Comparison of multiple typing methods for *Aspergillus fumigatus*

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ABSTRACT

As part of studies on the spread of infections, risk factors and prevention, several typing methods were developed to investigate the epidemiology of *Aspergillus fumigatus*. In the present study, 52 clinical isolates of *Aspergillus fumigatus* from 12 airway specimens from patients with invasive aspergillosis (hospitalized in three different centers) were typed by variable number of short tandem repeat (VNTR) typing and multilocus sequence typing (MLST). These isolates were previously typed by random amplified polymorfic DNA (RAPD), sequence-specific DNA polymorphism (SSDP), microsatellite polymorphism (MSP) and multilocus enzyme electrophoresis (MLEE). VNTR typing identified 30 genotypes and, for most patients all isolates were grouped in one cluster of the unweighted pair group method with arithmetic mean (UPGMA) dendrogram. Using MLST, only 16 genotypes were identified among 50 isolates, while two isolates appeared untypeable. RAPD, MSP, SSDP and MLEE identified 8, 14, 9 and 8 genotypes, respectively. Combining the results of these methods led to the delineation of 25 genotypes and a similar clustering pattern as with VNTR typing. In general, VNTR typing led to the same results as the combination of RAPD, SSDP, MSP and MLEE but had a higher resolution while MLST was less discriminatory and resulted in a totally different clustering pattern. Therefore, this study suggests the use of VNTR typing for research on the local epidemiology of *Aspergillus fumigatus*, which requires a high discriminatory power.

Key words: *Aspergillus fumigatus*, typing, variable number of short tandem repeat (VNTR) typing, multilocus sequence typing (MLST)

INTRODUCTION

Aspergillus fumigatus is an ubiquitous, saprophytic mold causing diseases such as invasive aspergillosis (IA), aspergilloma and alllergic bronchopulmonary aspergillosis. IA is currently the most important mold infection posing a threat to immunocompromised patients [1, 2]. *Aspergillus* species are also able to cause a number of other diseases in immunocompetent individuals [3] and hypersensitivity reactions to *A. fumigatus* are frequently occurring in patients with cystic fibrosis and asthma [4, 5, 6].

In order to study the epidemiology of *A. fumigatus*, multiple typing methods were developed. These methods can be based on phenotypic characteristics (e. g. enzyme patterns and antigenic profiles) or can directly or indirectly analyse differences in DNA sequences. Because of their technical complexity, poor reproducibility and/or low discriminatory power, the use of phenotype-based methods is decreasing [7]. Additionally, a given phenotype does not always accurately reflect the genotype of a micro-organism and may therefore not provide a reliable and stable epidemiological marker [8]. Several genotype-based methods such as randomly amplified polymorphic DNA (RAPD), sequence-specific DNA polymorphism (SSDP), restriction fragment length polymorphism (RFLP) and amplified fragment length polymorphism (AFLP) were described for *A. fumigatus* [9, 10, 11, 12, 13, 14]. However, comparison between results obtained in different laboratories with these pattern-based methods is difficult due to poor reproducibility of the patterns [15, 16].

Based on the genome sequence of *A. fumigatus* [17], a multilocus sequence typing (MLST) scheme and a typing scheme based on variable-number of short tandem repeats (VNTR) were developed [18, 19]. MLST is based on the principles of multilocus enzyme electrophoresis (MLEE) but the alleles are assigned to each locus

directly by nucleotide sequencing of housekeeping genes, rather than indirectly through the electrophoretic mobility of the corresponding enzymes. MLST has a high discriminatory power but, at the same time, can be used for studies on global epidemiology owing to the relatively slow change of nucleotides in housekeeping genes [20, 21, 22, 23]. For VNTR typing, alleles are assigned based on length polymorphisms of microsatellite loci. A high discriminatory power can be achieved because these typing targets are highly variable [24, 25].

In the present study, 52 *A. fumigatus* isolates from patients with IA were typed with VNTR typing and MLST. Typeability, discriminatory power, reproducibility, time to result, ease of use and cost were assessed for VNTR typing and MLST and compared to results obtained previously for the same set of isolates with microsatellite polymorphism (MSP), MLEE, RAPD and SSDP [26].

