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OBM Genetics

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Review

Genotyping *Pneumocystis jirovecii*: Impacting Our Understanding of Interhuman Transmission

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Academic Editors: Andrés Moya, Enrique J. Calderón and Luis Delaye

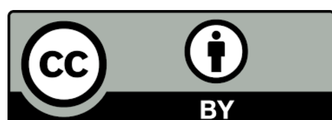
Special Issue: *Pneumocystis*: A Model of Adaptive Coevolution

OBM Genetics
2019, volume 3, issue 1
doi:10.21926/obm.genet.1901060

Received: December 04, 2018
Accepted: January 08, 2019
Published: January 18, 2019

Abstract

Pneumocystis jirovecii is an atypical fungus transmitted via the airborne route between humans. This fungus is exclusively associated with humans and almost each individual has encountered it at least once before reaching the age of two. *P. jirovecii* can be cleared and spontaneously resolutive in immunocompetent, whereas it can be responsible for severe *Pneumocystis* pneumonia (PCP) in immunocompromised patients. In the next decades, the putative increase of the population of immunocompromised patients is likely to lead to the rise of PCP infections. The detection of low fungal loads has been improved with the advances of molecular technologies and polymerase chain reaction (PCR) assays. This improvement in detection raised several questions. Is PCP consecutive of a persistent colonizing strain multiplication or acquisition of a new one? What is the variety of strains in PCP and non-PCP patients? Are outbreaks due to a specific strain? Genotyping approaches could bring some answers. In this review, we reviewed the main typing methods developed



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for *P. jirovecii* characterization, with an emphasis on the typing of low fungal loads, a subject rarely underlined. Secondarily, we present the main results obtained and the importance of including patients with low fungal load in the analyses because of their possible role as reservoirs and their impact on *P. jirovecii* transmission. Finally, we consider systematic treatment of the patients with low fungal load not only to prevent full-blown PCP, but also to block the transmission chain.

Keywords
Pneumocystis jirovecii; genotyping; methods; review

1. Introduction

Pneumocystis jirovecii is considered a commensal fungus of the pulmonary alveoli and is peculiar in the fungal world because of the direct interhuman transmission in its life cycle with no known environmental reservoir [1, 2]. This atypical fungus is exclusively associated with humans and almost each individual has encountered it at least once before reaching the age of two [3-6]. *Pneumocystis jirovecii* can exhibit spontaneously resolutive symptoms in infants and is also responsible for severe pneumocystis pneumonia (PCP) in immunocompromised patients, such as HIV-positive patients, solid organ transplant patients, patients with hematological malignancies, or patients given high dose steroids or anti-TNF drugs [7-10]. The increase of the population of immunocompromised patients is likely to lead to the rise of PCP infections in the coming decades.

With the advances of molecular technologies and polymerase chain reaction (PCR) assays, the detection of low fungal loads has been improved compared with microscopy, leading to the concept of carriage when detection was not clinically associated with pulmonary symptoms [11].

Consequently, detection of *P. jirovecii* DNA in non-PCP patients has raised several physiopathological questions. For instance, is PCP due to reactivation of a colonizing strain or acquisition of a new one? Are the strains in PCP and non-PCP patients similar? Are outbreaks due to a specific strain? Most of these questions can be addressed using genotyping, which must be performed directly on clinical specimens because of the absence of reliable culture methods [12].

P. jirovecii genotyping strategies have been debated in several reviews [13, 14]. Recently, Alanio et al. also proposed a review on fungi genotyping strategies with a focus on *P. jirovecii* [15]. Other reviews were published earlier on this question [16-18].

To continue on this topic, we firstly consider the main typing methods developed for *P. jirovecii* characterization and their pros and cons, with an emphasis on the typing of low fungal loads, a subject rarely underlined. Secondarily, we present the main results obtained and the importance of including patients with low fungal load in the analyses because of their possible role as reservoirs and their impact on *P. jirovecii* transmission. Finally, we open the debate on systematic treatment of patients with low fungal load not only to prevent full-blown PCP, but also to block the transmission chain.

