Exact Molecular Typing of *Aspergillus fumigatus*

Methods and Applications

ISBN 978-90-9023562-2
© Hanneke de Valk, 2008
All rights reserved. No part of this thesis may be reproduced or transmitted in any form or by any means without written permission of the author.

Cover: Corina Bens

Printed by Ponsen & Looijen bv, Wageningen, The Netherlands

Exact Molecular Typing of *Aspergillus fumigatus*

Methods and Applications

Een wetenschappelijke proeve op het gebied van de
Medische Wetenschappen

Proefschrift

ter verkrijging van de graad van doctor
aan de Radboud Universiteit Nijmegen
op gezag van de rector magnificus prof. mr. S.C.J.J. Kortmann,
volgens besluit van het College van Decanen
in het openbaar te verdedigen op dinsdag 25 november 2008
om 13:30 uur precies

Door

Hanneke Johanna Anna de Valk-van Haren
geboren op 6 december 1976
te Nijmegen

Promotor: Prof. dr. A. Voss

Co-promotores: Dr. C.H.W. Klaassen, Canisius Wilhelmina Ziekenhuis, Nijmegen
Dr. J.F.G.M. Meis, Canisius Wilhelmina Ziekenhuis, Nijmegen

Manuscriptcommissie: Prof. dr. P.E. Verweij (voorzitter)
Prof. dr. B.J. Kullberg
Prof. dr. G.S. de Hoog, Universiteit van Amsterdam

Financial support for publication of this thesis was kindly provided by:
Center for Pharmacodynamic Research
Pathoscreen BV
Pfizer BV
MSD BV
Gilead BV
Lab Land BV
Cephalon BV
Schering/Plough BV
BioMérieux Benelux BV
Roche Diagnostics Nederland BV

Contents

Chapter 1	Introduction and outline of the thesis	9
Part 1	**Technical aspects of the STR*Af* assay**	
Chapter 2	Use of a novel panel of nine short tandem repeats for exact and high-resolution fingerprinting of *Aspergillus fumigatus* isolates	39
Chapter 3	Comparison of two highly discriminatory molecular fingerprinting assays for analysis of multiple *Aspergillus fumigatus* isolates from patients with invasive aspergillosis	61
Chapter 4	Retrotransposon insertion-site context (RISC) typing: a novel typing method for *Aspergillus fumigatus* and a convenient PCR alternative to restriction fragment length polymorphism analysis	77
Chapter 5	Microsatellite based typing of *Aspergillus fumigatus*: Strengths, pitfalls and solutions	93
Chapter 6	Interlaboratory reproducibility of a microsatellite based typing assay for *Aspergillus fumigatus* through the use of allelic ladders: Proof of concept	107
Chapter 7	Stability of microsatellite markers in *Aspergillus fumigatus*	125

Part 2 **Clinical applications**

Chapter 8 Utility of a microsatellite assay for identifying clonally related isolates of *Aspergillus fumigatus* 137

Chapter 9 Molecular typing and colonization patterns of *Aspergillus fumigatus* in patients with cystic fibrosis 149

Chapter 10 Detection and direct genotyping of *Aspergillus fumigatus* in tissue and serum samples in patient with invasive aspergillosis 163

Chapter 11 Summary and general discussion 175

Nederlandse samenvatting 187

Dankwoord 199

List of Publications and Curriculum Vitae 201

Chapter 1

Introduction and outline of the thesis

Adapted from:

Hanneke A. de Valk, Jacques F.G.M. Meis en Corné H.W. Klaassen
Moleculaire genotypering van *Aspergillus fumigatus*-isolaten
Nederlands Tijdschrift voor Medische Microbiologie 2005, 2: 40-43

Hanneke A. de Valk, Corné H.W. Klaassen and Jacques F.G.M. Meis.
Molecular typing of *Aspergillus species*.
Mycoses 2008 Digital published doi:10.1111/j.1439-0507.2008.01538.x

Introduction

Aspergillus species occur most frequently in soil, water, decaying vegetation and organic debris. *Aspergillus* is the most ubiquitous fungus with airborne conidia. It sporulates abundantly and the spores are simply dispersed into the environment by air. As a result of this ubiquitous presence, people are probably constantly exposed to *Aspergillus* spores. Several species have been described as human pathogens.

There are severe clinical manifestations in which *Aspergillus* plays an important role. In immunocompromised individuals it can cause invasive aspergillosis (IA), which is a life-threatening disease. Pulmonary infections are thought to arise by local tissue invasion, and eventually disseminate to other deep seated organs. IA is a growing problem in hospitals, because of the increase in the number of patients undergoing bone marrow or solid organ transplantations and because of therapy with corticosteroids. The incidence of IA has increased dramatically in recent years [37, 50]. The main causes of nosocomial *Aspergillus* outbreaks are attributable to construction, renovation, demolition and excavation activities. This is plausible since renovation and demolition work have been shown to dramatically increase the amount of airborne fungal spores and consequently increase the risk for *Aspergillus* infections in susceptible patients [105].

