detection in pulmonary specimens using microscopic examination [Wright-Giemsa, immunofluorescence assay (MONOFLUO kit *P. jirovecii* Bio-Rad) and/or toluidine blue stain], positive results of *P. jirovecii* DNA detection using a PCR assay amplifying the mtLSUrRNA gene as described elsewhere,[3,4] and alternative diagnoses enabling us to rule out PcP diagnosis. The criteria for PcP diagnosis were based on positive results of *P. jirovecii* detection in pulmonary specimens using both microscopic examination and PCR combined with compatible clinical symptoms such as dyspnea, cough, and fever, and radiological signs such as round glass opacities or overt interstitial pneumonia. Biological, sociodemographic, and clinical data were collected using both hospital information online software and general laboratory systems. The patients' characteristics are described in Table 1.

Extracted DNA samples from pulmonary specimens were initially stored at −80°C for further investigation. Two pulmonary specimens per patient were examined, the first one at the time of colonization diagnosis and the second one at the time of PcP diagnosis. Then, they were examined at three loci, mtLSUrRNA, cytochrome b (CYB), and superoxide dismutase (SOD) genes, using direct sequencing of the two strands, as described elsewhere.[5] Consensus sequences were aligned with reference sequences [GenBank accession numbers M58605 (mtLSUrRNA), AF074871 (CYB), and KT592355 (SOD)][6–8] using the BioEdit software with the Clustal® W program. A multilocus genotype (MLG) consisted of the combination of mtLSU, CYB, and SOD alleles. *P. jirovecii* alleles and MLG in specimen pairs that consisted of the specimen at the time of colonization diagnosis and the other one at the time of PcP diagnosis were compared. To avoid contamination, each step of the PCR assays was performed in different areas of the laboratory with different sets of micropipettes. Mix reagents were prepared in a laminar flow cabinet. To monitor for possible contamination, negative controls were included in each experiment and PCR round.

The study was noninterventional, which did not require ethical approval according to French laws and regulations (CSP Art L1121e1.1).

The results of genotyping are described in Table 1. CYB and mtLSU loci were successfully amplified and sequenced from the five pairs of pulmonary specimens. The SOD locus was successfully amplified and sequenced in the four pairs from patients P1, P3, P4, P5, and only from the second specimen from patient P2. Indeed, the SOD locus was not amplified in the first specimen from patient P2. This observation could be due to the low fungal burden and the fact that this gene is present in a single copy in the nuclear genome.

A full match of MLGs, that is, with an identical combination of alleles mtLSU, CYB, and SOD, was observed in the pairs from patients P1 and P3. MLG analysis could not be performed for patient P2 since the SOD locus was not amplified in his first specimen. Nonetheless, a full match of mtLSU and CYB alleles was observed, since alleles mtLSU4 and CYB2 were identified in the first and second specimens, respectively.

A partial match of MLG was observed in the pair from patient P4. Both alleles mtLSU3 and CYB2 were identified in the first and second specimens. Allele CYB4 was associated with allele CYB2 in the second specimen, suggesting a mixed infection. In contrast, the presence of a mix of SOD alleles in the two specimens did not permit us to precisely identify MLGs. MLGs did not match in the pair from patient P5 as alleles mtLSU2, CYB1, and SOD2 were identified in the first specimen and alleles mtLSU2, CYB2, and a mix of SOD alleles were identified in the second specimen.

We obtained the first data on longitudinal follow up of *P. jirovecii* genotypes from patients who were initially deemed to be colonized by the fungus and who later developed PcP. We chose a highly discriminant typing scheme (Hunter index > 0.95).[9–11] The sequencing was performed using the Sanger method, which is easy to perform. Mixed alleles were identified in two of five patients (40%). This rate is lower than 85–92%, the rates reported in studies using molecular high-throughput methods.[12,13] Thus, minor genotypes may have been missed. This may be a potential weakness of our method. Nevertheless, our method is suitable for examining specimens with low fungal burdens,[5,11] a specific characteristic of colonized patients, the topic of this study.

