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# Simple and highly discriminatory microsatellite-based multiplex PCR for *Aspergillus fumigatus* strain typing

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## Abstract

The answers to important questions concerning *Aspergillus fumigatus* pathogenicity, transmissions routes and efficacy of treatments require highly discriminating and reproducible genotyping methods. The present study was aimed at improving microsatellite methodology for *A. fumigatus* typing by reducing the task of strain identification to a single multiplex reaction and by selecting highly accurate short tandem repeat polymorphisms. A set of eight primer pairs was used for the genotype determination of 116 clinical isolates of *A. fumigatus* obtained from three healthcare centres. A new, automated and highly discriminatory typing method is described for *A. fumigatus* strains. The optimized multiplex PCR was successfully performed with all tested clinical strains and showed a discriminatory power of 0.9997 among presumably unrelated isolates. The comparison of groups of strains from different health centres showed that 99.6% of the genotypic variation was present within groups. Strains with the same genotype were isolated from the same patient, sometimes recovered more than 1 year later. A few cases of patients at the same clinic unit carrying strains of identical genotype strongly suggested colonization by *A. fumigatus* during their hospitalization. Specific measures must therefore be taken in order to prevent and restrict such incidents.

**Keywords:** *Aspergillus fumigatus*, *Aspergillus* genome, invasive aspergillosis, molecular typing, nosocomial infection, STR, tandem repeat
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## Introduction

Invasive aspergillosis remains one of the most common fungal infections among immunocompromised patients. *Aspergillus fumigatus* is responsible for almost all cases and results in a high mortality rate among patients even after administration of antifungal therapy [1,2]. Although *A. fumigatus* causing nosocomial infection may come from diverse sources, airborne conidia are considered to be the most relevant [2,3].

Multiple genotyping methods have been described for *A. fumigatus*, e.g. random amplified polymorphic DNA [4–6], amplified fragment length polymorphism analysis [4,7,8], microsatellite typing [7,8,10–13], repetitive sequence-based PCR [14] and multilocus sequence typing [15]. All these methods have very distinct discriminatory power and reproducibility. Microsatellite typing has been applied for a long time for the discrimination among, and recognition of, microbial agents or populations [16,17], and is also widely used in human forensics [18]. More recently, this method was applied to *A. fumigatus*, and showed higher discriminatory power than other typing methods [8,10,11,15]. Important questions concerning *A. fumigatus* pathogenicity, transmission routes and efficacy of treatments of infections due to this fungus may be answered if this highly discriminatory and reproducible method is used widely [17].

The present study was aimed at improving microsatellite methodology for typing *A. fumigatus* isolates, particularly by reducing the task of strain identification to a single multiplex reaction and by selecting the most accurate short tandem repeat (STR) motifs (preferentially trinucleotides, tetranucleotides and pentanucleotides), which minimize typing ambiguities and facilitate interlaboratory comparison of the results. This multiplex procedure proved to be a practical and accurate method that may be extremely useful in providing a better understanding of the transmission of *A. fumigatus*

infections and their acquisition by susceptible patients in clinical units and in the community.

## Materials and methods

### *A. fumigatus* strains and DNA extraction

In total, 116 clinical isolates of *A. fumigatus*, obtained from two university hospitals, Hospital S. João (100 isolates) and Hospital S. António (five isolates), and an oncological hospital (nine isolates), all located in Porto, Portugal, and two reference strains (ATCC 46645 and MYA 772) were used in this study.

Most clinical isolates were collected from the lower respiratory tract and from surgical wounds during the last 5 years (2003–2008) and identified on the basis of macroscopic and microscopic morphological characteristics and standard mycological procedures. Two strains of each of the following species, *Aspergillus flavus*, *Aspergillus niger*, *Aspergillus nidulans*, *Aspergillus terreus*, *Aspergillus glaucus* and *Aspergillus versicolor*, were used as controls. The isolates tested were obtained from primary cultures, from which, in each case, a single colony was selected. Prior to DNA isolation, moulds were grown for 5 days on Sabouraud dextrose agar slants (Difco, Detroit, MI, USA) at 30°C. DNA was extracted from conidia with a method using sodium hydroxide, as previously described [19]. DNA (50–250 ng) was suspended in 50 μL of sterile water and stored at −20°C.