MATERIALS AND METHODS

Isolates. *A. fumigatus* isolates were retrieved from 12 airway specimens from 12 IA patients, leading to a collection of 52 isolates conserved in the BCCM/IHEM Culture Collection of the Scientific Institute of Public Health (Brussels, Belgium) [26]. The patients were hospitalized in three different European medical centers (Grenoble, France; Lyon, France and Milan, Italy) where clinical, radiologic and mycologic investigations were performed to confirm the diagnosis of probable IA according to previously published criteria [1]. An overview of the isolates included in the present study is given in Table 1. We also subjected two *A. lentulus* strains (IHEM 22458 and IHEM 22459) to VNTR and MLST typing.

Identification of the isolates. Isolates were identified as *A. fumigatus* according to standard morphological criteria and their ability to grow at 48°C.

DNA isolation. Genomic DNA was prepared as described previously [27].

VNTR typing. For typing based on DNA repeats, a recently developed method was used [19]. Three dinucleotide loci, three trinucleotide loci and three tetranucleotide loci were amplified using three multiplex PCR's. Subsequently, a 1:50 dilution of the PCR products combined with the ROX400-HD size standard (Applied Biosystems) was analysed by capillary electrophoresis on an ABI3130xl sequencer (Applied Biosystems). Fragment sizes of the nine markers were calculated with Peakscanner (Applied Biosystems) and based on the fragment size a locus type was assigned. Combination of these types resulted in an overall genotype (designated by an arbitrary number) for each isolate. Dendrograms were constructed with START2 software [28] using the UPGMA algorithm.

MLST. A previously published MLST scheme based on the sequence of seven gene fragments [18] was used to type all isolates. Forward and reverse DNA sequence

chromatograms were analysed with DNAstar software to identify interstrain single-nucleotide polymorphisms (SNPs). Based on the sequences found for all seven gene fragments, a genotype was assigned in accordance with previously published results [18].

Statistical analyses. For each typing method, the discriminatory index (DI) was calculated by using Simpson's index of diversity [29]. The DI is the probability that two unrelated strains sampled from the test population will be placed into different typing groups and was calculated by the following equation:

$$DI = 1 - 1/[N(N - 1)] \sum_{j=1}^{s} n_j (n_j - 1)$$

in which N is the total number of isolates, s is the total number of types described and n_j is the number of isolates belonging to the jth type. Confidence intervals (CI, 95%) for each DI were calculated with a method described by Grundman et al. [30], applying the following formula:

$$CI = [DI - 2\sqrt{\sigma^2}, DI + 2\sqrt{\sigma^2}]$$

in which σ^2 is the variance with which the values of DI are distributed about the true diversity of a population based on a sample of N individuals. This variance can be estimated with the following equation:

$$\sigma^2 = 4/N [\sum_{j-1}^{s} \pi_j^3 - (\sum_{j-1}^{s} \pi_j^2)^2]$$

in which π_j is the frequency n_j/N.

RESULTS

VNTR typing. Reproducibility was checked by typing five isolates (IHEM numbers 9418, 9419, 9420 and 9600 included in the present study and IHEM 5141 included in a previous study [19]) in three independent experiments. The maximum difference between the fragment sizes obtained in the three assays was 0.15, 0.27 and 0.31 base pairs for the dinucleotide, trinucleotide and tetranucleotide repeats, respectively. Additionally, the locus type could always be assigned unambiguously, as fragment sizes consistently differed by a multiple of the repeat size. VNTR typing of the 52 *A. fumigatus* isolates resulted in the identification of 30 distinct genotypes (Table 1), leading to a DI of 0.97 (Table 2). When a cut-off value of 0.1 (genetic distance) was used, ten clusters (1-10), each consisting of the isolates of a single patient, could be delineated while eight isolates (retrieved from patients GR/04, MI/02 and LY/25) occupied a separate position (Fig. 1). For the *A. lentulus* strains, no amplicon was obtained with the dinucleotide and tetranucleotide primers, whereas with the trinucleotide primers, fragments with sizes which have not been encountered with any of the *A. fumigatus* isolates were obtained.

MLST. All 52 isolates were typed with MLST using a previously developed method. Despite repeated attempts, we were not able to obtain good quality sequences for the LIP fragment for two isolates (IHEM 9508 and 9029), leading to a typeability of 96% for this method (Table 2). Among the remaining isolates, 16 genotypes were identified (Table 1), resulting in a DI of 0.86 (Table 2). Using a cut-off value of 0.1, six clusters could be distinguished in the UPGMA dendrogram, while *A. fumigatus* IHEM 9378 occupied a separate position (Fig. 2). For the *A. lentulus* isolates, no amplicon was obtained for any of the genes.