2. Genotyping Methods

There is no ideal method for genotyping fungi, the one that shows the most potential would be technically accessible and easy to perform, achievable at a reasonable cost, and have good reproducibility [15]. An additional feature would be performance, defined as the ability to target a marker that remains stable during the study period and is testable in every sample [19, 20]. The selected methods should also be capable of detecting mixtures, and in particular, have the ability to detect minority alleles. Detection of mixtures is an important aspect in the study of *P. jirovecii* transmission and epidemiology, considering that up to 92% of patients' respiratory samples present coinfections [17, 21-25].

Finally, the method should be discriminant. The discriminatory power of a method can be determined using Simpson's Diversity Index, which uses probability to assign a value of diversity to two unrelated strains sampled randomly from the population of a given species [26].

Several genotyping methods have been used to study epidemiology, strain variation, and resolve transmission events of *Pneumocystis* in humans. A brief chronological summary of the usual methods is given in Table 1.

2.1 Single-Strand Conformation Polymorphism (SSCP)

SSCP detects single-strand conformation polymorphisms created by a single nucleotide polymorphism [27]. This nucleotide polymorphism leads to a change of mobility of single-stranded DNA under non-denaturing electrophoretic conditions. The method is easy to perform, presents a low cost, and allows identification of nucleotide variation in amplicons ranging from 100 to 500bp. Moreover, this method can detect mixtures with a detection threshold around 10% [28, 29].

Several loci have been targeted for *P. jirovecii* typing with this method: internal transcribed spacer 1 (ITS1) region, the 26 rRNA gene (26S), the mitochondrial 26S rRNA gene (mt26S), a partial portion of the β-tubulin gene as proposed by Hauser in 1997, and also the dihydropteroate gene (*dhps*), which was added to this list by Ma and Kovacs in 2001 [28-31]. SSCP results are limited by the quality of the migration of the DNA fragments and has been progressively abandoned.

2.2 Restriction Fragment Length Polymorphism Analysis (RFLP)

RFLP typing has been widely used for many pathogens. RFLP includes an amplification of the selected loci followed by DNA digestion using restriction enzymes, which excludes loci lacking of restriction sites. The digested and amplified fragments are then analyzed by gel electrophoresis. This method is limited by the need for a high amount of fungal DNA in the patients' sample, as a low amount could lead to a poor signal [32]. Sensitivity could be increased with the use of hybridization with a probe specific for the amplified locus, which requires the use of radioactivity. The band patterns obtained allow the determination of sequence similarities or differences. This method is fast, cheap, and easy to perform. Nonetheless, result analysis is challenging; slight differences in band patterns could be missed. This difficulty in assigning a definite size limits data exchange between laboratories.

Several targets have been used for *P. jirovecii* typing with this method. RFLP analysis targeting the major surface glycoprotein family A1 gene (*msg*-RFLP) was proposed in 2009 and successfully used to investigate outbreaks in two centers receiving renal transplant patients [32, 33]. The *msg*-RFLP assay targets a 1300 bp fragment of the A1 *msg* gene subfamily, which is described as highly

polymorphic [14, 34, 35]. This method was also used in addition to a multilocus sequence typing (MLST) scheme of 3 loci to investigate an outbreak in a Danish transplant center [36].

The dhps gene of *P. jirovecii* has also been used for RFLP typing, but the polymorphism is limited to only two single nucleotide polymorphism (SNP) positions at nucleotides 165 and 171 [37-40]. As a consequence, this marker cannot be used alone for genotyping.

2.3 Direct DNA Sequencing

2.3.1 Single Locus

Sanger sequencing of selected loci is easily accessible for most medical facilities. Several loci have been tested which can be divided into genomic and mitochondrial markers.

Internal transcribed spacer ITS1 and ITS2 are very polymorphic genomic loci and have been used in numerous studies [16, 18, 41, 42]. At least 60 unique genotypes have been deposited in GenBank. Of note, ITS1 and ITS2 frequently present poly (T) and poly (A) repetitions of variable lengths within the same strain. Such polymorphisms could hamper alignment of downstream sequences [31, 42, 43]. ITS markers are often included in MLST schemes and NGS studies as described hereafter.