### Microsatellite selection and PCR primer design

A search in the *A. fumigatus* genome sequences, available in the databases from the US National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/sites/entrez/), was performed in order to identify sequences with microsatellite repeats. Five microsatellites were selected from a previous report [11]. The search was limited to trinucleotide, tetranucleotide and pentanucleotide motifs with at least eight simple and complete units located in distinct chromosomes. The sequences that were identified and selected for locus-specific amplification are given in detail in Table 1. The name of each marker indicates the chromosome on which it is located (e.g. MC3 denotes a microsatellite located on chromosome 3). Primers for each locus were designed by using the software Primer3, available at http://frodo.wi.mit.edu.

### PCR amplification and DNA sequencing conditions

*Singleplex amplification.* For all microsatellite loci, singleplex PCRs were performed with several different strains in order to evaluate locus amplification specificity and to obtain PCR-amplified alleles for sequence analysis. The PCRs were performed using 1 μL of genomic DNA (1–5 ng/μL), 2.5 μL of Qiagen multiplex PCR master mix (Qiagen, Hilden, Germany) and 0.5 μL of each primer (final concentration of each primer: 0.2 μM), in a final volume of 5 μL. After a 95°C pre-incubation step of 15 min, PCRs were performed for a total of 30 cycles, using the following conditions: denaturation at 94°C for 30 s, annealing at 60°C for 90 s and extension at

**TABLE 1. Primers, consensus repeat structures and discriminatory power of microsatellite markers**

| Marker | Primer[a] | Short tandem repeat[a] | DP value | Ref.[b] |
|---|---|---|---|---|
| MC3 | Forward: *VIC*-GTACACAAAGGGTGGGATGG<br>Reverse: ATCGAGGTTACATGGCTTGG | Primer—17 bp—$(TAC)_{6–63}$—10 bp—primer | 0.935 | STR*Af*3C |
| MC1 | Forward: *VIC*-GCCGTTTTGGTAGTGGACAT<br>Reverse: ATGGGTCAGATCGTGCTTCTC | Primer—92 bp—$(AAAAG)_{5–18}$ $(GGA)_{0,1}$ $(AAAAG)_{0,2}$—129 bp—primer | 0.877 | New |
| MC8 | Forward: *NED*-CGATGATTTAGCACCTTGCTT<br>Reverse: GGGAAACCCACTCGGAAT | Primer—1 bp—$(CATA)_{5–34}$—7 bp – $(ACATCCAT)_{0,1}$—56 bp—primer | 0.826 | STR*Af*4C |
| MC5 | Forward: *NED*-GCCACATTCTCGCTTCTTTC<br>Reverse: AAGGACAGGATGGACGAAGA | Primer—137 bp—$(TTTAT)_{5–26}$ $((TWY/-AT)^{c}$ $(TTTAT)_{3,4,7})_{0–2}$ $(TTAT)_{2,3,5}$—TTCTTTAT[d]—79 bp—primer | 0.905 | New |
| MC2 | Forward: *PET*-GCCCTCTTCCGTTATTCCTT<br>Reverse: GCGCATTGATAGCTACCTCA | Primer—30 bp—$(AAAG)_{0,2}$ $(AAAA)_{0,1}$ $(AAAG)_{7–23}$—15 bp—primer | 0.838 | New |
| MC6a | Forward: *PET*-CCACGTCGTACCTCGATCTT<br>Reverse: AACTCAACTCTCGCCCATGT | Primer—144 bp[e]—$(AAG)_{8–38}$—66 bp[f]—primer | 0.842 | STR*Af*3B |
| MC7 | Forward: *6FAM*-GTACGACTCTCGGTTCTAAGACAA<br>Reverse: TGAGCCTTCACTAAGAGATCATTG | Primer—4 bp—$(TAGA)_{5–26}$—24 bp—primer | 0.810 | STR*Af*4B |
| MC6b | Forward: *6FAM*-AAGCAGACGAGGAATTGACG<br>Reverse: CGGTGAAGACAAGCACAGAA | Primer—7 bp—$(GAAA)_{0,2}$ $(GGAA)_{0,1}$ $(GAAA)_{4–25}$—32 bp $(A/-)^{g}$ 116 bp—primer | 0.868 | STR*Af*4A |