Different MLGs were identified in the two pulmonary specimens from patient P5. It is noteworthy that the two pulmonary specimens were obtained 586 days apart; no intermediate specimens were retrieved. The patient may have been colonized by a strain of *P. jirovecii* that was later cleared from the lungs, and have further acquired another strain which was involved in PcP development. In this case, the colonization appears different from PcP.

Two different hypotheses may explain the results of genotyping in patient P4. CYB4, a putative minor allele, was effectively present in the first specimen but was not detected or the patient may have secondarily acquired this allele, which was detected in the second specimen when PcP was diagnosed. This remains an open question.

Identical MLGs or alleles at mtLSUrRNA and CYB genes were identified in the pairs from patients P1, P2 and P3. This observation suggests that at the time of PcP development the patients were not infected secondarily with another *P. jirovecii* strain. The same *P. jirovecii* strains were present during the infection process, the colonization being considered as the incubation period of the forthcoming PcP. These misdiagnoses may have led to poor prognosis, specifically for patient P2 who could have been treated earlier and unfortunately died secondarily. In other respect, in these three cases, the second pulmonary specimen was obtained 63, 41, and 7 days after the first one. These intervals are consistent with PcP incubation duration, which varies greatly.[14–16]

To sum up, despite the small number of patients retrospectively enrolled in the study, the results of *P. jirovecii* genotyping suggest that the colonization state can differ from PcP (eg, patient P5) but can also represent the incubation period of PcP. Consequently, clinicians should not systematically rule out the treatment of putative colonized patients and should at least discuss initiation of prophylaxis on a case-by-case basis.

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Conflicts of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and the writing of the paper.

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Letter to the Editor

A proposal for pragmatic investigation of possible clonal clusters of pneumocystis pneumonia cases

Sir,

Pneumocystis jirovecii is an uncultivable and human-specific ascomycete that causes severe pneumonia in immunocompromised patients [1]. Interindividual transmission of *P. jirovecii* via the airborne route has been highly suspected in humans considering the occurrence of clusters of pneumocystis pneumonia (PCP) cases in hospitals and the exhalation of *P. jirovecii* by infected patients in their surrounding aerial environment [2–5]. PCP outbreak incidence appears to have increased in the last 20 years [2–4].

Recently, the burden of pneumocystis infection outbreaks in France has been evaluated through a retrospective nationwide survey [6]. Over 12 years, 16 outbreaks occurred in solid-organ transplantation units [6]. Usually, the investigation of PCP clusters involves drawing up a transmission map to identify patient encounters, combined with genotyping of *P. jirovecii* isolates from patients involved in this cluster. Identification of identical genotypes shared by these patients supports the occurrence of a nosocomial transmission of *P. jirovecii*. However, this investigative approach is fastidious and time-consuming. We propose herein a more pragmatic approach consisting first in *P. jirovecii* genotyping, and second, depending on the results of genotyping, in analysing possible patient encounters through a transmission map. Our proposal relies on our own experience of *P. jirovecii* genotyping in putative clonal outbreaks in medical wards and illustrated here in light of the investigation of a cluster of four PCP cases that recently occurred within the Department of Respiratory Medicine of Nantes University Hospital, Nantes, France.

The four PCP cases occurred from May 20th, 2020 to June 2nd, 2020 in a conventional pneumology unit transiently dedicated to suspected COVID-19 patients, whereas only two cases of PCP occurred in patients monitored in this Department from November 2019 to May 2020. In these circumstances a nosocomial outbreak of PCP was suspected. The patients' characteristics are detailed in Table I.