Allergic bronchopulmonary aspergillosis (ABPA) is an uncommon but serious respiratory condition characterized by chronic airway inflammation and airway damage resulting from persistent colonization by and sensitization to *Aspergillus*. ABPA is a severe complication in children, adolescents and adults with cystic fibrosis (CF) [23]. In 57% of these patients the respiratory tract is colonized with *Aspergillus* species, in up to 11% of these patients sensitization to *Aspergillus* allergens may lead to the development of ABPA [66].

Of all aspergilli, *A. fumigatus* is the organism most frequently isolated from human infections [52]. *A. flavus* is the second most common etiologic agent of human aspergillosis [43]. Besides these two, other aspergilli like *A. niger*, *A. nidulans* and *A. terreus* can also be the causative agents of various forms of infections.

Some *Aspergillus* species produce mycotoxins, which are a serious problem as they have adverse effects on human health. Aflatoxins are produced by some isolates from the section *Flavi* such as *A. flavus*, *A. parasiticus*, and *A. nomius*. These are highly carcinogenic, mutagenic and teratogenic secondary metabolites [9, 46]. Another important mycotoxin is ochratoxin A (OTA), this has been implicated in a diverse range of toxicological effects, including renal toxicity, mutagenicity, teratogenicity and immunotoxicity in humans and it is a possible human carcinogen. OTA is produced by *A. ochraceus* and *A. carbonarius* belonging to *Aspergillus* section *Nigri*, also known as black aspergilli [32, 38, 71]

By typing *Aspergillus* isolates the understanding of the genetic and epidemiological relationships between environmental and clinical isolates can be improved. Understanding pathogen distribution and relatedness is essential for determining the epidemiology of nosocomial infections and aiding in the design of rational pathogen control methods [85]. Typing techniques can also give a deeper understanding of the colonization pattern of patients. Whether colonization of the respiratory tract is due to one or several isolates or if frequent recolonization takes place can be evaluated by typing cultured isolates from sequential sputum samples [104]. The genetic diversity in patients with IA can also be investigated to visualize the dissemination routes of isolates from the respiratory tract to the lung tissue and other organs [26, 37].

Several phenotypic and genotypic studies have been used to distinguish between individual *Aspergillus* isolates. With the development and implementation of new typing technologies based on DNA rather than on phenotypic characteristics such as multilocus enzyme electrophoresis (MLEE), the way of typing has dramatically changed [85].

In this review a summary is given of the different available typing methods for *Aspergillus* species. The advantages and disadvantages of the different typing techniques will be evaluated in terms of their discriminatory power and applicability, as well as technical aspects and interpretation. We will also address the issue which typing method best serves the purpose of epidemiological studies.

Random amplified polymorphic DNA (RAPD)

In a standard PCR reaction two primers will anneal to genomic DNA under highly stringent conditions. RAPD is based on a single primer (8 to 20 base pairs (bp) long) in combination with low annealing temperatures during the PCR reaction. Under such conditions a primer can anneal to multiple positions on the genome in an aspecific fashion. In this way a number of arbitrary fragments will be formed. Following amplification, the resulting fragments are separated by agarose gel electrophoresis. The number and sizes of the obtained fragments can be determined by the number and location of annealing sites. Unrelated isolates usually yield different banding patterns.

The RAPD technique is sometimes also called arbitrarily primed PCR (AP-PCR). The repetitive extragenic palindromic PCR (rep-PCR) [100] or interrepeat PCR [41] are techniques similar to RAPD with only little modifications. The rep-PCR primers are based on specific repetitive sequences present in certain microbial genomes.

Aufauvre-Brown *et al*. [6] tested a total of 44 different primers on three different *A. fumigatus* isolates. An often used primer is R108, which also seems suitable for other *Aspergillus* species [7, 31, 48, 59, 73 , 75, 77]. Several other primers suitable for typing *Aspergillus* species have later been described by other groups as well [54, 58, 80, 103].

Restriction Fragment Length Polymorphism without hybridization

When genomic DNA from an *Aspergillus* isolate is cut with a restriction enzyme with a six base pair (bp) recognition sequence, up to 10,000 different DNA fragments may be obtained. These DNA fragments are on average some 4,000 bp long. The vast majority of these fragments cannot be distinguished from each other. By restricting the analysis to only the large fragments (10 - 50 kbp), different isolates may show different banding patterns. Differences in banding patterns originate by substitution, insertion or deletion of nucleotides in the recognition sequence of the restriction enzymes or by insertions and deletions in the region between the recognition sites. Denning *et al*. [30] tested different restriction enzymes for their ability to type a collection of 31 *A. fumigatus* isolates. The enzymes XhoI and SalI seemed to yield the highest discriminatory power with this approach. This technique was originally referred to as restriction enzyme analysis (REA).

This RFLP technique has also been used for *A. flavus* on mitochondrial DNA. It clearly identified *A. flavus* in a heterogeneous group of *Aspergillus*, but isolates from identical species could not be distinguished from each other [64].