Typing with pattern-based methods. In a previous study, 52 *A. fumigatus* isolates were typed with RAPD, MSP, SSDP and MLEE [26] and 8, 14, 9 and 8 genotypes were identified, respectively (Table 1). The DIs for these methods and the corresponding 95% CIs (Table 2) ranged from 0.77 (0.68, 0.86) to 0.90 (0.87, 0.93). Combining the results from these methods led to the identification of 25 genotypes and to a combined DI of 0.96 (0.94, 0.98). A dendrogram based on the results obtained with these four methods is shown in Fig. 1. With a cut-off value of 0.1 (genetic distance), nine clusters were identified (designated 1' to 10'), while six isolates occupied separate positions in the dendrogram.

DISCUSSION

Previously, RAPD, SSDP, RFLP, AFLP, MSP and MLEE were used to type *A. fumigatus* isolates [3, 9, 14, 31, 32]. More recently, MLST and VNTR typing schemes were developed [18, 19, 24] and these latter methods have not been included in comparative studies yet. In the present study, results obtained with VNTR typing and MLST were compared with previous results obtained with four pattern-based methods.

In general, a high concordance was found between the results obtained with VNTR typing and with the combination of the four pattern-based methods RAPD, MSP, SSDP and MLEE. All clusters delineated in the VNTR dendrogram with a 0.1 (genetic distance) cut-off had corresponding clusters in the dendrogram obtained with the four pattern-based methods using the same cut-off value (Fig. 1). However, in the latter dendrogram, isolates from VNTR clusters 1 and 7 (with the exception of *A. fumigatus* IHEM 9451) were merged in one large cluster (1'/7') and *A. fumigatus* IHEM 9601 (occupying a separate position in the VNTR dendrogram) was part of cluster 4'. Furthermore, the results obtained with VNTR typing and the combination of pattern-based methods were generally in agreement with the epidemiology, as all ten clusters delineated in the VNTR dendrogram consist of the isolates retrieved from one single patient each (Fig. 1).

When the clusters found after analysis of the results obtained with VNTR typing were compared to clusters found in the dendrogram based on MLST results, little agreement was noticed. Several factors caused this totally different clustering pattern. While VNTR typing and the combination of the four methods both led to the identification of patient specific genotypes, several genotypes identified with MLST were shared between patients. Genotype 24 was identified in seven of the twelve

patients and genotypes 5 and 9 were identified for isolates retrieved from two different patients. In addition, isolates from the same patient were often not more similar to each other than isolates retrieved from different patients when MLST was used. Using VNTR typing however, most genotypes identified for isolates from one patient displayed only few differences.

The discriminatory power (DP) of a method is defined as the ability to assign a different type to two unrelated strains sampled randomly from the population. It can be expressed as a probability using Simpson's index of diversity (DI) [29]. Although a group of unrelated strains is necessary to accurately determine the DI of a given typing method, we calculated this index using the 52 strains incorporated in this study for all typing methods and used it as a comparative characteristic rather than as an absolute reflection of the DP of a method. In recently published guidelines for typing methods [8], a DI of 0.95 or higher is recommended. In the present study, only VNTR typing with a DI of 0.97 reached this level (Table 2). The four pattern-based methods and MLST all had a lower DI and additionally, the CIs are relatively large compared to VNTR typing (Table 2). However, combination of the results obtained with the pattern-based methods led to a similar discriminatory power (0.96) as VNTR typing (Table 2). Because a very high DP is found with VNTR typing, this method can be used for research of the micro-variation of *Aspergillus fumigatus* strains. This phenomenon was recently observed in CF patients [33] and in strains from an outbreak of aspergillosis [34].For MLST the low DP may be improved by using a different set of genes (only 27 genes were tested during the development of the MLST scheme [18]). However, previous results obtained with MLEE demonstrated that the level of inter-strain variation of genes is rather low in *A. fumigatus* [31, 32]. Therefore, it is unlikely that a higher DP can be obtained with a different set of genes.

Typeability refers to a method's ability to assign a type to all isolates tested [8]. VNTR typing and the four pattern-based methods all had a typeability of 100% (Table 2). Despite repeated attempts (including several DNA preparations), we were not able to obtain high-quality sequences for the LIP fragment of two strains, resulting in a typeability of 96% for MLST. Whether poor quality DNA or whether differences in the sequence of the primer binding site are responsible for this remains to be determined.

