**SNaPAfu: A Novel Single Nucleotide Polymorphism Multiplex Assay for Aspergillus fumigatus Direct Detection, Identification and Genotyping in Clinical Specimens**

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**Abstract**

**Objective:** Early diagnosis of invasive aspergillosis is essential for positive patient outcome. Likewise genotyping of fungal isolates is desirable for outbreak control in clinical setting. We designed a molecular assay that combines detection, identification, and genotyping of *Aspergillus fumigatus* in a single reaction.

**Methods:** To this aim we combined 20 markers in a multiplex reaction and the results were seen following mini-sequencing readings. Pure culture extracts were firstly tested. Thereafter, *Aspergillus*-DNA samples obtained from clinical specimens of patients with possible, probable, or proven aspergillosis according to European Organization for the Research and Treatment of Cancer/Mycoses Study Group (EORTC/MSG) criteria.

**Results:** A new set of designed primers allowed multilocus sequence typing (MLST) gene amplification in a single multiplex reaction. The newly proposed *SNaPAfu* assay had a specificity of 100%, a sensitivity of 89% and detection limit of 1 ITS copy/mL (~0.5 fg genomic *Aspergillus*-DNA/mL). The marker A49_F was detected in 89% of clinical samples. The *SNaPAfu* assay was accurately performed on clinical specimens using only 1% of DNA extract (total volume 50 μL) from 1 mL of used bronchoalveolar lavage.

**Conclusions:** The first highly sensitive and specific, time- and cost-economic multiplex assay was implemented that allows detection, identification, and genotyping of *A. fumigatus* strains in a single amplification followed by mini-sequencing reaction. The new test is suitable to clinical routine and will improve patient management.

**Introduction**

*Aspergillus fumigatus* is the major filamentous fungal pathogen in severely ill patients, causing disseminated infections [1]. Invasive aspergillosis (IA) is associated with mortality above 50% [2]. Early and reliable diagnosis of invasive fungal infections (IFIs) can lower patients’ mortality and reduce treatment expenses, due to an early targeted therapy, but this is often difficult and might be in part also due to the limitations of conventional diagnostic techniques (culture and histology), which often lack sensitivity, promptness, and efficiency [3–5]. As an example, detection of circulating galactomannan (GM) has become commonly used but false-positive and false-negative results are still a major limitation to this auxiliary method for an earlier IA diagnosis [6]. Nucleic acid-based assays have the capacity to diagnose fungal diseases in an early stage. False-positive results represent drawbacks, as a direct consequence of the high sensitivity of molecular assays and to ubiquitous presence of *A. fumigatus* in the environment [3,7].

Molecular assays are presently recommended for correct identification of *A. fumigatus* within *Aspergillus* section *Fumigati* and evaluation of its genetic diversity, a critical issue for outbreak controlling [8–11]. Current gold standard in microbial genotyping is Multilocus sequence typing (MLST), developed for *A. fumigatus* by Bain et al. [12]. The present MLST panel combines seven housekeeping genes showing a discriminatory power of 0.93, not as high as microsatellite genotyping, but with the advantage of providing a free online database which improves data transferability and genetic analyses. MLST proved to be specific for this fungal species within section *Fumigati* [13–15], but remains...
expensive, laborious, and unfeasible for routine examination due to multiple sequencing.

The implementation of single nucleotide polymorphism (SNP) markers has been suggested as an attractive alternative for the detection of specific bacterial lineages and/or identification purposes [16–18]. A SNP based strategy has recently been developed for genotyping of Pseudomonas aeruginosa presenting a discriminatory power of 0.9993 comparing with MLST [19].

The aim of this study was to design an assay that combines detection, identification and genotyping in a single reaction and that performs directly on DNA extracts gained from clinical specimens. To this aim, we selected a panel of discriminatory-informative SNPs that are variable enough to accurately detect, identify and genotype A. fumigatus isolates and combine those markers in a single multiplex reaction named SNaPAfu

Materials and Methods

Ethics Statement

The Ethics commission of Innsbruck Medical University had approved retrospective usage of residual materials from diagnostics. A written informed consent was provided by study participants and/or their legal guardians.