DP, discriminatory power.
[a]All primers and short tandem repeats are given from 5′ to 3′.
[b]Name of short tandem repeat according to de Valk *et al.* [11]; only MC3 primers were similar to the primers reported by de Valk *et al.* [11].
[c]The sequences TATAT, TTCAT, TACAT or TTAT were found.
[d]The sequence TTCTTTAT appears as a duplicated sequence in the published complete genome sequence of *A. fumigatus* but it was found to be a single sequence in all strains analysed here.
[e]The published sequence TGT, upstream from the repeat, was not found in all sequenced strains.
[f]The second nucleotide downstream from the repeat was C in all sequenced strains, while the published complete genome sequence of *A. fumigatus* showed T.
[g]Indel of A.

72°C for 1 min, with a final extension step of 10 min at 72°C.

*Multiplex amplification.* Multiplex PCRs were performed similarly to singleplex amplifications, but using 0.5 μL of a mix with eight pairs of primers (final concentration of each primer: 0.2 μM). PCR conditions were similar to those previously described for singleplex PCR. The internal size standard GeneScan 500 LIZ (Applied Biosystems, Foster City, CA, USA) (0.5 μL) and HiDi formamide (Applied Biosystems) (12 μL) were added to the PCR-amplified products and run in an ABI PRISM 3100 genetic analyser 16-capillary electrophoresis system (Applied Biosystems). Fragment size and allele determinations were performed automatically, using Genemapper software 4.0 (Applied Biosystems) and by comparison with allelic sequence ladders including the most frequent alleles for each marker. The alleles were designated by the number of repeat units determined after sequencing, and partial repeats were designated by the complete repeat followed by a decimal point and the number of additional bases [20].

*Sequencing.* Several alleles at each locus were sequenced. The PCR-generated fragments were purified on Microspin S-300 HR columns (Amersham Pharmacia Biotech, Quebec, Canada). The sequencing reaction included a 95°C pre-incubation step of 2 min, PCR for 35 cycles with denaturation at 96°C for 15 s and annealing at 50°C for 9 s, and then 60°C for 2 min and 60°C for 10 min. Sequencing reactions were carried out using the ABI Big Dye terminator cycle sequencing ready reaction kit (Applied Biosystems). The products were purified using AutoSeqG-50 columns (Amersham Pharmacia Biotech). The DNA fragments were dissolved in 8 μL of HiDi formamide and separated using an ABI PRISM 3100 genetic analyser 16-capillary electrophoresis system. The results were analysed using Sequencing 5.2 analysis software (Applied Biosystems).

### Reproducibility and stability

The reproducibility of the method and the stability of the microsatellite markers were assessed following 75 successive cultures on Sabouraud agar slants (i.e. conidia → vegetative mycelia → conidia, repeated 75 times) of two randomly selected strains and, additionally, the reference strain ATCC 46645. The reproducibility of the method was also evaluated by comparing the results obtained using the singleplex and the multiplex PCR procedures.

### Statistical analysis

Genotype frequencies were estimated by genotype counting of all presumably unrelated isolates (collected from different patients; different genotypes of isolates from a single patient were considered). Cluster analysis was performed using the NCSS 2001 program (NCSS, Kaysville, UT, USA) and Euclidean distances (without rescaling of the data) with the linkage-type unweighted pair group method (group average) and cluster cut-off of 1.0. Statistical analysis for pairwise linkage disequilibrium was performed by an exact test using a Markov chain (chain length of 10 000 and dememorization of 1000), and the statistical software package Arlequin 3.1 (http://cmpg.unibe.ch/software/arlequin3/). Genotype diversity (discriminatory power) analysis of molecular variance (AMOVA) and fixation indices were also tested using Arlequin software.

## Results

### Multiplex specificity

The results obtained with the singleplex and the multiplex PCRs were similar. A graph with eight distinct peaks, each one corresponding to a marker, resulted from the multiplex PCR defining the genotype for each isolate. The multiplex PCR was successfully performed with all tested clinical strains. Cases of mixed strains, resulting in more than a single peak for each marker, were not found. *Aspergillus* isolates other than *A. fumigatus* did not yield amplicons of any of the tested STRs, confirming the *A. fumigatus* specificity of these microsatellite markers. Additionally, the genotypes were found to be identical after 75 successive cultures of three strains, including the reference strain *A. fumigatus* ATCC 46645. Thus, these genomic regions were considered to be fairly stable and the particular markers to be reliable for use in genotyping studies.