Mitochondrial markers, such as the mitochondrial small (mtSSU) and large subunit rRNA (mtLSU) genes, were used for direct DNA sequencing, phylogeny studies, and genotyping, with at least 25 unique mtSSU and 5 unique mtLSU genotypes reported in GenBank [13, 44, 45]. mtSSU or mtLSU genes appear less discriminant than ITS1 and ITS2 [18, 46]. Nevertheless, complete mitochondrial genome analysis revealed a 1-kb noncoding region rich in polymorphic sites, including both tandem repeats and single nucleotide polymorphisms [47]. This 1-kb polymorphic region discriminated at least 20 unique *P. jirovecii* genotypes in 23 clinical samples [47].

Other genes have been used for genotyping studies: mitochondrial genes, such as genes encoding cytochrome b (*cob*); nuclear genes such as β-tubulin; thymidylate synthase (*ts*); dihydropteroate synthase (*dhps*); dihydrofolate reductase (*dhfr*); superoxide dismutase (*sod*); the multifunctional product of *arom*; kexin (*kex1*); and thioredoxin reductase 1 (*trr1*) [44, 48-59].

Although Sanger sequencing is easily affordable, a single given locus is not sufficient to represent the polymorphism of *P. jirovecii* in a clinical sample. Therefore, several markers have to be associated in an MLST scheme (see below), which limits the throughput and increases the cost. Additionally, Sanger sequencing cannot detect minority alleles below a 20-30% threshold.

2.3.2 Allele-Specific PCR

In this method, described by Keely et al., an allele-specific PCR assay is designed for identifying genotype switching in patients presenting two PCP episodes [60]. The target is the mtrRNA large subunit (mtLSU) locus already known to be polymorphic at site 85. This allele-specific assay requires two steps: the first step is a pre-amplification of the targeted 346 base pair mtrRNA amplicon. The second step consists of parallel PCRs using a specific primer designed to amplify DNA with a C at site 85, or using a specific primer designed to amplify DNA with an A at site 85. Amplified DNA is then subjected to electrophoresis in agar gel and hybridized with a labelled probe for detection. This approach allowed the comparison of the mt85 profile (A or C) between PCP episodes. This allele-specific PCR provided arguments for the re-infection hypothesis versus

reactivation of latent microorganisms. This method was used to investigate several episodes in a given individual but is not fully adapted to discriminate between genotypes to investigate outbreaks.

2.3.3 DNA Sequencing Using a Multilocus Sequence Typing

The multilocus sequence typing (MLST) method involves PCR amplification and DNA sequencing of a set of loci [61]. Thereafter, an allele is assigned to the sequence of each gene in a given sample and the combination of alleles defines the sequence type corresponding to the specific allelic profile [62]. Numerous markers have been evaluated in MLST schemes for *P. jirovecii* genotyping (*ITS* region*, 26S rDNA*, *mt26S*, *β-tubulin*, *sod*, *cytochrome b*, *mtLSU rRNA* gene, as well as *dhps* and *dhfr* genes) and different combinations of genetic loci have been proposed [17, 63-68]. A common nomenclature for the various alleles of these MLST markers has led to the creation of a specific web page (http://mlst.mycologylab.org/pjirovecii) to harmonize the results. Each of the different sets proposed had a high discriminatory power, but there were few comparative studies of the different MLST schemes and the choice and the number of the loci are not standardized. In 2013, Maitte et al. evaluated different combinations in regard to their discriminatory power on 33 epidemiologically unrelated patients. The discriminatory power of the different combinations ranged from 0.751 using one marker to 0.996 using eight markers, and the authors proposed an optimized MLST scheme restricted to three loci (*ITS*, *mt26S*, and *cyb*) which achieved a discriminatory power of 0.996 [63]. The MLST approach was used in outbreak investigations and confirmed the presence of identical genotypes recovered from different patients during outbreaks [69-71]. Nevertheless, MLST remains labor-intensive to achieve amplification and sequencing of multiple individual loci from individual respiratory samples. Esteves et al. skirted these disadvantages by using DNA pooling of samples before genotyping. In Esteves's study, DNA pooling enabled cost reduction by using fewer PCR reactions before sequencing [72].