Biological diagnoses of PCP were based on positive results of *P. jirovecii* detection in bronchoalveolar lavage (BAL) specimens using microscopic examination (modified Gomori–Grocott methenamine silver staining), and a real-time polymerase chain reaction assay amplifying the mtLSUrRNA gene [7]. DNA samples from BAL specimens were stored at −80°C for further *P. jirovecii* genotyping. Genotyping was performed by multi-locus sequence typing (MLST) at three distinct loci, namely the mtLSUrRNA gene, the cytochrome *b* (CYB) gene, and superoxide dismutase (SOD) gene, as previously described [7,8]. The results are presented in Table I. No patients shared an identical mtLSUrRNA allele with any other patient. Allele CYB1 was shared by patients 1 and 2, and allele CYB2 was shared by patients 1 and 4, whereas allele CYB8 was identified only in patient 3. Allele SOD2 was shared by patients 2 and 3, allele SOD1 was identified in patient 4, and a mix of SOD alleles was observed in patient 1. No multi-locus genotype (MLG) identified using MLST was shared by the four patients, ruling out a possible clonal cluster.

This MLST scheme was chosen in light of its high discriminative power with Hunter index (H) values >0.95; specifically, H values of 0.987 and 0.99 as we reported elsewhere [7,8]. Since the mtLSUrRNA genotypes as well as the MLGs in the four patients were different, a common source of *P. jirovecii* and interindividual transmission of the fungus were excluded. These results support our strategy for exploring a PCP cluster. Without genotype similarity, it is unnecessary to go ahead, which saves time and effort by avoiding a superfluous transmission map.

Contrary to what is widely believed, the tracing of patient encounters is the most time-consuming step in a cluster investigation, whereas genotyping can be performed rapidly by a team with specific genotyping skills.

Consequently, if MLGs are identical or if a major MLG is identified in the patients involved in a cluster, analysis of patient encounters should be performed. In addition, *P. jirovecii* genotyping of a control group which consists of unlinked PCP patients, e.g. patients from the same hospital but monitored in different units, is also warranted. In cases of clonal clusters, it is expected that this control patient group may harbour different genotypes or a lower proportion of the major genotype involved in the nosocomial cluster [6]. Hence, as illustrated here, in the course of a putative cluster

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Table I
Characteristics of the four patients involved in a pneumocystis pneumonia (PCP) cluster occurring in the Respiratory Medicine Department in Nantes University Hospital, Nantes, France

Patient no.	Sex	Age (years)	Underlying diseases	Hospital admission	Date of PCP diagnosis	*P. jirovecii* genotyping	Treatment	Outcome, date of discharge
1	M	62	Heart transplantation	May 15th, 2020	May 22nd, 2020	mtLSU1, mtLSU2; CYB1, CYB 2; mix of SOD alleles	TMP/SMX	Recovery Jun 3rd, 2020
2	M	84	Rheumatoid arthritis	May 19th, 2020	May 20th, 2020	mtLSU3; CYB1; SOD2	Atovaquone	Recovery May 28th, 2020
3	M	66	Bronchopulmonary cancer	May 20th, 2020	May 25th, 2020	mtLSU5; CYB8; SOD2	TMP/SMX	Recovery Jun 17th, 2020
4	M	69	Liver transplantation	May 28th, 2020	Jun 2nd, 2020	mtLSU4; CYB2; SOD1	TMP/SMX	Death Jun 10th, 2020

MtLSU, mitochondrial large subunit rRNA; CYB, cytochrome; SOD, superoxide dismutase; TMP/SMX, trimethoprim/sulfamethoxazole.
[a]Mix of SOD alleles, presence of several different alleles that were not identified.
The alleles were identified with reference to changes at nucleotide positions described elsewhere by Maitte *et al.* [7] and Nevez *et al.* [8]. A multi-locus genotype consisted of the combination of mtLSU, CYB, and SOD alleles.

investigation, promoting rapid genotyping has the potential to save time and effort.

Conflict of interest statement
None declared.