### Genotype diversity

The diversity indexes for each marker are shown in Table 1. The multiplex PCR showed a discriminatory power of 0.9997 (standard deviation of 0.0017), taking into account all the presumably unrelated isolates (91 strains were considered, as shown in Fig. 1). Moreover, MC3 was the most discriminating marker (31 different alleles), followed by MC5 (21 alleles), and MC6a and MC6b (17 alleles), whereas MC7 was a less discriminating marker, with 11 different alleles, followed by MC1 and MC2 (14 alleles) and MC8 (16 alleles). The new marker MC5 proved to be the second most discriminatory, whereas MC1 and MC2 (also new markers) were least useful in this dataset. A significant departure from pairwise linkage disequilibrium was found ($p < 0.05$), considering unrelated strains for most pairs of markers, with the exception of the pairs of markers MC3–MC8, MC3–MC2

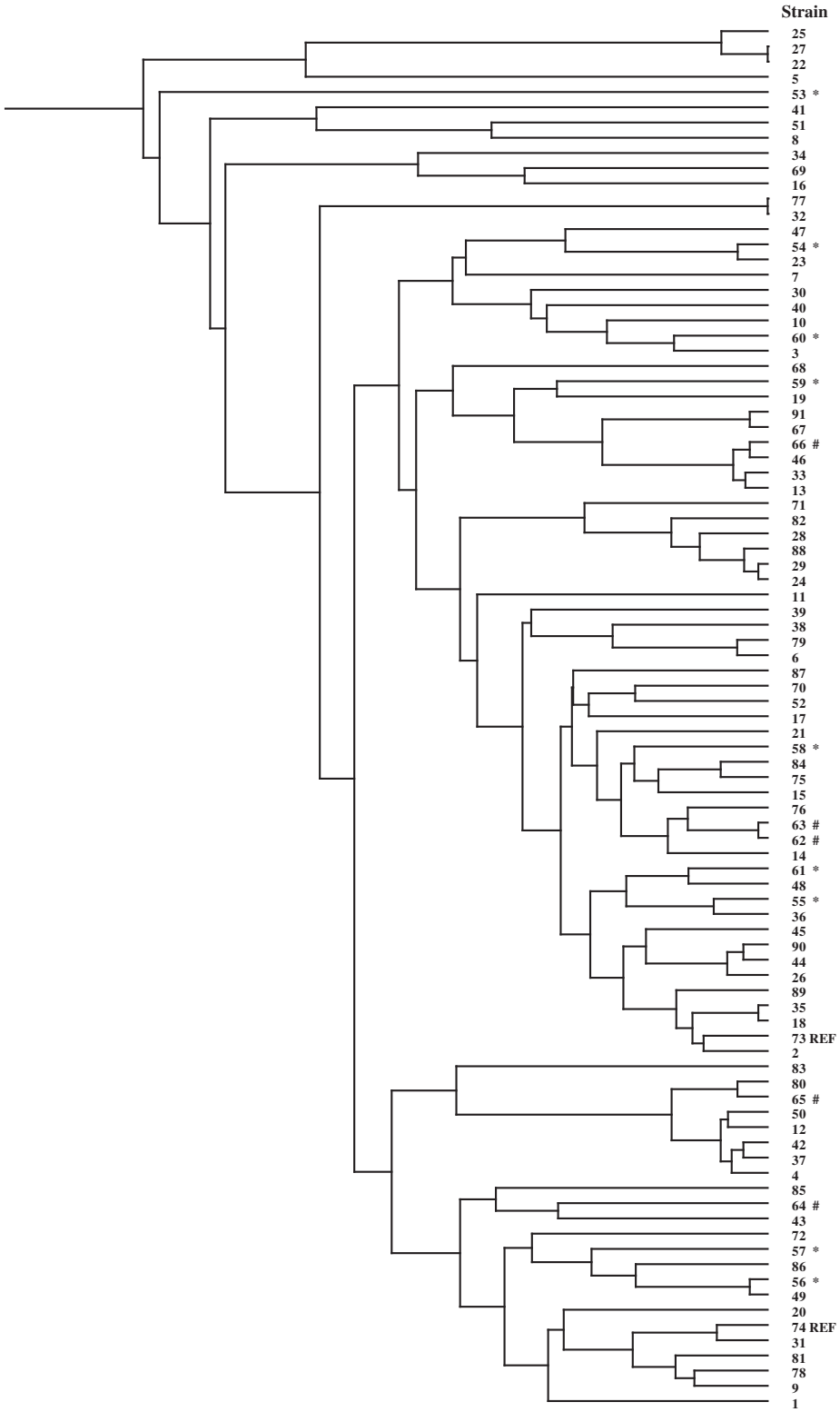


**FIG. 1.** Dendogram of dissimilarity among the 91 presumably unrelated strains. The cluster analysis was performed with NCSS 2001 software using Euclidean distances, without rescaling of the data and the linkage-type unweighted pair group method (group average). REF: reference strains; *: strains from patients at the oncological hospital; #: strains from patients at Hospital S. António. All the other strains were from patients at Hospital S. João.

and MC1–MC8. The comparison of groups of strains from different healthcare centres (two university hospitals and one oncological hospital), using the AMOVA test, showed that there were no significant differences among the groups and that 99.6% of the genotypic variation was intrapopulational. Fixation indices among populations were not significant at the single-locus level (data not shown).

### Genotype persistence in the same patient

Table 2 shows the details of all strains found in this study that were similar to each other.

Almost all such similar isolates were recovered from the same patient and collected on the same day or a few days apart; sometimes, isolates of the same strain were recovered more than a year apart (e.g. isolates of strains 39, 79 and 82).

