Establishment and optimization of a combined MLST scheme for *Pneumocystis jirovecii*

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Introduction

Pneumocystis jirovecii is an unicellular fungus causing Pneumocystis Pneumonia (PCP), associated with high morbidity and mortality rates, especially in certain patient groups¹. Although it is the most prevalent opportunistic disease in AIDS patients, it may be more life-threatening in patients who are otherwise severely immunocompromised². Prevention is essential but not always possible, since the ecological niche and route of transmission of *P. jirovecii* remain uncertain³. Multilocus sequence typing (MLST) is currently the preferred approach for the analysis of *P. jirovecii* genetic diversity as it compares polymorphisms in a number of independent genetic loci applicable to clinical samples⁴. Currently, there are 17 loci and 40 primer sets used in different MLST schemes to study the allelic polymorphisms of *P. jirovecii* isolates⁵. However, there is still no consensus MLST scheme, leading to the fact that laboratories/hospitals are unable to compare isolates, investigate outbreaks and construct a global genotyping network for *P. jirovecii*. Adding further complications is the inability of the fungus to grow in culture⁶. This difficulty places particular emphasis on the source of the *P. jirovecii* sample, the quantity collected, and sample type, to ensure successful typing.

Objectives

The aims of the study were:

- (A) Comparison of all MLST schemes currently used worldwide and establishing the discriminatory power of each scheme.
- (B) Establishing a MLST scheme combining the loci with the highest amplification efficiency and discriminatory power.
- (C) Analysing various factors influencing MLST genotyping: source, effectivity of sample site of source, extraction and amplification techniques.

Methods

A literature review was performed to select the most appropriate loci. The discriminatory power for each loci was determined using the H-index⁷. Sputum, BAL and pharyngeal swab *P. jirovecii* isolates were used to test DNA extraction methods to establish the optimum conditions for maximum DNA yield. Based on the number of primer and discriminatory power, three MLST schemes were chosen for comparison of primer success rate and amplification efficiency. Application of the combined MLST schemes is currently underway.

Results

From the 17 loci available, the 8 most common ones were chosen for comparison: mt26S, 26S rDNA, ITS1, β -TUB, SOD, CYB, DHPS, and DHFR. As expected the H-index indicated that the scheme with the highest discrimination used all 8, but implementing an 8-loci scheme for *P. jirovecii* study is not practical. We therefore tested three MLST schemes (Table 1) on 24 *P. jirovecii* isolates to evaluate the efficiency of the schemes in a real-life setting, analysing the primer efficiency and DNA sequencing success.

Table 1. Performance of four MLST schemes of *P. jirovecii* evaluated by the Hunter index.

MLST Scheme	No. of samples	H-index
ITS1/2, β -tub, 26S, mt26S, CYB, SOD	24	0.998
CYB, mt26S, SOD ⁴	23	0.996
CYB, ITS1, mt26S ⁴	23	0.987
β -tub, DHPS, ITS1/2, mt26S	24	0.996

Primer success was analysed visualisation of the obtained PCR products on agarose gels (Figure 1). The scheme which produced the most successful amplification bands, amplifying 24/24 was the MLST scheme containing the loci SOD, CYB, and mt26S (Table 2), with the primer set for the loci SOD and CYB according to C. Maittea *et al.*, and the mt26S locus was amplified with the primer pair “PneumoLSU” herein designed.

We also analysed other factors influencing MLST genotyping, such as: source, the effectivity of sample site, extraction and amplification schemes. From the 311 published (source GenBank), we found that successful DNA sequencing most often was obtained from bronchoalveolar lavage (BAL) samples, while the most common sample site was induced sputum (IS). This was due to ease of access and patient comfort. The least common and least successful sample site to yield DNA were laryngeal swabs. Since we encountered IS most commonly, a DNA extraction protocol to maximise DNA levels was established. DNA extraction was optimised by adapting the Sputum DNA Isolation Kit (Norgen, Cat. 46200) with the addition of a liquid nitrogen grinding step, resulting in a consistent robust high yield of DNA.

Table 2. PCR success rate for each investigated locus

Primer Pair	SOD ⁴	CYB ⁴	mt26S ⁴	PneumoLSU	ITS1 ⁴	PneumoITS	PneumoBTUB	PneumoDHPS
Success Rate	24/24	24/24	18/24	24/24	8/24	9/24	13/24	24/24

Figure 1. (A) Success rate of each primer set used, based of visualized amplification of PCR bands. (B) The left band is an example of a successful amplification, any bands fainter than this where not considered (e.g. right band)

Conclusions

This study highlights the importance of an effective DNA extraction method to minimise yield lost during DNA preparation for MLST analysis. Additionally, it determined a new MLST scheme with the highest discriminatory power, for *P. jirovecii*, which is being suggested to become the globally consensus MLST scheme for this important human pathogen, to improve genotyping consistency, facilitate worldwide collaborations and to establish a global genotype map associated with standardized database of *P. jirovecii*.

References
1. Catherineot E, Lanternier F, Bougnoux M-E, Lecuit M, Couderc L-J, Lortholary O. Pneumocystis jirovecii Pneumonia. Infectious disease clinics of North America. 2010;24(1):107-38. 2. Krajcek BJ, Thomas CF, Limper AH. Pneumocystis pneumonia: current concepts in pathogenesis, diagnosis, and treatment. Clinics in chest medicine. 2009;30(2):265-78. 3. Thomas CF, Limper AH. Current insights into the biology and pathogenesis of Pneumocystis pneumonia. Nature Reviews Microbiology. 2007;5(4):298-308. 4. Maittea C, Leterrier M, Le Pape P, Miegville M, Morio F. Multilocus sequence typing of Pneumocystis jirovecii from clinical samples: how many and which loci should be used? Journal of clinical microbiology. 2013;51(9):2843-9. 5. Esteves F, Montes-Cano M, De La Horra C, Costa M, Calderon E, Antunes F, et al. Pneumocystis jirovecii multilocus genotyping profiles in patients from Portugal and Spain. Clinical Microbiology and Infection. 2008;14(4):356-62. 6. Harris J, R. S. A. Balajee, et al. (2010). "Pneumocystis jirovecii Pneumonia: Current Knowledge and Outstanding Public Health Issues." Curr Fungal Infect Rep 4(4): 229-237. 7. Hunter, Paul R., and Michael A. Gaston. "Numerical index of the discriminatory ability of typing systems: an application of Simpson's index of diversity." Journal of clinical microbiology 26.11 (1988): 2465-2466.