MLST genotyping employing a multiplex strategy

The MLST sequence alignment generated by multiplex reaction is needed as basis for the development of the SNaPAfu assay, as this assay is built on SNPs located in MLST genes. A set of seven housekeeping genes was studied in 20 clinical and unrelated A. fumigatus isolates as suggested by Bain et al. [12]. These strains were obtained from sputum samples collected from patients admitted at Hospital S. João (Oporto, Portugal) and had been previously genotyped by microsatellite-based strategy [8,9]. Singleplex amplification was performed as previously described [12]. These primers were found unsuitable for a multiplex reaction as they formed dimers. Therefore new primers were designed using Primer 3 (http://frodo.wi.mit.edu/) and the absence of hairpins and dimers verified in silico using Auto-dimer software (http://www.ncbi.nlm.nih.gov/sra/base/AutoDimerHomepage/AutoDimerProgramHomepage.htm); newly selected primers are listed in Table 1. Multiplex PCR amplification reaction consisted of: 0.5 µL of genomic DNA (50 ng-250 ng), 2.5 µL of My Taq™ HS MIX (Bioline) and 0.5 µL of primer mix (each primer at 2 µM), in a final volume of 5.0 µL. The multiplex PCR reaction was run at the following conditions: initial denaturation step at 95°C for 15 min, followed by 35 cycles of denaturation at 95°C for 30 s, annealing at 61°C for 1 min and extension at 70°C for 1 min, followed by a final extension step at 70°C for 10 min. Amplification products of multiplex PCR products were confirmed on polyacrylamide gel and visualized by silver staining.

Sequencing analysis. Amplification products were purified with ExoSAP-IT (USB Corporation); 1.0 µL of ExoSAP-IT was added to 1.5 µL of amplification product. After purification, sequencing reaction was conducted as previously described [9]. Sequencing data were processed and analyzed with Sequencing 5.2 analysis software (Applied Biosystems).

SNaPAfu assay

The methodic strategy used for the SNaPAfu assay is based on targeting only SNPs present in MLST genes instead of amplifying full MLST gene sequences. In the SNaPAfu assay these SNPs are amplified in a multiplex reaction and subsequently analyzed by mini-sequence reaction.

### Table 1. Primers used for MLST genotyping of Aspergillus fumigatus in a multiplex reaction.

| Locus | Primer sequence (5’ to 3’) | Amplicon size (bp) |
|-------|--------------------------|------------------|
| ANXC4 | Forward GCAAAAGCTTGATGACAGAAA | 582 |
|       | Reverse* TAGTGGCAACAGGTTG | |
| BGFT | Forward* AGCGATGGCGCTAATAA | 654 |
| CAT1 | Forward CTTGATACAGCACTCTCAAG | 822 |
| CAT1 | Reverse* AGCGATGGCGCTAATAA | 654 |
| CAT1 | Reverse* CTGTGCCTCTCCTAGT | |
| LiP | Forward AGCGATGGCGCTAATAA | 822 |
| LiP | Reverse* GTTGCTGCTTTAGCAGCA | |
| MAT1-2 | Forward* TCGACTTTCCAGAGCATG | 311 |
| MAT1-2 | Reverse* TTCCTCACGCTTCCAGTT | |
| SOD8 | Forward CAAATGGCTTCCGTCTCAA | 826 |
| SOD8 | Reverse* GTTGCTGCTTTAGCAGCA | |
| ZNF2 | Forward GAGGACCTTGAGGATCGTC | 698 |
| ZNF2 | Reverse* GATGATACCTTCGACCCATGA | |

*Primer used for sequencing.

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MLST sequences were added into a database that included all the information available at MLST database (http://pubmlst.org/afumigatus/). Sequence alignment of target genes was performed (throughout the 3036 bp MLST concatenated sequences) and a final set of 20 SNPs was selected. Primers were designed for those positions, combined in a multiplex reaction, and a non-homologous tail was added at the 5’ end of extension primers to ensure separation by capillary electrophoresis (Table 2).

A genotyping reference set of 113 clinical and environmental A. fumigatus strains previously genotyped by microsatellite-based strategy isolated in Portugal were tested used for the establishment of the SNaPAfu assay [8,9]. Clinical strains (n = 76) were mainly obtained from sputum samples collected from patients admitted at Hospital S. João (Oporto, Portugal). Environmental strains (n = 37) were collected from soil, water, and air samples at Oporto region. A reference strain of A. fumigatus (ATCC46646) was also included in the strains set. A second group of fungal strains belonging to the section Fumigati was obtained from Centraalbureau voor Schimmelcultures (CBS): Aspergillus fumigatus (CBS117186), A. lentulus (CBS116380), A. fumigatus (CBS117182, and CBS117885), A. nofonutius (CBS117519), A. suliformis (CBS126.56 and CBS283.66), A. viridinutans (CBS121.95), Neoaspergillus fischeri (CBS316.89), N. hirsutae (CBS124073), A. pseudofischeri (CBS208.92 and CBS110899), and N. udagavorae (CBS114217). In addition, eight strains of Aspergillus belonging to other sections (A. flavus, A. niger, A. nidulans, and A. terreus) were also tested to verify the specificity of the assay. DNA was extracted from conidia using the sodium hydroxide method as previously described (http://www.aspergillus.org.uk/indexhome.htm?secure/laboratory_protocols). DNA extracts with concentrations reaching from 50 ng to 250 ng were suspended in 50 µL of ultrapure water (Qiagen) and stored at −20°C.