In various previous studies the reproducibility of results obtained with pattern-based methods was questioned and this lack of reproducibility between laboratories hampers the large-scale implementation of these typing schemes [10, 11, 12, 16]. An important advantage of MLST is that it provides unambiguous sequencing data which can easily be archived, shared between various laboratories and/or stored in an online database (http://pubmlst.org/afumigatus). Despite previous problems with reproducibility of VNTR typing [35], fragment sizes were very similar in independent assays in the present study. In addition, an allelic ladder was recently developed to further improve interlaboratory compatibility of this VNTR typing scheme (H. A. de Valk, J. F. G. M. Meis and C. H. W. Klaassen, presented at the Thirth Advances Against Aspergillosis conference, Miami, FL, 16 to 19 January 2008).The increasing use of this technique highlights the need for and added value of a centralised database similar to the one for MLST data but such a database for VNTR profiles of *A. fumigatus* is yet to be established.

The ease of use of a method not only encompasses the technical simplicity but also the workload, suitability for processing large numbers of isolates and the ease of scoring and interpreting the results [8]. As specific enzyme staining procedures are included in the MLEE assay, this method is more laborious. Although data analysis of

240 the newer methods (VNTR typing and MLST) might require some training, all
241 techniques used in this study are fairly easy to perform (Table 2). A disadvantage of
242 MLST is its rather time-consuming nature.

243 The resources necessary to implement a method depend both on the initial cost
244 for equipment, the labour costs and the cost per isolate. Only the latter cost is
245 calculated for all studied methods and presented in Table 2, while costs for equipment
246 and labour were not taken into account. MLST is by far the most expensive technique
247 compared to the other methods used in this study. As we used core facilities, charges
248 for sequencing and capillary electrophoresis are included in the cost per isolate for
249 MLST and VNTR typing (Table 2), while this is not the case for the pattern-based
250 techniques.

251 **Conclusion.** In the present study, 52 *A. fumigatus* isolates from patients with IA
252 were typed with VNTR typing and MLST and results were compared to those
253 obtained previously with MSP, MLEE, RAPD and SSDP [26]. As previously
254 suggested [18], a low sequence variability between *A. fumigatus* isolates restricted the
255 use of MLST for high-level strain discrimination, although MLST might still be
256 useful for population studies. VNTR typing resulted in a higher discriminatory power
257 and an epidemiologically more relevant clustering pattern than MLST. Additionally,
258 VNTR typing proved to be more simple and reproducible and cheaper than the
259 combination of four pattern-based methods. In conclusion, our results suggest that
260 VNTR is a superior tool for typing of *A. fumigatus* isolates in studies concerning local
261 epidemiology.
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TRANSPARENCY DECLARATION

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Figure legends:

Fig. 1: UPGMA dendrograms of the isolates typed with VNTR (left) and with a combination of 4 pattern-based methods (RAPD, MSP, SSDP and MLEE) (right). The scale bar presents the genetic distance between the isolates. Clusters were delineated with a cut-off value of 0.1 genetic distance for both methods as indicated by the vertical lines. Clusters found in the VNTR dendrogram are designated with the numbers 1 to 10 and correspond to clusters 1' to 10' in the right dendrogram. In both dendrograms the patient codes for the isolates in each cluster are shown.

Fig. 2: UPGMA dendrogram of the isolates typed with MLST. The scale bar presents the genetic distance between the isolates. Clusters were delineated with a cut-off value of 0.1 genetic distance as indicated by the vertical line. Clusters found in the dendrogram are designated with the letters A to F.