However, it was also possible to find different strains in the same patient, e.g. strains 39 and 79 (patient O), and strains 48 and 91 (patient S), as shown in Table 2. Interestingly, two strains (25 and 27; shown in Fig. 1) that were recovered from the same patient differed with respect to just a single marker (MC8), suggesting a micro-evolution event. The observation of other cases of micro-evolution might be anticipated, because some of the strains were closely related and differed from other genotypes in a single marker and in only a few repeat units (Fig. 1).

**TABLE 2. Origins of the strains of identical genotype among 116 clinical isolates of *Aspergillus fumigatus* studied (strain numbers are those shown in Fig. 1)**

| Strain | Patient | Unit | Date |
|---|---|---|---|
| 9 | A | Intensive care | September 2003 |
| | A | Intensive care | September 2003 |
| 12 | B | Emergency care | September 2003 |
| | B | Emergency care | September 2003 |
| | B | Emergency care | October 2003 |
| 14 | C | Neurosurgery | November 2003 |
| | C | Neurosurgery | November 2003 |
| 15 | D | Medicine | December 2003 |
| | D | Medicine | December 2003 |
| 18 | E | Pneumology | August 2005 |
| | E | Pneumology | April 2006 |
| 19 | F | Haematology | June 2004 |
| | F | Haematology | June 2004 |
| 22 (=27) | G | Pneumology | July 2005 |
| | H | Intensive care | August 2005 |
| 32 (=77) | I | Intensive care | July 2005 |
| | J | Intensive care | July 2005 |
| | K | Intensive care | July 2005 |
| | L | Emergency care | July 2005 |
| 81 | M | Surgery | March 2006 |
| | M | Surgery | March 2006 |
| 33 | N | Infectious diseases | December 2005 |
| | N | Infectious diseases | December 2005 |
| | N | Infectious diseases | December 2005 |
| | N | Infectious diseases | December 2005 |
| 39 | O | Paediatrics | November 2004 |
| | O | Paediatrics | January 2008 |
| 47 | P | Emergency care | January 2005 |
| | Q | Emergency care | April 2005 |
| | R | Emergency care | April 2005 |
| 48 | S | Paediatrics | January 2005 |
| | S | Paediatrics | January 2005 |
| 52 | T | Emergency care | May 2005 |
| | T | Emergency care | May 2005 |
| 65[a] | U | HSA | April 2004 |
| | U | HSA | April 2004 |
| 66[a] | V | HSA | April 2004 |
| | V | HSA | April 2004 |
| 78 | W | Infectious diseases | January 2006 |
| | W | Infectious diseases | January 2006 |
| 79 | O | Paediatrics | January 2005 |
| | O | Paediatrics | July 2005 |
| | O | Paediatrics | March 2006 |
| | O | Paediatrics | January 2008 |
| 82 | Y | Paediatrics | January 2005 |
| | Y | Paediatrics | April 2006 |
| 91 | S | Paediatrics | March 2005 |
| | S | Paediatrics | March 2005 |
| | S | Paediatrics | March 2005 |