Restriction Fragment Length Polymorphism with hybridization

The major difference of the RFLP technique with hybridization is that a specific subset of restriction fragments is analyzed. Genomic DNA is cut by a restriction enzyme. The obtained fragments are separated by size using agarose gel-electrophoresis, and are subsequently transferred to a nylon filter by Southern blotting. A subset of the restriction fragments is then detected by hybridization with a labeled probe. The application of repeated sequences as probes will yield a multiple banding pattern. Differences in patterns between unrelated isolates can be expected because of variations in copy-number and/or flanking sequences of the target regions.

Different restriction enzyme and probe combinations have been analyzed for discrimination between isolates from several *Aspergillus* species. Girardin *et al*. [35] looked for multicopy elements in the *A. fumigatus* genome and tested them in combination with the restriction enzyme EcoRI for their utility as an effective typing technique. This probe, called Afut1, possesses specific characteristics of a retrotransposon-like element [68] and has been used in most RFLP studies so far [47, 52, 104]. Other probes used for fingerprinting of *A. fumigatus*, which are based on retrotransposon-like elements are Afut2 [69] and Af4A [84].

McAlphin *et al*. constructed and characterized a species specific probe for *A. flavus* named pAF28 [56]. This probe has been applied to type clinical and environmental isolates in a neonatal intensive care unit [43]. To discriminate *A. flavus* isolates from outbreak and non-outbreak related situations, a RFLP analysis of Sma I-digested DNA using RAPD generated probes was performed [19].

Non-species specific probe sequences such as the intergenic spacer from the ribosomal RNA gene of *Aspergillus nidulans* pMN1 [45, 79], plasmid pFOLT4R4 harboring *Fusarium oxysporum* telomeric sequences [92] or southern hybridization with labeled bacteriophage M13 [4] also displayed complex patterns of hybridization bands but have only occasionally been reported.

For *A. terreus* a repetitive sequence probe was used to type 12 isolates, six patterns could be distinguished [76]. For the determination of a strain belonging to the group of black aspergilli, RFLP analysis was used with three different genes as probes. The RFLP method is used in here to confirm the differences of this new species with known species. Unique RFLP patterns were obtained for this isolate and it was therefore designated as a novel species [28].

In a simpler version of this technique, the analysis is performed on only one specific PCR amplified target. This fragment is then cut by a (several) restriction enzyme(s) and the obtained fragments can be separated by gel-electrophoresis. This is often referred to as PCR-RFLP. This approach is generally suitable to discriminate between different species, but has insufficient discriminatory power to distinguish between unrelated isolates within a species [2, 88].

Amplified Fragment Length Polymorphism (AFLP)

In AFLP-analysis, first described by Vos *et al*. [106], genomic DNA is usually cut with two restriction enzymes, one with an average cutting frequency and a second one with a higher cutting frequency. Synthetic double stranded DNA fragments are ligated to the obtained sticky ends to serve as primer binding sites in a successive PCR reaction. The ligated fragments are subsequently amplified in a PCR reaction using stringent PCR annealing temperatures. The number of fragments that will be generated can be modulated by extending the amplification primer(s) at the 3' site with one or more selective nucleotides. The addition of each nucleotide reduces the number of amplified fragments by a factor four. The PCR primer that spans the average-frequency restriction site is labeled. At first the primers were radioactive labeled, a major improvement is the switching from radioactive to fluorescently labeled primers for detection of fragments on a high-resolution electrophoresis platforms [81]. A highly informative complex DNA banding pattern of 50 to 500 bp is obtained. Variations between different isolates originate by differences in the number and location of restriction enzyme recognition sites in the genome.

Warris *et al*. [107] investigated a collection of 96 clinical and environmental *A. fumigatus* isolates using the restriction enzymes EcoRI and MseI with three selective nucleotides. The different isolates could be separated from each other. With the same enzyme combination but with other selective nucleotides, the genetic diversity of *Aspergillus* sections *Flavi* and *Nigri* was examined [63, 70]. All these studies demonstrated AFLP to be an excellent typing method to differentiate at and below the species level in these groups of *Aspergillus* species.

Schmidt *et al*. [83] used AFLP patterns to detect specific markers for *A. carbonarius*. In another study specific markers for *A. ochraceus* were developed based on AFLP patterns [82]. Specific primers were designed, based on the sequences of the obtained markers to detect the species, which yielded specific products for their respective target organism. The species belonging to the *Aspergillus* section *Nigri* are regularly isolated from food. The latter species are responsible for the production of the mycotoxin OTA in coffee and grape-derived products. The developed PCR assays allow specific and sensitive detection and identification of *A. carbonarius* and *A. ochraceus*, which make these assays a powerful tool to improve quality control and consumer safety in the food processing industry [83].