2.4 Single-Base Extension Methods (SBE)

These methods are based on already known SNP detections at different loci [73]. In single-base extension methodologies (SBEs), the first step consists of the amplification of the selected loci (with multiplex PCR or not) containing the known SNPs. This step is followed by the SBE assay using specific primers designed to hybridize to the targeted DNA sequence just immediately upstream of the polymorphic site of interest. These specific primers can be paired with tags for further discrimination if multiplex analysis is needed. The specific primer hybridizes with the targeted complementary region and this hybridization allows the enzymatic extension by a single base in the presence of the four ddNTPs. Thus, the ddNTP complementary to the polymorphic base could be incorporated, the elongation stopped, and then be identified. The identification of this incorporated base can be determined by fluorescence labelling, isotope labelling, or size determination using electrophoresis.

Esteves et al. used this method on *P. jirovecii* in 2011 on three genetic targets (*dhfr*, *mtLSU rRNA*, and *sod*) with four SNP positions (dhfr312, mt85, sod110, and sod215) [74]. The concordance for genotyping was 94% using capillary electrophoresis when compared to direct sequencing. Esteves et al. then successfully used this method with high throughput abilities [75]. In Esteves's multicenter study, SBE enabled the clustering of genotypes according to their

geographical origin (samples from Portugal, USA, Spain, Cuba, and Mozambique) and a specific haplotype (DHFR312T/SOD110C/SOD215T) appeared as associated with severe AIDS-related PCP.

Alanio et al. also used the SBE approach on the mt85 locus (analyzing three alleles) and found a correlation between allele type and the place of hospitalization [76] . This approach also enables the detection of minor variants in mixed samples. This method is suited for studying minority variants but is limited as a genotyping method to investigate outbreaks, considering the use of a restrictive number of SNP positions. Moreover, this method remains costly when compared to Sanger sequencing.

2.5 Short Tandem Repeat (STR)

Short tandem DNA repeats (or microsatellites) are found in numerous loci in eukaryotes and one of their features is a high mutation rate [77]. Due to slipped strand mispairing during the DNA replication process, STR loci have been found to mutate at 10 to 100,000 times more frequently than non-repetitive elements in eukaryotic genomes [78, 79]. The method to determine the size of the repetitive element consists of the amplification of the target locus followed by a capillary electrophoresis. The combination of several STRs have already been used for several pathogens [80-83].

The first STR locus used for *P. jirovecii* is located in the intron of the upstream conserved sequence (UCS) of the *msg* gene. This locus presents a 10-bp repetitive motif with three sequence types observed, and a variety for each sequence type determined according to the number of repeats of the motif [24, 58, 84, 85]. In 2012, the availability of *P. jirovecii* genome published by Cisse et al. allowed the identification of numerous loci as possible STRs [86]. The first study using STR analysis published by Parobek and colleagues in 2014 is based on eight STR markers and was validated using 91 *P. jirovecii*-positive respiratory specimens collected in patients from Uganda, the United States, and Spain [87]. The discriminatory power reached in this study was 0.999. Another STR analysis scheme (with one locus in common with Parobek's study) was proposed in 2015 and was based on six STR markers [22]. In studying 106 respiratory specimens (corresponding to 91 patients), the discriminatory power was 0.992 after the exclusion of putative epidemiologically related samples. This method was subsequently used to investigate *P. jirovecii* strains' population structure via a collaboration between European teams [23].

Regarding the ability to determine mixtures of *P. jirovecii* genotypes in samples, STR typing studies reported 70% (Parobek) and 68% (Gits-Muselli) of genotype mixtures, which was confirmed with a European study that analyzed 249 samples (68% of mixtures) [22, 23, 87]. The maximal ratio of mixture detection using simulated mixed samples was 1/50 [22].

Although this method is easy to use, fast, cheap, and without need of sequencing, STR typing is not standardized and selection of the best STR markers remains to be established. As a consequence, no database for reporting and inter-laboratory comparison is currently available.

2.6 Next-Generation Sequencing (NGS)

NGS encompasses a lot of different applications from genome assembly to amplicon analyses. These applications do not require the same equipment and the same procedures.