[a]Strains from patients admitted at Hospital S. António (HSA). All the other strains were from patients admitted at Hospital S. João.

### Identical genotypes in strains isolated from different patients

Some strains collected from several patients admitted to the same hospital unit showed the same genotype, e.g. strain 32 (from patients I, J and K; intensive care) and strain 47 (from patients P, Q and R; emergency care) (Table 2). Although these situations were not very frequent, they strongly suggest that the strains were acquired by patients during admittance to the respective unit. There were also two groups of strains (22 and 27; and 32 and 77) that were recovered from distinct patients admitted to distinct clinical units but located at the same hospital and with very close admission dates. The patients with identical strains were not neutropenic (only a discrete leucopenia was seen in patients H and Q, with a total white blood cell count between 3500 and 4000/μL).

## Discussion

Different genotyping methods have been developed and employed in order to better evaluate and recognize nosocomial infections, but also to help in their prevention. Microsatellite typing was previously suggested as the best method for *A. fumigatus* typing, showing high discriminatory power and excellent reproducibility [11]. However, microsatellite methodology for *A. fumigatus* typing could be substantially improved, particularly by limiting PCR to a single reaction and avoiding the use of dinucleotide repeat motifs, which are more prone to lead to DNA polymerase slippage during amplification [16].

The new method for *A. fumigatus* STR typing described here showed a very high discriminatory power and overcame some of the previously encountered methodological difficulties of microsatellite genotyping. The reported lack of consistency between the results from different laboratories [21]

was easily compensated for by the construction of allelic ladders for each of the tested markers.

Thus, the method can be used for diverse studies, particularly those concerning genetic population diversity and the evaluation of routes of infection and outbreaks, as previously proposed [10,16,17].

*A. fumigatus* strains have always shown great genetic diversity, even when different typing methods were used [4,6,9,11,13]. Genotypically similar strains have rarely been found, particularly when methods with high discriminatory power have been employed [8,9,11]. The reliable detection of genotypically similar strains at several sites or in several biological products of the same patient, or of different patients, is of high medical relevance, particularly for a better understanding of the dissemination of the agent, for the improvement of the efficacy of treatment, and even for the prevention of outbreaks in clinical units.

The large group of strains included in this study confirmed the high genetic diversity of the *A. fumigatus* population. As observed similarly in previous studies [4,6,9,11,15], most of the strains presented a distinct genotype. In particular cases, strains of identical genotype were found in samples from the same patient. Interestingly, in some patients the same strain persisted for several years. Further attention should be paid to this possibility in order to understand in which set of patients persistence of *A. fumigatus* strains may be expected and to prevent its occurrence. Micro-evolution or microvariation may result from adaptation to harsh environments that involves minor genotype changes, usually in a single marker [10,16].

The case of the strains showing a single difference in marker MC8 may be described as one of micro-evolution. Furthermore, it was possible to identify distinct *A. fumigatus* genotypes in strains from the same patient, as previously described [6,13], and this fact calls for additional care to be taken during the evaluation of antifungal susceptibility. Although *A. fumigatus* is usually susceptible to antifungal agents [22,23], a few strains with reduced susceptibility have been identified [23], and it should be considered that strains with distinct susceptibility patterns may be simultaneously found in the same patient.

Outbreaks due to particular *Aspergillus* strains have been increasingly reported [5,10,12]. The employment of highly discriminating typing methods may avoid false results and help in locating the origin of outbreak episodes. Several cases of patients harbouring one and the same *A. fumigatus* strain are reported here, suggesting patient colonization during admission to the hospital. Clinical units should take action in order to restrict, if not prevent, similar incidents. Greater attention should be given to quality control programmes, particularly in units admitting critical-care and immunosuppressed patients.

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