Microsatellites or Short Tandem Repeats (STR's)

Microsatellites or STR's are short repetitive sequences that are abundantly present in the genomes of most higher organisms and to a lesser extent in several prokaryotic genomes as well [3, 55, 72, 86, 99]. Different isolates can be distinguished from each other based on differences in repeat numbers. Microsatellite markers are easily amplified by PCR using primers based on their flanking sequences. If one of the primers is fluorescently labeled, they can be sized very accurately using high-resolution electrophoresis platforms. The number of repeats in each marker can be deduced from the sizes of the fragments. All repeat numbers of the analyzed markers form a genotype for each individual isolate. These genotypes are easily compared to each other. Microsatellite based typing schemes yield unambiguous typing- and highly portable data.

Bart-Delabesse *et al*. [14] used microsatellites to type *A. fumigatus*. Four different dinucleotide markers were used to discriminate between unrelated isolates. With this panel of markers, some 30.000 different genotypes can be identified. Microsatellite based assays are sometimes also referred to as MLP (microsatellite length polymorphism) [14], MSP (microsatellite polymorphism) [17] or PMM (polymorphic microsatellite marker) [47].

For *A. flavus* and *A. parasiticus* seven polymorphic microsatellite loci have been characterized [96]. Searches for microsatellite motifs were performed on genomic sequences of *Aspergillus* section *Flavi*. These 7 markers are tested on 20 *A. flavus* and 15 *A. parasiticus* isolates and yielded 2 to 11 alleles for *A. flavus* and 1 to 9 alleles for *A. parasiticus* [96]. For *A. niger* six polymorphic loci were developed to analyze 28 isolates. These markers displayed 6 to 13 alleles, which demonstrated diversity within *A. niger* [33]. For *Emericella nidulans* (teleomorph of *A. nidulans*) five to 12 alleles were found with 7 microsatellite loci on a collection of 44 isolates [42].

Multilocus Sequence Typing (MLST)

MLST, originally developed for characterizing isolates of bacterial species, compares the nucleotide sequence of internal 400-500 bp regions of a series of genes (typically six to ten) which are present in all isolates of a particular species [93]. For all unique sequences an arbitrary allele number will be assigned (usually in the order of discovery). Each isolate is defined by the alleles at each of the sequenced housekeeping loci, which together comprise the allelic profile or sequence type (ST). Relationships among isolates are apparent by comparisons of allelic profiles: closely related isolates have identical STs, or STs that differ at only a few loci, whereas unrelated isolates have unrelated STs. MLST is the molecular alternative of MLEE (multilocus enzyme electrophoresis) by targeting the variation present in multiple housekeeping loci [98]. The advantage of MLST compared to MLEE is that as DNA is more variable than protein encoded (due primarily to synonymous mutations) fewer loci need to be examined in MLST. Furthermore, MLST data is portable.

MLST is increasingly applied as a routine typing tool that enables international comparison of isolates. MLST data have also been exploited in evolutionary and population analyses that estimate recombination and mutation rates and investigate evolutionary relationships among bacteria that are classified as belonging to the same genus [98]. In the *Aspergillus* section *Fumigati*, the MLST method has led to the description of new pathogenic species, among which is *A. lentulus*. [10]

Recently, Bain *et al*. described an MLST scheme for *A. fumigatus*, where seven genes were selected to genotype a panel of 100 clinical and environmental *A. fumigatus* isolates. Among the 100 isolates analyzed, 30 STs were distinguished [8].

Other less-frequently reported PCR based typing techniques

Random amplified microsatellites (RAMS) is based on PCR and uses primers containing microsatellite sequences and degenerate anchors at the 5' site. The DNA between the distal ends of two closely located microsatellites is amplified. The resulting PCR products are separated by electrophoresis. RAMS marker polymorphism is due to the length differences in the sizes of amplified products between different isolates. Since it has not been shown that the amplified fragments are indeed flanked by microsatellites, this technique fundamentally differs little from RAPD. This method has been used to fingerprint 24 *A. fumigatus* and 13 *A. flavus* isolates from respectively 14 and 10 patients [40].

Another PCR based method is sequence specific DNA primers (SSDP), described for the first time by Mondon *et al*. [62]. RAPD fragments that differentiate unrelated *A. fumigatus* isolates were selected and sequenced. Primer sets are formulated on the basis of these sequences and the resulting PCR products are scored as either present or absent. A total of 18 different fragments obtained with three different RAPD-primers, were analyzed. Five of these primer combinations made it possible to distinguish between nine unrelated isolates. These five SSDP primers have been applied to investigate 43 clinical isolates [62]. Theoretically, with these primer combinations 32 genotypes can be distinguished.

Intergenic spacer-analysis (IGS-PCR) is a PCR based method in which the primers flank the region between the small and large subunit of the ribosomal RNA-gene. The genome of a fungus contain about 100 highly conserved copies of this gene, which are tandemly repeated head to tail with spacer regions in between. Since these intergenic spacer-regions may vary in length, unrelated isolates may yield a different banding pattern. Radford *et al*. [74] tested this technique on a population of 119 patient- and environmental isolates from one hospital; in this collection 11 different types were recognized.