P. jirovecii whole genome assembly, Cisse et al. performed whole genome sequencing on a single bronchoalveolar lavage (BAL) sample. Before sequencing assays, the BAL sample was

enriched in *P. jirovecii* by immunoprecipitation. Nucleic acids were then extracted and sequenced using high throughput sequencing methodologies. Roche's 454 and Illumina's paired-end approaches were used to construct the libraries. Indeed, the lack of culture systems for *P. jirovecii* in a global approach to sequencing resulted in a huge quantity of undesirable DNA from humans and other microorganisms present in the BAL fluid; a dedicated software was used to eliminate human sequences [86].The first assembly was followed by a second one in 2015, and this allowed genome comparison of two different strains [34]. A recent study by Cissé et al. compared the whole genome sequence of 33 *P. jirovecii* strains to determine genomic variation and population structure [88]. This study found that the natural population maintains a high level of genetic variation despite low levels of recombination.

Nonetheless, comparison of several clinical strains using whole genome sequencing (WGS) for genotyping is currently limited by the cost and the complexity of some analyses requiring bioinformaticians.

However, amplicon analysis using NGS can answer some questions on genotyping and does not require specific training in bioinformatics. Alanio et al. used the Roche GS Junior System to evaluate the diversity of *P. jirovecii* strains associated with acute infection [21]. Two nuclear (DHFR and ITS2) loci and one mitochondrial (mtLSU) locus were targeted using ultra-deep pyrosequencing. NGS revealed that 92% of patients harbored mixtures of genotypes with variable proportions during the course of infection. This study also highlighted the possibility of heteroplasmy, with the observation of more variants for the mtLSU locus than for nuclear loci.

Recently, few studies have been published using NGS amplicon analysis for *P. jirovecii* outbreak investigations. In 2016, Urabe et al. [89] published an outbreak investigation study using a MLST scheme on four loci: B-tubulin, 26S, SOD, and CYB. The MLST interpretation was limited by the overlapping of certain bases. In this study, MLST was followed by MiSeq NGS that allowed the identification of the ratio of these overlapping bases observed in the samples by Sanger sequencing. The NGS showed almost identical ratios of the different types of bases in the tested samples. The limit of the MLST approach with detection of overlapping bases at SNP positions was avoided by the addition of NGS.

The same approach was used by Charpentier et al. [90] to investigate an outbreak in Grenoble University Hospital. Three MLST loci (mtLSU, SOD, and CYB) were used and the GS Junior System was used to sequence modified amplicons of 700-800 bp fragments. The limit of variant detection was 1:100. This study confirmed the previous result of Alanio et al. on mitochondrial marker diversity, with a higher number of haplotypes compared to the nuclear markers.

NGS methodologies are useful to study diversity and mitigate the limits of other genotyping methods, however, they are costly and somewhat time-consuming.

3. Main Results of *P. jirovecii* Genotypes

3.1 Mixtures of **P. jirovecii** *Genotypes*

To date, all typing methods have reported the presence of mixtures of two or more genotypes in a single pulmonary sample but with different frequencies, as mixture detection depends on the depth of the analysis. Thus, the range of mixture detection varies according to the method used, from 30% using Sanger DNA sequencing to 70% using SSCPs [24, 28, 32, 45, 68, 76, 91, 92] and STRs [23, 87] to 90% using pyrosequencing [21]. Recently, implementation of STR analysis

methods and next-generation sequencing (NGS) has allowed easy detection of low amounts of coinfecting genotypes, with ratios of about 1:50 and 1:1,000, respectively [22, 90]. Mixtures are generally encountered with *P. jirovecii* infections, and it does not seem to depend on the underlying disease since mixture detection was as high in AIDS patients as in solid organ transplant recipients [22, 23].

Mixture detection complicates epidemiological investigations when needing to attribute a given genotype to a given patient when mixtures are detected. Moreover, mixture detection not only depends on the method used but also on the fungal load. If the fungal load is too low, amplification of some single copy loci can fail, possibly hampering the analysis of genotyping and the detection of mixtures. This could introduce biases if low fungal loads are associated with specific genotypes [76].