The mini-sequence assays were carried out in a final volume of 5.0 µL containing 1.5 µL of purified PCR product, 1.0 µL of the SNaPAfu primer mix (each primer at 1 µM), 1.0 µL of SNaPsHot™ Ready Mix (Applied Biosystems), and 1.5 µL of ultrapure water (Qiagen). The mini-sequence reaction was performed as recommended by the manufacturer. A post-purification treatment
of SNaPshot™ products was performed with 1 U of SAP (USB Corporation) at 37°C for 1 h, followed by a cycle of 85°C for 15 min. The products (0.5 μl) were mixed with 9.0 μl of HiDi™ formamide (Applied Biosystems) and 0.5 μl of GeneScan-120 LIZ size standard (Applied Biosystems). Capillary electrophoresis was performed on a 3130xl Genetic Analyzer and the size standard (Applied Biosystems). The DNA extracts gained from the clinical samples were used for DNA extraction. BAL and biopsy samples were conserved with liquid nitrogen (−80°C) and stored until use. DNA was extracted using a modified cetyltrimethylammonium bromide protocol [13,15,22] and finally resuspended in 50 μl of ultrapure water. Extracted DNA was detected with an in-house Platelia test (Bio-Rad Laboratories).

The DNA extracts gained from the clinical samples were exposed to an enrichment pre-cycle with illutra™ GenomiPhi V2 DNA Amplification Kit (GE Healthcare) by following manufacturer’s instructions before multiplex gene amplification. SNaPAfu assay was applied in these samples as described above.

### Data and statistical analysis

MLST sequencing data were deposited on an in-house record that included the information of 29 profiles attainable at online databases: 27 profiles accessible by MLST database (http://pubmlst.org/afumigatus/) and sequences of AF293 and AF1163 strains available at ENSEMBL (http://fungi.ensembl.org/index.html). The programs BioEdit sequence alignment editor (available at http://www.mbio.ncsu.edu/bioedit/bioedit.html) and Geneious™ Pro 5.3.4 (Biomatters Ltd, Auckland, New Zealand) were used to align and detect polymorphic positions in DNA sequences. Simpson’s diversity index was applied to determine the discriminatory power of the proposed strategy. Data and statistical

### Application on clinical samples

A defined and well-described reference set of 37 clinical specimens (bronchoalveolar lavages (BAL) and biopsy materials) from Innsbruck Medical University were studied retrospectively using the new SNaPAfu assay (see Table 3). The samples were obtained from 21 patients with possible (n=8), probable (n=7), and proven (n=6) aspergillosis according to the European Organization for the Research and Treatment of Cancer/Mycoses Study Group (EORTC/MSG) criteria [21]. The samples had been previously tested by culturing, direct microscopy, GM, and pan-fungal PCR. Aspergillus Plateia test (Bio-Rad Laboratories) was performed for BAL samples with sufficient sample volume according to manufacturers instructions, regarding samples with values ≥0.5 as positive. An amount of 1 mL of BAL (total amount received for microbial diagnostics 50 mL) and 2.5 mg of lung biopsy was used for DNA extraction. BAL and biopsy samples were conserved with liquid nitrogen (−80°C) and stored until use. DNA was extracted using a modified cetyltrimethylammonium bromide protocol [13,15,22] and finally resuspended in 50 μl of ultrapure water. Extracted DNA was detected with an in-house pan-fungal PCR as previously described [22,23]. Following DNA extraction, the DNA was stored at −20°C for few months (in this study the oldest samples were stored for 6 months). Sequencing was done with Big Dye terminators and a capillary sequencer (3500 Genetic Analyzer; Applied Biosystems).

The DNA extracts gained from the clinical samples were exposed to an enrichment pre-cycle with illutra™ GenomiPhi V2 DNA Amplification Kit (GE Healthcare) by following manufacturer’s instructions before multiplex gene amplification. SNaPAfu assay was applied in these samples as described above.