Recently a novel single-locus sequence based typing method was developed based on the presence of tandem repeats in a gene encoding a putative cell surface protein. This method is referred to as CSP typing [12]. Since the repeat is not fully conserved, differences in genotype are the result of both repeat number variation and nucleotide sequence variation. The discriminatory power of this technique was evaluated on a collection of 55 epidemiological linked *A. fumigatus* isolates from six different outbreak investigations.

Comparison of the typing techniques

The performance of typing techniques can be compared to each other in respect of their practical feasibility. Specific advantages and disadvantages are evaluated in terms of applicability, the ease of use, the exchangeability and the reproducibility within a laboratory and between laboratories. The discriminatory power and interpretation of the typing methods will be evaluated in separate paragraphs. Table 1 gives an overview of the different aspects for all typing techniques discussed before.

Firstly the ease of use of methods will be compared to each other. Most of the techniques described in this review are PCR based methods. PCR based techniques require only small amounts of amplifiable DNA, which are easily obtained [15]. In contrast, RFLP either with or without hybridization, as non-PCR based methods, need relatively large amounts of highly purified DNA which make them time-consuming and labor intensive [52, 47].

Several PCR based methods like AFLP, CSP, MLST, microsatellites and RAMS need special equipment to separate obtained fragments with high resolution electrophoresis conditions. On the other hand the RAPD method can be performed with relatively inexpensive equipment and is therefore the most widely used method for typing *Aspergillus* isolates [47].

Not all techniques are able to generate a genotype for any given organism. Microsatellite based approaches appear to be species specific [14, 252, 63] as are SSDP, IGS and MLST [8, 24, 62, 741]. For RFLP both species specific and non-species specific probes have been described. Probes based on repetitive chromosomal DNA sequences, like pAF28 [56] and Afut1 [35] or probes based on RAPD patterns [19] hybridize strongly only to the species for which the probe has been developed. Southern blotting in combination with labeled bacteriophage M13, however, has also successfully been used to differentiate isolates of several fungal species from different genera [61]. In contrast, the AFLP technique is basically applicable to any organism's genome without the need for prior sequence information [81]. Besides aspergilli, AFLP fingerprinting has already successfully been used with a large variety of other microorganisms [65, 81] including other fungi [97], but also in plants and animals [16]. This is also the case for RAPD, which has been applied to many bacteria [39, 78, 101], parasites [20], viruses [5] and fungi [57]. The RAMS technique has been used to determine genetic diversity in plants and animals and, more recently, also in other fungi [40].

Reproducibility refers to the ability of a technique to yield the same result when a particular isolate is repeatedly tested. For large scale and longitudinal epidemiological studies, stable fingerprinting techniques are required. The main source of ambiguity in band-based approaches like RAPD, RFLP, RAMS, IGS, SSDP and AFLP concerning reproducibility is the variable intensity of bands, which is probably due to small variations in the various steps of the procedures that may affect the final peak intensity [15, 26, 81]. In contrast, exact techniques, like CSP, microsatellites and MLST are potentially 100% reproducible [9, 25].

Another important aspect in evaluating typing techniques is the ability to exchange data between different laboratories. As a consequence of the easy exchange of data, genotypes obtained from all over the world can be compared to each other, which would facilitate our understanding of the global epidemiology of microorganisms. Therefore exact techniques seem to be most suitable, because the data generated with these techniques can be fully expressed in a simple, digital format. For MLST, schemes for several microorganisms are publicly available at http://www.mlst.net/ and on http://pubmlst.org, where sequence data for MLST alleles can be uploaded to ascertain allele genotypes and strain STs [44, 98]. The data obtained with CSP typing also allows the creation of unambiguous data sets that can be readily exchanged between labs [12]. Digital fingerprinting data in the shape of banding patterns is more complex and therefore difficult to exchange. In addition, standard procedures have to be followed by all laboratories to avoid differences in the banding patterns [81].

Table 1. Overview of different aspects of typing techniques used for Aspergilli

Typing technique	Discriminatory Power	Reproducibility	Exchangeability	Ease of use	Species specific	Interpretation
AFLP	High	Good	Poor	Moderate	No	Difficult
CSP typing	Limited	Excellent	Excellent	Moderate	Unknown	Easy
IGS-PCR	Unknown	Limited	Poor	Easy	Yes	Moderate
Microsatellites	High	Good	Poor	Moderate	Yes	Easy
MLST	Limited	Excellent	Excellent	Moderate	Yes	Easy
RAMS	Unknown	Good	Poor	Easy	No	Moderate
RAPD	Limited	Limited	Poor	Easy	No	Moderate
RFLP without hybridization	Limited	Limited	Poor	Moderate	No	Difficult
Afut1-RFLP	High	Good	Poor	Difficult	Yes	Difficult
SSDP	Limited	Excellent	Good	Easy	Yes	Moderate

Interpretation

All techniques mentioned in this review, can roughly be divided in pattern-based approaches and exact techniques. With respect to the interpretation of these kinds of results, different pitfalls can be encountered. The techniques used for distinguishing *Aspergillus* isolates rely on electrophoretic separation of DNA fragments of different lengths, which results in a complex pattern of bands. The following techniques described for fingerprinting of *Aspergillus* species are band-based approaches: RAPD, RFLP, RAMS, IGS, and AFLP. Exact typing techniques yield unambiguous typing results, like the peak length (bp) typing results obtained with the MLP assay, and sequence data obtained with MLST and CSP typing. The SSDP technique could be an exact technique since bands are either scored present or absent. However, Lasker found that some SSDP-primers sporadically generate faint bands, which undermines the potential exact character of this technique [47].