3.2 Resistance to Co-Trimoxazole

A specific clinical issue has emerged with the use of trimethoprim-sulfamethoxazole (co-trimoxazole) prophylaxis of patients with PCP and AIDS. Although *dhps* mutations have been reported in PCP treatment failure (suggesting selection by drug pressure), the same mutations have also been detected in PCP patients not receiving co-trimoxazole, and no difference was evidenced regarding mortality in patients harboring wild type or mutant strains [38, 93]. Therefore, rather than selection pressure by co-trimoxazole prophylaxis, the presence of *dhps* mutations could be explained by incidental interhuman transmission and may serve as an epidemiological marker rather than a resistance marker [94, 95]. For instance, in a French study evaluating *dhps* mutation frequency from different parts of the country, *dhps* mutants were reported only in Paris, where exchanges between people could have been facilitated by population density rather than a pressure by co-trimoxazole prophylaxis [40].

3.3 Outbreak Investigations

Outbreak investigations have been the main application of genotyping. All the methods reported above have been used. To date, there is no definitive demonstration of superiority of one method over another. To demonstrate that two genotypes are different, there is no need for a very discriminant method after the difference is observed. In contrast, to demonstrate that two genotypes are identical, the most discriminant method should be used. The increased access of NGS technologies will probably change the way we perform outbreak investigations in the future, acknowledging that the presence of mixtures of *P. jirovecii* make the analysis of generated sequences difficult.

Outbreaks or clustered cases of PCPs have been reported in different settings, including kidney transplant units, liver transplant units, pediatric oncology wards, hematology wards, and wards of other medical specialties [66, 96-99]. These outbreaks confirmed the currently accepted hypothesis of airborne transmission between humans, with the ascus (previously called the cyst) as the most probable infecting agent [100, 101].

The search for index cases was also part of the outbreak investigations. The genotypes from PCP patients and carriers did not differ, and carriers can harbor *P. jirovecii* for long periods [102]. Thus, all carriers, and probably all individuals, can potentially act as a reservoir and transmit the fungus to immunocompromised hosts, as extrapolated from transmission experiments in mice

[103, 104]. Thus, transmission could occur between healthy individuals (such as healthcare workers) and immunocompromised patients as already suggested [105-107]. Indeed, health care workers would be able to transmit *P. jirovecii* for quite a long time, since *P. jirovecii* was detected in specific healthy individuals for up to 10 weeks [107].

Immunocompromised patients without full-blown PCP can also serve as a transmitter. The recent study of Robin et al. In stem cell transplant patients using STRs evidenced transmission in daycare centers between patients without clinical signs of PCP that harbored low fungal loads in their respiratory samples [98]. This underlies the need of prophylaxis in different clinical situations of immunosuppression using co-trimoxazole [9, 108]. Prophylaxis could not only prevent PCP in a patient at risk, but would also decrease the fungal load of colonized patients and therefore potential transmission to other immunocompromised patients who may share or visit the specialized wards. Prophylaxis would then have a broader protective ability than individual protection.

For outbreak investigations, genotyping alone cannot ascertain transmission and contact between patients must also be included in the analysis [15]. The timing between exposure and disease (incubation time) has not been clearly defined. Therefore, when PCP occurs in a hospital setting, it remains unanswered when a possible transmission occurred. Recently, transmission of a specific genotype between renal transplant recipients, hematology patients, and cancer patients of a specific hospital have been studied over a period of 4 years. The median time between suspected exposure and PCP was 197 days (interquartile range: 57-342.5), suggesting that the incubation time of PCP is variable and can be as long as 3 months, even in the hospital and in immunocompromised individuals [22].

3.4 Choice of Genotyping Markers

When considering the choice of markers to use for genotyping, the current debate is not in favor of using mitochondrial markers, since mitochondrial and nuclear genomes drift at different speeds [109]. Notably, Alanio et al. described that mixed mitochondrial genotypes were associated with the highest fungal loads [76]. This either suggests coinfections with several genotypes or the accumulation of mutations due to a high replication rate of the microorganism rather than specific geographical localizations. The observation suggesting heteroplasmy with mixtures of mitochondrial DNA and unique nuclear markers also supports a precocious use of mitochondrial markers for studying population genetics [21, 110].