### Table 2. The primers used in SNaPAfu assay.

| Primer* | Primer sequence | Tail added to the primer (bp) | Final primer size (bp) | Targeting base | Expected peak size (bp)* | Expected base* |
|---------|-----------------|------------------------------|-----------------------|---------------|-------------------------|---------------|
| A45_R   | CGTATGAGGAGTCCCTGGGAT | 20                           | 40                    | G/A           | 45–46                   | C/T           |
| A49_F   | CCTCGTCTCGTATCTGAGTAG | 44                           | 67                    | G/A           | 70–71                   | G/A           |
| A313_R  | ACCCGGACAGTTCCAGATTG | 68                           | 88                    | C/T           | 91                       | G/A           |
| A392_F  | GACGCGGGCGACAGGCGC   | 0                             | 17                    | G/A           | 19–20                    | G/A           |
| B129_F  | CAGGACCCTTCGCTCTCTCTTC | 16                           | 35                    | G/A           | 40                       | G/A           |
| B164_F  | CGCTGCTGCTCCCTCAAGAA | 6                             | 24                    | T/C           | 35–36                   | T/C           |
| C185_F  | GGATTATCTGACGGGTGTCG  | 51                           | 71                    | T/G           | 75                       | T/G           |
| C193_R  | GACATGAGGGACAGGCGTC   | 13                           | 30                    | G/A           | 36–37                   | C/T           |
| C403_R  | ACCACGTACGGAAATTGCC  | 65                           | 85                    | C/G           | 87–90                   | G/C           |
| C428_R  | AGACAGATGTACGTTGTGC   | 28                           | 48                    | G/A           | 53–54                   | C/T           |
| C540_R  | GATCTTTGTGACTACTCTG  | 54                           | 75                    | G/C           | 77                       | C/G           |
| L54_R   | CTGCCCAATTAGCGAGG     | 0                             | 18                    | C/G           | 31–32                   | G/C           |
| L164_R  | TTTGCTTCGGCTACTCTTC  | 35                           | 55                    | G/A           | 58–59                   | C/T           |
| L456_R  | CTGCGTACGGAAACCAATT  | 15                           | 35                    | T/C           | 41                       | A/G           |
| L487_R  | GACATCAATTCCGAGTCTGT  | 19                           | 40                    | T/C           | 47                       | A/G           |
| M15_R   | GTATTGGCTGCTGAGGATG   | 40                           | 61                    | G/A           | 63                       | C/T           |
| S276_R  | TTTCAAGCTGGCATGTCCTCTC | 43                           | 64                    | A/G           | 66                       | T/C           |
| S329_R  | TGACGACGGAGGTGAGCC    | 61                           | 82                    | C/A           | 87                       | C/A           |
| Z198_F  | GCACCGAGGAGTGGAGGCC   | 27                           | 48                    | T/C           | 52–53                   | A/G           |

*Primer nomenclature and incorporated information on the targeting polymorphic position (e.g. A45_R means that a reverse primer was designed in the polymorphic position 45 of the gene ANXC4 of MLST panel).

Expected peak size in the electropherogram (see Figure 2); bp means base pairs.

Base expected in MLST and SNaP profiles; a complementary base is expected to be seen on the SNaPfu electropherogram for primers designed for reverse sequence.
### Table 3. Clinical samples considered in this study and methods used for diagnosis of invasive aspergillosis (ARDS: Acute Respiratory Destress Syndrome; BAL: bronchoalveolar lavages; BS: skin biopsy samples; nm: not enough material to be tested; GM: galactomannan; PF-PCR: Pan-fungal PCF; (+) and (−) represent positive and negative results).