The patterns obtained with band based techniques can be extremely complex, because they are composed of both strong and faint bands which makes the interpretation of these methods difficult [15, 81]. In order to compare different fingerprints to each other, and to determine the genetic relatedness between multiple isolates, one has to rely on specialized computer software. Computerized analyses are usually performed using either band based or pattern based algorithms. Band based algorithms, where individual bands are either scored as present or absent, still need visual inspection, which makes it more laborious and subjective. However, pattern based approaches, which use all the information from all bands in the fingerprint, hardly ever give 100% identical values for the same samples although they may appear to be identical upon visual examination. When relying solely on the computer-based comparison, wrong conclusions could be drawn. This requires a visual inspection of the fingerprints to be included in the final interpretation.

In contrast, exact fingerprinting techniques are much easier to interpret. Artifacts that may compromise correct interpretation of microsatellite data can be avoided by choosing appropriate reaction conditions [14, 27]. Sequence data obtained with MLST can be interpreted and compared in a central web database (http://pubmlst.org [44]). The sequence of any new allele is entered into the database when the forward and reverse trace files are submitted to the MLST scheme curator, before a new allele number is assigned by the curator for the novel sequence [1]. This is to avoid that the database is corrupted by nonexistent alleles and sequence types.

Another problem among the analysis of general typing data is the subjective interpretation of a "genotype". The assignment of a genotype is user dependent when using band-based approaches, whereas the genotype resulting from exact techniques is unambiguous. Unfortunately, at present there are no established guidelines for defining a genotype for the methods described here (such as the criteria of Tenover *et al*. [94] for the interpretation of pulsed-field gel electrophoresis fingerprints) [53, 81]. By comparing banding patterns with each other it is assumed that bands with the same size contain the same gene fragments. This can be misleading, because bands with the same size can consist of completely different gene fragments and thus give false identical strains [53]. This is avoided in exact genotyping methods, where the target is well defined and primers can be designed very specific [53]. With exact approaches unambiguous data can be obtained, to determine if isolates with different genotypes may be related.

Discriminatory power

Apart from the advantages and disadvantages of practical feasibility and the interpretation of the different techniques, another important factor, the discriminatory power, must be evaluated. One way to assess whether a DNA fingerprinting method is sufficiently discriminating, is to compare the data generated by one method with those generated by a completely unrelated DNA fingerprinting method for the same set of test isolates [87]. For *A. fumigatus*, several studies have been performed to compare different typing techniques.

RAPD, *Afut*1-RFLP and MLP have been compared to each other in two comparative studies [15, 47]. In both investigations RAPD yielded the lowest discriminatory power, while *Afut*1-RFLP and MLP proved to be equally discriminatory in one study [47], in another study RFLP appeared to be superior [15].

The choice or design of a specific probe is essential for high discriminatory power for RFLP assays. Several probes have already been used to type *A. fumigatus*. Although the M13 probe has the advantage that it is readily available from commercial suppliers, it seems that the *Afut*1 probe, which is based on a moderately repetitive sequence from *A. fumigatus*, discriminates best between unrelated isolates [21, 29].

AFLP analysis can be used both for identification of isolates to the species level as well as for discriminating of the intraspecies level, more commonly referred to as "typing". This was already demonstrated for the *Aspergillus* section *Nigri* group [70]. The AFLP method was also used for determining the epidemiological relatedness between 96 clinical and environmental *A. fumigatus* isolates. The AFLP could differentiate most of the isolates, indicating the high discriminatory power of this technique [107].

Sequence based typing of genes can be used to distinguish new species in the section *Fumigati* [10, 11]. Differentiation between *A. fumigatus* isolates can also be accomplished by MLST. Bain *et al.* applied seven gene fragments on a collection of 100 isolates from diverse sources. This yielded different types amounting to approximately one-third of the number of isolates tested [8], which renders the discriminatory power less discriminating than other exact fingerprinting methods. If the level of nucleotide variation is not discriminating enough to answer epidemiological questions, more variable loci have to be tested.

The SSDP method makes use of five primer combinations, therefore this method is limited to discriminate between only 32 (2^5) genotypes. Cimon *et al*. examined 109 isolates from 7 different CF patients with SSDP. In 6 of the 7 patients identical genotypes were found [22]. Isolates from six different locations tested by Symoens *et al*. showed also identical genotypes [91]. The genotypes found in CF-patients [22] and the genotypes found in the environment of IA-patients [91] appeared to be identical.