3.5 Latent Versus Recently Acquired Infections

With the constant airborne circulation of *P. jirovecii*, PCP could not only be due to reactivation of latent disease acquired in childhood as in *Cryptococci*, but could also be the consequence of recent exposure [3, 111, 112]. This latter hypothesis comes from the observation of mixtures (see above), which can reach from 70% to more than 90% of cases, depending on the detection method used [4, 21, 113]. These mixtures can come from mutations of the initial organism contracted in infancy, resulting from microevolution in a single host. On the other hand, these mixtures can originate from continuous inhalation of new genotypes of varying frequency, given that different genotypes can be inhaled simultaneously or sequentially. Detection and genotyping of *P. jirovecii* DNA in the air surrounding individuals with or without active PCP reveals the

connection between the human source and the acquisition of a specific genotype from other individuals in a specific period of time and at a given location [18, 64].

The description of outbreaks or clustered cases in different settings (see above) favors the hypothesis of constant inhalation of different genotypes. Indeed, the sharing of a given genotype suggests recent acquisition and proliferation of a particular *P. jirovecii* genotype responsible for the outbreak in potentially *P. jirovecii*-naïve patients. Another argument for recent acquisition is that genotypes found during PCP were more closely related to the place of PCP diagnosis rather than to the place of birth, reinforcing the hypothesis of newly acquired *P. jirovecii* infection in hospital settings [114, 115].

However, both mechanisms could occur simultaneously or sequentially: reactivation of *P. jirovecii* from prior exposure and multiplication from recent *P. jirovecii* exposure. The organism acquired in the past could be controlled by the immune system, resulting in equilibrium between host response and multiplication of the microorganisms, which becomes undetectable using the current PCR methods since around 70-80% of the BAL fluids tested in microbiology laboratories are negative. When a new microorganism with different surface proteins is inhaled, this new microorganism can cause a new illness as long as the immune response has not reached a new equilibrium as already suggested [13, 116, 117].

The polymorphism of the surface proteins prompts us to understand whether some microorganisms, possibly linked to different genotypes, are more prone to induce infection or more prone to proliferate compared to others, or if some genotypes are more specific to a given host's background. Our multicenter study on European genotyping suggested that some specific genotypes could be linked to the patient's background. A specific genotype (named Gt123) was observed in kidney transplant recipients and could be associated with a particular virulence for these patients presenting the same type of immunosuppression [23].

3.6 "Colonization" or "Carriage" of **P. jirovecii**

The concept of "colonization" or "carriage" of *P. jirovecii* was introduced as soon as *P. jirovecii* DNA was detected using PCR in patients without any symptoms, although at risk for PCP [118]. This concept has been extensively reviewed [11]. HIV infection, malignancies, solid organ transplantation, or immunosuppression secondary to immunosuppressive drugs including steroids, increases the prevalence of *P. jirovecii* detection in asymptomatic patients. However, the prevalence of this colonization is very variable from one study to another; this may result from the PCR method used or also from the patient cohorts studied with different levels of immuno-depression [11]. *P. jirovecii* DNA has also been detected in non-immunosuppressed patients, such as those with chronic lung diseases, cigarette smokers, or in pregnant women [11]. Until recently, most have assumed that the concept of "colonization" owns a specific status which have been brought in opposition with PCP [11] In contrast, colonized patients (either immunocompetent or immunocompromised individuals) can be considered as recently exposed patients with *P. jirovecii,* and thus serve as the reservoir of the organism, at least transiently, as discussed above. Consequently, we proposed that "colonization" could be considered as a situation where people have recently encountered *P. jirovecii*. In those individuals, only the immune status will predict if infection with symptoms will occur. Thus, what is called "colonization" or "asymptomatic carriage" could be the starting point for PCP in immunocompromised patients and therefore should not be

neglected, as a possibility in addition to recent acquisition or reactivation of previous infection. For instance, Mori et al. demonstrated that patients receiving immunosuppressive therapy for rheumatoid arthritis tested positive for *P. jirovecii* DNA without respiratory symptoms and then developed PCP in the next 2 to 4 weeks [119]. Upon *P. jirovecii* exposure or reactivation in immunocompromised hosts, the fungal load (which is initially not detectable) increases until it plateaus at the stage of PCP [120]. The rapidity of this increase varies according to the immune status and the degree of immunosuppression.