| Patient | Gender | Age | EORTC | Disease | Hospital | Sample | Microscopy | Culture | GM | PF-PCR | MLST | gel | SNaP 4fu | A49_F | B129_F | L164_R | M15_R | All markers |
|---------|--------|-----|-------|---------|----------|--------|------------|---------|----|---------|------|-----|----------|-------|--------|----------|------|-------------|
| 1       | M      | 58  | Possible | Pneumonia | Hematology | Lung biopsy | NEG        | NEG     | nm  | POS     | (+)  | (+) | (−)     | (+)  | (+)    | (−)     | (+) | (−)         |
| 2       | F      | 63  | Possible | Pneumonia | ICU       | BAL     | NEG        | Candida sp. | nm  | POS     | (+)  | (+) | (−)     | (+)  | (−)    | (−)     | (+) | (−)         |
| 3       | F      | 70  | Proven  | Pneumonia | Pneumology | Lung biopsy | POS        | A. fumigatus | nm  | POS     | (+)  | (+) | (+)     | (+)  | (+)    | (+)     | (+) | (−)         |
|         |        |     |         |          |           |         |            |          |     |         |      |     |          |      |        |          |     |             |
| 3       |        |     |         |          |           | BAL     | POS        | Mycelium sterilium | nm  | POS     | (−)  | (+) | (−)     | (+)  | (−)    | (−)     | (+) | (−)         |
| 3       |        |     |         |          |           | BAL     | NEG        | Mycelium sterilium | nm  | POS     | (+)  | (+) | (+)     | (+)  | (+)    | (+)     | (+) | (−)         |
| 3       |        |     |         |          |           | BAL     | NEG        | Mycelium sterilium | nm  | POS     | (+)  | (+) | (+)     | (+)  | (+)    | (+)     | (+) | (−)         |
| 3       |        |     |         |          |           | BAL     | POS        | NEG       | nm  | POS     | (+)  | (+) | (−)     | (+)  | (+)    | (+)     | (+) | (−)         |
| 4       | M      | 42  | Proven  | Pneumonia | Transplant | Abscess | POS        | A. fumigatus | nm  | POS     | (+)  | (+) | (−)     | (+)  | (−)    | (−)     | (−) | (−)         |
| 4       |        |     |         |          |           | Abscess | POS        | A. fumigatus | nm  | POS     | (+)  | (+) | (−)     | (+)  | (−)    | (−)     | (−) | (−)         |
| 5       | M      | 54  | Possible | ABPA    | Transplant | BAL     | NEG        | A. niger | POS  | POS     | (+)  | (+) | (+)     | (+)  | (+)    | (+)     | (+) | (−)         |
| 6       | F      | 67  | Possible | Pneumonia | ICU       | BAL     | NEG        | NEG      | POS  | POS     | (+)  | (+) | (+)     | (+)  | (+)    | (+)     | (+) | (−)         |
| 7       | F      | 62  | Probable | Pneumonia | ICU       | BAL     | POS        | C. albicans, A. fumigatus | POS  | POS     | (+)  | (+) | (−)     | (+)  | (+)    | (+)     | (+) | (−)         |
| 7       |        |     |         |          |           | BAL     | POS        | Candida sp. | POS  | POS     | (−)  | (+) | (−)     | (+)  | (−)    | (−)     | (−) | (−)         |
| 8       | M      | 65  | Possible | Pneumonia | ICU       | BAL     | NEG        | NEG      | POS  | POS     | (+)  | (+) | (−)     | (+)  | (+)    | (+)     | (+) | (−)         |
| 8       |        |     |         |          |           | BAL     | NEG        | NEG      | POS  | POS     | (+)  | (+) | (+)     | (+)  | (+)    | (+)     | (+) | (−)         |
| 9       | M      | 56  | Possible | Pneumonia | Transplant | BAL     | POS        | A. terreus | POS  | POS     | (+)  | (+) | (+)     | (+)  | (+)    | (+)     | (+) | (−)         |
| 10      | M      | 55  | Proven  | Pneumonia | Transplant | Lung biopsy | POS        | NEG      | POS  | POS     | (+)  | (+) | (+)     | (+)  | (+)    | (+)     | (+) | (−)         |
| 11      | M      | 54  | Proven  | Pneumonia | Transplant | Lung biopsy | POS        | A. fumigatus | nm  | POS     | (+)  | (+) | (+)     | (+)  | (+)    | (+)     | (+) | (−)         |
| 12      | M      | 60  | Probable | ARDS    | ICU       | BAL     | POS        | Mycelium sterilium | POS  | POS     | (+)  | (+) | (+)     | (+)  | (+)    | (+)     | (+) | (−)         |
| 13      | F      | 63  | Probable | Pulmonary infection | ICU | BAL | POS | C. albicans, A. fumigatus | POS | POS | (+) | (+) | (+) | (+) | (+) | (+) | (−) |
| 13      |        |     |         |          |           | ICU     | BAL | POS | A. fumigatus | POS | POS | (+) | (+) | (+) | (+) | (+) | (+) | (−) |
| 13      |        |     |         |          |           | ICU     | BAL | POS | A. fumigatus | POS | POS | (+) | (+) | (+) | (+) | (+) | (+) | (−) |
| Patient | Gender | Age  | EORTC | Disease          | Hospital | Sample | Microscopy | Culture | GM | PF-PCR | MLST gel | SNaPPafu | A49_F | B129_F | L164_R | M15_R | All markers |
|---------|--------|------|-------|------------------|----------|--------|------------|---------|----|--------|----------|----------|--------|--------|--------|-------|-------------|
| 13      |        |      |       |                  | Medicine | BAL    | POS        | A. fumigatus | POS | POS   | (-)      | NEG      | (-)    | (-)    | (-)    | (-)    | (-)         |
| 13      | M      | 47   |       | Probable | Pulmonary infection | ICU     | BAL    | POS        | NEG     | POS   | (+)      | POS      | (+)     | (-)    | (+)    | (+)    | (+)         |
| 15      | M      | 52   |       | Probable | Pneumonia | ICU     | BAL    | POS        | NEG     | POS   | (-)      | NEG      | (-)     | (-)    | (-)    | (-)    | (-)         |
| 16      | M      | 68   |       | Proven | Pneumonia | Lung biopsy | BS    | POS        | NEG     | nm    | POS      | (+)   | (-)    | (+)    | (+)    | (+)         |
| 17      | F      | 32   |       | Possible | Pneumonia | ICU     | BAL    | NEG        | Candida sp. | NEG | POS    | (-)      | POS      | (+)    | (-)    | (+)    | (-)    | (-)         |
| 18      | F      | 49   |       | Probable | Pneumonia | Pneumology | BS    | POS        | A. fumigatus, Candida sp. | POS | POS   | (-)      | NEG      | (-)    | (-)    | (-)    | (-)    | (-)         |
| 19      | F      | 47   |       | Possible | Pneumonia | Transplant | BAL   | NEG        | A. fumigatus | nm  | POS    | (-)      | POS      | (+)    | (-)    | (-)    | (-)    | (-)         |
| 19      |        |      |       |                  | Transplant | BAL    | POS        | C. albicans | POS  | POS    | (-)      | POS      | (+)    | (+)    | (+)    | (+)    | (+)         |
| 20      | M      | 69   |       | Probable | Pneumonia | Medicine | BAL   | NEG        | NEG     | POS   | (-)      | POS      | (+)     | (+)    | (+)    | (+)    | (+)         |
| 21      | M      | 73   |       | Proven | Aspergilloma | Medicine | Lung biopsy | A. fumigatus | nm  | POS    | (-)      | POS      | (+)    | (+)    | (+)    | (+)    | (+)         |