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Titre : Diversité des gènes de *Pneumocystis jirovecii* codant pour les cibles de molécules ayant une activité anti-*Pneumocystis* potentielle ou démontrée

Mots clés : *P. jirovecii*, atovaquone, méthotrexate, échinocandines, diversité génétique

Résumé : *Pneumocystis jirovecii* est un champignon transmissible responsable de pneumonies sévères (pneumonies à *Pneumocystis* [PPC]) chez les patients immunodéprimés. Les échinocandines et l'atovaquone ciblant respectivement la 1,3-bêta-D-glucane synthase et le cytochrome b sont utilisées comme thérapies de deuxième intention de la PPC en France. Le méthotrexate (MTX) est utilisé pour traiter les maladies auto-immunes ou les cancers. Cependant, il possède également une activité anti-*Pneumocystis* potentielle puisqu'il pourrait cibler la DHFR de *Pneumocystis*. L'objectif de cette thèse était d'évaluer la pression de sélection potentielle exercée par ces trois médicaments sur *P. jirovecii*. Le gène *gsc1* codant pour la 1,3-bêta-D-glucane synthase, le gène *dhfr* et le gène *CYB* chez *P. jirovecii* provenant de patients infectés ont été amplifiés et séquencés. Les souches issues de patients avec ou sans exposition à l'un des trois médicaments ont été comparées. La diversité génétique des gènes *gsc1* et *dhfr* était similaire chez les patients avec ou sans exposition aux échinocandines et au MTX, respectivement. La diversité du gène *CYB* différait entre les patients avec ou sans exposition préalable à l'atovaquone. Plus précisément, les deux mutations faux-sens C431T (Ala144Val) et C823T (Leu275Phe) situées sur le site actif du cytochrome bc1, codé par *CYB*, étaient associées de manière significative à une exposition à l'atovaquone ($P < 0,001$). Nos résultats suggèrent que la pression de sélection globale exercée par les échinocandines, le MTX et l'atovaquone sur *P. jirovecii* en France reste faible. A l'inverse, la pression de sélection exercée par l'atovaquone apparaît élevée chez les patients directement exposés.

Title : Diversity of *Pneumocystis jirovecii* genes encoding the targets of molecules with putative or known antifungal activity

Keywords : *P. jirovecii*, atovaquone, methotrexate, echinocandins, genetic diversity

Abstract : *Pneumocystis jirovecii* is a transmissible fungus responsible for severe pneumonia (*Pneumocystis* pneumonia [PCP]) in immunocompromised patients. The echinocandins and atovaquone targeting 1,3-beta-D-glucan synthase and cytochrome b respectively are used as second line therapies of PCP in France. Methotrexate (MTX) is used to treat autoimmune diseases or cancers. However, it also has a putative anti-*Pneumocystis* activity since it may target the DHFR of *Pneumocystis*. The aim of this PhD thesis was to evaluate the putative selective pressure exerted by these three drugs on *P. jirovecii* organisms. The gene *gsc1* encoding the 1,3-beta-D-glucan synthase, the gene *dhfr* and the gene *CYB* of *P. jirovecii* isolates from infected patients were amplified and sequenced. In chapter 1, 2, and 3, patients with or without exposure to one of each drug were compared. The genetic diversity of the gene *gsc1* was similar in patients with or without prior exposure to echinocandins. The diversity of the *dhfr* gene was similar between patients with or without prior exposure to MTX. The diversity of the *CYB* gene was different between patients with or without prior exposure to atovaquone. Specifically, both missense mutations C431T (Ala144Val) and C823T (Leu275Phe) located at the active site of the enzyme cytochrome bc1, encoded by *CYB*, were significantly associated with prior atovaquone exposure (Fisher's exact test, $P < 0.001$). Our results suggest that the overall selective pressure exerted by echinocandins, MTX, and atovaquone on *P. jirovecii* organisms in France remains low. Conversely, selective pressure exerted by atovaquone appears high in patients directly exposed to this drug.