438 Table 1: Genotypes obtained by VNTR typing, MLST, RAPD, MSP, SSDP and

439 MLEE for the studied isolates

Center, patient and IHEM no.	Sample type [b]	Isolation date	Genotype by [a]:						
			VNTR typing	MLST	Pattern-based methods				
					RAPD	MSP	SSDP	MLEE	Combined
Grenoble									
GR/02	BAL	01/13/1995							
9418			17	24	1	1	24	7	1
9419			17	24	1	1	24	8	2
9420			17	24	1	1	24	7	1
GR/04	BA	04/18/1995							
9600			6	9	6	2	32	1	3
9601			16	24	7	3	24	1	4
9602			15	3	4	1	32	1	5
9603			5	24	4	4	31	1	6
9604			7	13	6	4	24	1	7
GR/06	BA	05/29/1995							
9720			12	24	3	5	24	1	8
9721			11	24	3	6	24	1	9
9722			11	24	3	7	24	1	10
9723			11	24	3	8	24	1	11
9724			11	24	3	8	24	1	11
GR/01	BAL	10/04/1994							
9347			13	7	3	3	24	1	12
9348			14	7	3	3	24	1	12
9349			13	24	3	3	32	1	13
9350			13	7	3	3	24	1	12
9351			13	24	3	3	32	1	13
Lyon									
LY/07	BAL	11/16/1994							
9378			3	32	2	9	30	3	14
9379			4	24	2	9	30	3	14
9380			1	5	2	9	30	3	14
9381			1	5	2	9	30	3	14
9382			2	5	2	9	30	3	14
LY/20	BAL	02/17/1995							
9508			19	-	3	10	26	4	15
9509			19	24	3	10	28	4	16
9510			19	24	3	10	28	4	16
9511			19	24	3	10	28	4	16
9512			19	33	3	10	28	4	16
LY/25	BAL	04/04/1995							
9595			27	11	2	11	28	2	17
9596			107	11	2	11	28	2	17
9597			28	11	2	11	28	2	17
9598			70	11	2	11	28	2	17
LY/28	Sputum	01/10/1995							
9447			21	5	4	9	30	3	18
9448			20	5	4	9	30	3	18
9449			22	34	4	9	30	3	18
9450			21	35	4	9	30	3	18
9451			21	5	4	8	30	3	19
Milan									
MI/02	Sputum	03/25/1994							
14317			26	36	3	12	28	3	20
14318			18	36	3	3	30	4	21
MI/05	BA	05/09/1996							
10054			25	25	5	13	25	3	22
10055			25	37	5	13	25	3	22
10056			25	25	5	13	25	3	22
MI/03	BA	04/21/1994							

9025			8	24	8	14	29	3	23
9026			8	14	8	14	29	3	23
9027			9	14	8	14	29	5	24
9028			8	38	8	14	29	3	23
9029			10	-	8	14	29	3	23
MI/12	RA	10/24/1997							
14202			24	9	3	1	18	6	25
14203			24	9	3	1	18	6	25
14204			23	9	3	1	18	6	25
14205			23	38	3	1	18	6	25
14206			24	9	3	1	18	6	25

[a]: An arbitrary number was designated to the genotypes presented in this Table. Genotype numbers displayed for the pattern-based methods and MLST correspond to previously published results [18, 26].

[b]: BAL, bronchoalveolar lavage; BA, bronchial aspiration; RA, rhinopharyngeal aspirate

447 Table 2: Characteristics of the typing methods for *A. fumigatus* used in this study.

Typing method	Typeability (%)	DI (95% CI)	Reproducibility [a]	Ease of use [b]	Estimated cost/isolate ($ / €) [c]
VNTR	100	0.97 (0.96, 0.99)	High	High	12/7
MLST	96	0.86 (0.78, 0.93)	Very high	Moderate	66/42
RAPD	100	0.77 (0.68, 0.86)	Low	High	± 1.5/± 1
MSP	100	0.90 (0.87, 0.93)	Low	High	± 2.5/± 1.5
SSDP	100	0.85 (0.81, 0.89)	Moderate	Very high	± 5/± 3
MLEE	100	0.82 (0.75, 0.88)	Low	Moderate	± 9.5/± 6
Combination of RAPD, MSP, SSDP and MLEE	100	0.96 (0.94, 0.98)	Low	Moderate	± 18/± 11.5

448

449 [a]: Reproducibility was scored from "low", over "moderate" and "high", to "very high" based on both the reproducibility of fragment size,
450 sequence or marker pattern, and the reproducibility of the final results (i.e. genotype assignment).

451 [b]: Ease of use of the methods was scored from "moderate" over "high" to "very high" based on technical simplicity, workload, suitability for use
452 in large-scale studies, ease of interpreting the results and amount of training required.[c]: As core facilities were used for MLST and VNTR
453 typing, charges for sequencing and capillary electrophoresis are included in the cost per isolate, while this is not the case for the pattern-based
454 techniques. Costs for equipment and labour were not taken into account.