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analyses were performed using Arlequin software v.3.0 (http://cmpg.unibe.ch/software/arlequin3/).

Results

Multiplex for amplification of MLST sequences

The MLST sequences served as basis for SNaPf assay, as the same gene fragments were targeted. To this aim we designed a new set of primers for MLST amplification in order to get MLST genes in a single multiplex reaction (see Figure 1). An optimal annealing temperature of 61°C was determined by performing gradient PCR (data not shown). The PCR multiplex co-amplified seven fragments, corresponding to the MLST housekeeping genes, with sizes ranging from 311 bp to 826 bp for all A. fumigatus strains.

SNaPf assay

For this aim, sequences generated with the MLST assay were compared with online MLST data. We created an alignment for the seven genes. On the basis of this alignment we identified 61 polymorphic positions as possible targets for the new molecular assay. Non-polymorphic positions were eliminated, as well as redundant polymorphism positions, and a final set of 20 SNPs was selected. SNPs were selected for the SNaPf assay based on their differentiation power. Primers were tested independently in the A. fumigatus reference strain to confirm its specificity (a single peak was observed for each primer confirming the mini-sequencing reaction). Comparing the sequence types distinguishable by MLST versus SNaP profiles obtained by SNaPf assay, two MLST profiles (ST14 and ST27) could not be separated; markers for those profiles were not included in our panel due to primers hairpin formation.

SNaPf assay was tested on 113 clinical and environmental strains of A. fumigatus, as well as on the non-Aspergillus fumigatus set of clinical relevant fungi. SNaPf assay was successfully tested in all A. fumigatus strains, while in non-fumigatus strains failed to provide the complete set of 20 markers. Interestingly, a few markers could still be identified in strains belonging to section Fumigati: a) B129_F and M15_R in all strains from the section Fumigati, b) C193_R in A. lentulus, A. viridinutans, and N. fischeri, c) C540_R in A. viridinutans, N. fischeri, and N. udagawae, and d) C403_R and L164_R in N. fischeri. Strains outside section Fumigati did not show the presence of a single marker (Table S1).