Unfortunately the discriminatory power of methods like IGS and RAMS is difficult to assess since the discriminatory power of these methods has not been compared to other typing techniques.

Clinical aspects

Aspergillus species are widely distributed fungi whose conidia are ubiquitous in their distribution and inhalation of spores is believed to be the major route of transmission [105]. Prior to invasion, colonization with *Aspergillus* species may be necessary. Colonization of the lower respiratory tract is a risk factor for patients, especially patients with chronic lung disease (e.g. chronic obstructive lung disease, cystic fibrosis (CF), inactive tuberculosis and α1-antitrypsin deficiency) to acquire pulmonary or disseminated infections [34].

To visualize the colonization pattern of *Aspergillus* isolates in CF patients, several studies where sequential isolates were typed, have been performed. Verweij *et al*. [104] presented two CF-patients with *A. fumigatus* isolates cultured in sequential sputum samples over a period of two years, which were analyzed with *Afut*1-RFLP and RAPD. The fingerprints of these samples showed three different genotypes in 11 samples for one patient, of which one genotype was predominant present in the sputum samples. In the other patient about 9 different genotypes were seen in 12 samples. In another study, six CF patients were followed over a period of two years. In total 412 *A. fumigatus* isolates, collected from sequential sputum samples from these patients, yielded 54 unique genotypes with the *Afut*1-RFLP method. In all samples of the six patients several *A. fumigatus* genotypes were found, whereas two patients harbored a predominated isolate. From one of these patients 10 different genotypes were identified in the first year, while in the second year a single genotype was found from 40 consecutive samples. This patient did not show any clinical symptoms of aspergillosis. However, the other patient, with a predominant genotype in nine sputum samples, developed a pulmonary infection [67]. Cimon *et al*. observed that two out of seven patients harbored a unique genotype, whereas several genotypes were simultaneously and/or successively displayed in the other patients.

The results of these studies are in agreement, CF patients appeared to have different patterns of colonization with *A. fumigatus*, since analysis of sequential isolates showed that some CF patients exhibited a predominant genotype and others had multiple genotypes [22, 67, 104]. In most of the CF patients airway colonization with *A. fumigatus* seems to be a recurrent event. The repeated isolation of one unique genotype in these patients does not necessarily forecast the occurrence of aspergillosis [67].

The presence of various genotypes in sequential isolates has also been shown in other patients, like lung transplant recipients [18 89], aspergilloma patients [36] and patients with invasive aspergillosis [13, 29]. In general, the variability of genotypes of isolates from patients with invasive aspergillosis or aspergilloma is lower than in CF patients [13, 29 36]. One must take into account that in most studies either the number of isolates or the time interval between the first and the last isolate is lower in patients with invasive aspergillosis compared to CF patients. In two studies, the genotypes of invasive *A. fumigatus* isolates, collected from deeper tissue samples, are compared to the genotypes of isolates from respiratory samples [25, 30, 37]. Overall results show that intrapatient samples of non-respiratory origin belonged to the same genotype. By contrast, multiple genotypes were detected in isolates originating from respiratory samples.

Aspergillus conidia are permanently present in the air [21]. The extent of genetic diversity of environmental and clinical isolates has been demonstrated in an Afut1-RFLP based study including 700 *A. fumigatus* isolates from hospital air and patients. Analyses of the environmental isolates represent in 85% of the isolates a single genotype. The remaining 15% of the isolates were found multiple times and persisted in the same hospital for several months [21]. In another study 879 *A. fumigatus* isolates from different hosts and geographical origins were analyzed with Afut1-RFLP. The patterns obtained with the Afut1 probe gave distinct and unique genotypes for 424 isolates. Only 14 matches were found between isolates of different origins [29]. The high genetic variability in the air together with the high genetic variability of *A. fumigatus* in respiratory samples confirm that individuals may well be constantly exposed to a large variety of different genotypes from the environment [21, 60]. Even if typing is helpful towards the epidemiology of *A. fumigatus,* it remains difficult to identify the origin of a nosocomial outbreak [91].

Genetic variability of *Aspergillus* species other than *A. fumigatus* is less pronounced [102] and spores of other *Aspergillus* species are less prominent present in the environment [21, 51, 91]. Therefore it is more difficult to identify a nosocomial source of contamination for other aspergilli than *A. fumigatus*. However, James *et al*. [43] indicated that a roll of adhesive tape was the probable source of the infecting isolate of *A. flavus* recovered from two low-birth-weight infants. Genotyping of these samples was performed with RFLP with the pAF28 repetitive DNA sequence probe. Fingerprinting of successive *A. terreus* isolates from two patients with CF and two with invasive aspergillosis yielded identical genotypes within patients [90]. RAPD analysis was also used to identify the source of *A. terreus* infections in a hematology unit [49] and was recently performed on a collection from three geographically distinct centers [48].