3.7 Geographical Distribution

The European STR study of *P. jirovecii* on 249 samples described wide genotype diversity across Europe, and highlighted local clusters of patients infected with a given genotype. The previous study by Parobek et al. using STRs also described a wide variety of genotypes and suggested that the genetic differences between samples from Uganda, the United States, and Spain is limited when evaluating genetic distance using Rst and Fst, two classical factors to measure population demographic history with microsatellite markers [121]. Fst is statistical measure used to examine the overall genetic divergence in allele repartition among subpopulations. Rst is associated with genetic distance of microsatellites. In the Parobek et al. study, R_{ST} and F_{ST} suggested that Ugandan samples were significantly divergent from the San Francisco and Spain populations, but the study is limited by reduced sampling. Nonetheless, genetic differentiation was limited when considering all three populations. The recent study of Cissé et al. using whole genome sequencing found no evidence of population structuring by geography [88].

4. Conclusion and Perspective

The current observations reinforce the hypothesis that immunocompromised infants or adults could be a reservoir of *P. jirovecii* as well as immunocompetent individuals, at least transiently. Thus, immunocompetent individuals could be a potential source of constant circulation of the fungal organism in the population with the risk of transmission to potentially immunocompromised hosts. There is a need to study the different surface proteins and how the fungus can switch from one to another to explain the maintenance of transmission, despite the response of the immune system.

To further investigate transmission in humans, genotyping tools should be able to characterize low to very low amounts of *P. jirovecii* DNA in order to reveal whether these low loads correspond to quiescent forms or present the same polymorphism as the general population. New diagnostic tools allowing a more precise description of the metabolic state of *P. jirovecii* in a given sample or patient would be also needed. Consequently, anti-*Pneumocystis* treatment, such as co-trimoxazole, could be postponed to avoid side effects if *P. jirovecii* is in a latent state.

Table 1 Summary Methods Advantages and Pitfalls.

Method	*Advantages*	*Pitfalls*	*Database*	*References*
Direct DNA sequencing	Easily accessible , fast and cheap Accuracy	Low throughput Poor detection	Genbank Possibilities of	Lu 1994 JCM Wakefield

one loci	depending on the loci Detection of all possible variant on the loci	of mixture	data exchange between Laboratories	1998 FEMS
Direct DNA sequencing MLST	Accessible , Cost depending of the number of selected loci Various scheme with three to eight loci High discriminatory power Good throughput	Poor detection of mixtures Costly	Yes with a specific scheme of loci Possibilities of data exchange between Laboratories	Maitte 2012 JCM Esteves 2012 Clin Microbiol
PCR-SSCP	Easily accessible , fast and cheap	Low throughput Poor detection of mixture	No data exchange between laboratories	Hauser 2004 Infect Genet Evol
RFLP and msg-RFLP	Easily accessible , fast and cheap High discriminatory power for *msg*-RFLP	Low throughput	No data exchange between laboratories	Helweg-Larsen 2000 J Scand Infect Dis Ripamonti 2009 JID
Allele specific PCR	Easily accessible, fast moderate cost	Only possible on known SNP Investigation of several episodes of given individuals Poor discriminatory power	Possibilities of data exchange between Laboratories	Keely AIDS 1996
Multiplex single based extension technology	Easily accessible , fast High discriminatory power	Moderate Throughput Already described SNP Accurate detection of mixture	Possibilities of data exchange between Laboratories	Esteves 2011 JCM Alanio 2015 Eukar Microbiol
VNTR	Fast and cheap Various scheme High discriminatory power	Moderate Throughput Accurate detection of mixture	No data exchange between laboratories	Ma 2002 JID Parobek 2014 JCM GIts-Muselli 2015 PloS One

NGS approaches'	High Throughput Very accurate detection of mixture and minority variant	Costly Labor intensive Data analyze	No data exchange between Laboratories	Alanio 2016 Frontiers Urabe 2016 CMI Charpentier 2017 EID

Acknowledgments

No specific acknowledgments for this review.

Author Contributions

MGM, SB and AA contributed equally to this work.

Funding

The authors have declared that no competing interests exist.

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