The final electropherogram for each A. fumigatus strain was converted into a SNaP profile (Figure 2). The SNaP profile represented the group of 20 polymorphisms obtained for each isolate (profiles were converted in accordance to MLST profiles to facilitate comparison of results, as shown in Figure 2). A set of 62 SNaP profiles was generated from our population of 113 strains; approximately 70% of the SNaP profiles were unique in the tested population of A. fumigatus. Few polymorphic positions included in our panel were exclusively described in the strain profiles available at MLST database (positions 129 of BGT1 and 403 of CAT1), while others were uniquely described in our strains set (positions 392 of ANCX4, 183 and 540 of CAT1, 164 and 456 of LIP, and 329 of SODB). Strains with similar SNaP profile were re-sequenced by MLST scheme in order to assess the location and number of polymorphisms that could not be detected by SNaPf assay. A few polymorphisms were found in a small group of strains, mainly in the genes ANCX4 and CAT1. Novel polymorphisms were uncommon and detected in strains easily differentiated by microsatellites. Those polymorphisms were not included later in the SNaPf assay, as they did not add new major discriminatory information.

For this newly developed method, a diversity index value of 0.99 was achieved when compared to MLST. These strains have been previously genotyped by a single microsatellite-based multiplex PCR [8,9]; furthermore, D was recalculated for the arrangement of both genotyping methods (SNaPf plus microsatellite multiplex) offering a combined discriminatory power of 0.9998.

Application of SNaPf on clinical samples

The detection limit of SNaPf assay was tested for two clinical strains. Serial dilutions of conidia were conducted as previously described [20] using ultrapure water (details in Table S2). The set of 20 markers was observed to be constantly obtained in samples with at least 10 A. fumigatus ITS copies/mL. Below this limit few markers failed. The value of 1 ITS copies/mL defined the detection limit of SNaPf assay as more than 5 markers can still be detected.

Clinical samples from 37 patients with possible/probable/proven according to the Aspergillus infection EORTC/MSG

Figure 1. Silver stained polyacrilamide gel showing the band patterns of MLST multiplex amplification. (AF1 to AF11 are A. fumigatus strains from Portuguese collection; reference A. fumigatus ATCC 46645 was added). doi:10.1371/journal.pone.0075968.g001
criteria were tested with SNaPfu assay. Marker A49_F, confirmed above to be specific for A. fumigatus, was observed in 33 of 37 clinical samples (89%) confirming once again its ability to detect the present of this species (Table 3). Complete SNaPfu profiles were observed in six samples (total DNA content ranging from 5 ng/µL to 375 ng/µL; among those were one lung biopsy and five BAL samples) retrieved from patients suffering from probable or proven aspergillosis according to the EORTC/MSG criteria (see Table 3). The positivity of direct microscopy and the other diagnostic tests already indicated the high fungal burden present in some samples, reinforced by the presence of highly marked bands on MLST electrophoresis. The results of direct microscopy, culture and GM showed agreement values of 62%, 46%, and 67%, respectively, with SNaPfu results, similarly to pan-fungal PCR. Samples with efficient amplification of MLST genes and showing intense bands in MLST electrophoresis resulted in clearer electropherograms and better correlation with SNaPfu results (Table 3). The samples with lower fungal DNA amount showed less intense bands or absence of bands in MLST electrophoresis and, therefore, only a few peaks (from 2 to 6) were observed on the final electropherogram. Markers B129_F, L_164R, and M15_R were commonly observed in clinical samples (were detected in around 70% of the samples), however, these markers were identified above in species from section Fumigati besides A. fumigatus.

Discussion

The new SNaPfu assay, an innovative method proficient to detect, identify and genotype A. fumigatus was established. This strategy of this assay is to target the most relevant polymorphisms present in MLST alignments. Instead of sequencing gene sequences like MLST, in the new SNaPfu assay only SNPs are targeted, amplified in a multiplex reaction and subsequent analyzed by mini-sequence reaction. MLST has major advantages as described earlier [7]; disadvantages are undoubtedly related with the final cost (over €50 per sample), and the extended time needed to apply the method to large collections of strains. SNaPfu assay offers informative data in a single amplification and mini-sequencing reaction. The SNaPfu assay requires less than a working day (8 h) to obtain results from clinical samples and estimated cost per sample is less than €10 (depending on supplier). This innovative method can detect the presence of single or multiple A. fumigatus strains in the DNA extracts of clinical specimens, a pioneering aspect regarding molecular tools with diagnostic purposes, especially in clinical samples’ studies such as...
those related to IA. Although ITS is currently recognized as the primary barcoding marker [24], it presents some limitations for identification of closely related species [25]. MLST genes are capable of reliable identification of several microorganisms and accumulate sufficient variability to genotype microbial isolates [13,26–28].

The ability of SNaPfu assay to detect the presence of A. fumigatus was confirmed on clinical samples. The specific molecular marker A49_F showed a great sensitivity (89%) in clinical samples. Some other markers included in the assay showed less sensitivity (60–70%) to detect A. fumigatus, but the presence of several markers in the multiplex platform guarantees the presence of some peaks in the final electropherogram, even if a very small amount of DNA is present in the extract gained from bronchoalveolar or biopsy materials. SNaPfu assay showed high sensitivity (89%) and good correlation with GM values (67%), similarly to previously described for other molecular assays [29,30].

The presence of a non-fumigatus Aspergillus species that belongs to section Fumigati can also be suggested by the present methodology. Six markers (B129_F, C193_R, C403_R, C540_R, L164_R, and M15_R) were detected in species from section Fumigati, while the remaining markers were specific for A. fumigatus. The presence of such markers and the absence of specific A. fumigatus markers may suggest the presence of a rare species belonging to section Fumigati, some of them clinically very relevant and even resistant to the commonly used antifungal compounds [12,13,15,31]. However, this ability to detect and confirm the presence of other species from section Fumigati still needs to be clarified. These species are rare and its genetic diversity is still poorly known. A higher number of species and strains need to be tested to confirm the persistent presence/absence of those molecular markers in their genome. No markers could be detected in other Aspergillus species.

The availability of molecular multiplexes for detection of several fungi has been reported of greater clinical interest, and it might be of major importance regarding molecular diagnosis approaches. An ideal molecular panel would be converted into an assay applicable to clinically complex samples and comprising a pool of markers suitable not only for detection, but also for identification and general overview of microbial population structure (if possible available through free online platforms). The novel SNaPfu may be used as a feasible, reliable and forceful alternative regarding this matter.

Major limitations of the present assay are related to the difficulties to detect some markers in clinical samples. A complete panel with 20 peaks was observed only in samples with high DNA quality and quantity. As it has already been reported [32], the efficiency of the Aspergillus PCR is limited by the extraction procedure, such as low recovery of fungal DNA complemented with bacterial and human DNAs and/or presence of PCR inhibitors, and not by PCR amplification itself. The usage of molecular assays in such samples results in the loss of some genotyping power and the ability to detect multiple strains. Further inclusion or replacement of specific and well-studied markers may improve this feature. As previously stated, our method had a detection limit of 0.5 fg genomic Aspergillus DNA/mL, which is similar to 1 ITS copy/mL. This assay was not tested in blood and/or serum samples. Some limitations can be faced in such samples as the presence of PCR inhibitors and the DNA concentration can be below the detection limit observed in this study. Biopsies and BAL samples used in this work are currently very important clinical samples to establish proven invasive infections [21]. Improvements that could lead to the application of this tool in such samples would be of great value to clinical laboratories.

One of the major benefits of this assay is the possibility to reach fungal diagnosis, A. fumigatus identification and genotype in a single reaction. A clearer and deeper understanding of the A. fumigatus epidemiology and routes of transmission is essential to develop coherent and effective control measures to prevent contact of critical patients. Employment of reliable genotyping methods becomes crucial for understanding the large genetic diversity of A. fumigatus [8–10,23,33], monitoring clinical outbreaks caused by A. fumigatus [34,35] and identifying contamination sources in hospitals. MLST is the current “gold standard” method for microorganism genotyping; SNaPfu assay herein described exhibits similar discrimination power with great improvements in practicability and cost. The inherent plasticity of the current assay allows the addition of these and other markers to the panel able to provide relevant information concerning the studied fungal strains (e.g. pathogenicity, susceptibility, mating-type, or population genetics of specific populations). Such information coded by SNPs can easily be managed and included in the present panel.

Molecular methods are efficient to detect multiple strains in daily clinical samples and assess the efficacy of antifungal treatments [9,36–38]. We strongly believe that SNaPfu comprises a great potential for clinical routine examination launching a new generation of diagnostic molecular tools. It represents a fast, standardized, inexpensive and interactive platform for detection, identification and population analysis of A. fumigatus in clinical specimens.

Supporting Information

Table S1 Results of SNaPfu assay tested in Aspergillus species.

Table S2 The detection limit of SNaPfu assay determined by testing serial dilutions of A. fumigatus conidia.

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Author Contributions

Conceived and designed the experiments: LG AA RA. Performed the experiments: RC ML RA. Analyzed the data: RC LG ML RA. Contributed reagents/materials/analysis tools: ML AA RA. Wrote the paper: RC LG ML AA RA.

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