Vonberg *et al*. [105] summarized numerous outbreaks of IA. From the 53 outbreaks that were included, genotyping of clinical and environmental isolates was performed in 11 studies. Construction work or renovation activities within the hospital or in the surrounding areas were most commonly considered to be the probable or possible source of the nosocomial *Aspergillus* outbreak, followed by a contaminated or defective air supply system [105]. This is plausible because renovation and demolition work have been shown to increase the amount of airborne fungal spores dramatically, and consequently increase the risk for *Aspergillus* transmission to susceptible patients [51, 95]. Routes of *Aspergillus* transmission in nosocomial outbreaks other than airborne spores are rarely described [105]. Detection of airborne aspergilli in high-risk patient care areas should always draw attention to the potential risk of a serious infection [105].

Conclusions

Several molecular methods have been described for typing various *Aspergillus* species. Most of the techniques are developed to analyze *A. fumigatus* isolates, which is the most common species. Fingerprinting isolates collected from respiratory samples and from the environment showed that a high extent of genetic variability can be found in *A. fumigatus*. Therefore typing techniques with high discriminatory power are necessary. Techniques which fulfill this criterion are RFLP in combination with the Afut1 probe and MLP. Another important aspect of typing methods is the reproducibility. Exact typing methods, like MLST and CSP, are highly reproducible, which make them eligible to analyze epidemiological relationships between large amounts of isolates over a longer period of time. Unfortunately, CSP typing and the loci described so far for MLST are probably not discriminating enough to identify a true source in an outbreak situation. The RAPD method has been used most, probably because this technique needs less equipment and no genomic sequence information is necessary to perform this assay.

References

1. Aanensen, D. M., and B. G. Spratt. 2005. The multilocus sequence typing network: MLST.net. Nucl Acids Res 33:W728-33.
2. Accensi, F., J. Cano, L. Figuera, M. L. Abarca, and F. J. Cabanes. 1999. New PCR method to differentiate species in the *Aspergillus niger* aggregate. FEMS Microbiol Lett 180:191-6.
3. Adair, D. M., P. L. Worsham, K. K. Hill, A. M. Klevytska, P. J. Jackson, A. M. Friedlander, and P. Keim. 2000. Diversity in a variable-number tandem repeat from *Yersinia pestis*. J Clin Microbiol 38:1516-9.
4. Anderson, M. J., K. Gull, and D. W. Denning. 1996. Molecular typing by random amplification of polymorphic DNA and M13 southern hybridization of related paired isolates of *Aspergillus fumigatus*. J Clin Microbiol 34:87-93.
5. Aubin, J. T., H. Collandre, D. Candotti, D. Ingrand, C. Rouzioux, M. Burgard, S. Richard, J. M. Huraux, and H. Agut. 1991. Several groups among human herpesvirus 6 strains can be distinguished by Southern blotting and polymerase chain reaction. J Clin Microbiol 29:367-72.
6. Aufauvre-Brown, A., J. Cohen, and D. W. Holden. 1992. Use of randomly amplified polymorphic DNA markers to distinguish isolates of *Aspergillus fumigatus*. J Clin Microbiol 30:2991-3.
7. Baddley, J. W., P. G. Pappas, A. C. Smith, and S. A. Moser. 2003. Epidemiology of *Aspergillus terreus* at a university hospital. J Clin Microbiol 41:5525-9.
8. Bain, J. M., A. Tavanti, A. D. Davidson, M. D. Jacobsen, D. Shaw, N. A. Gow, and F. C. Odds. 2007. Multilocus sequence typing of the pathogenic fungus *Aspergillus fumigatus*. J Clin Microbiol 45:1469-77.
9. Baird, R. E., R. N. Trigiano, G. Windham, P. Williams, R. Kelley, H. K. Abbas, J. K. Moulton, and M. L. Scruggs. 2006. Comparison of aflatoxigenic and nonaflatoxigenic isolates of *Aspergillus flavus* using DNA amplification fingerprinting techniques. Mycopathologia 161:93-9.
10. Balajee, S. A., J. L. Gribskov, E. Hanley, D. Nickle, and K. A. Marr. 2005. *Aspergillus lentulus* sp. nov., a new sibling species of *A. fumigatus*. Eukaryot Cell 4:625-32.
11. Balajee, S. A., D. Nickle, J. Varga, and K. A. Marr. 2006. Molecular studies reveal frequent misidentification of *Aspergillus fumigatus* by morphotyping. Eukaryot Cell 5:1705-12.
12. Balajee, S. A., S. T. Tay, B. A. Lasker, S. F. Hurst, and A. P. Rooney. 2007. Characterization of a novel gene for strain typing reveals substructuring of *Aspergillus fumigatus* across North America. Eukaryot Cell 6:1392-9.
13. Bart-Delabesse, E., C. Cordonnier, and S. Bretagne. 1999. Usefulness of genotyping with microsatellite markers to investigate hospital-acquired invasive aspergillosis. J Hosp Infect 42:321-7.
14. Bart-Delabesse, E., J.-F. Humbert, E. Delabesse, and S. Bretagne. 1998. Microsatellite markers for typing *Aspergillus fumigatus* isolates. J Clin Microbiol 36:2413-2418.

15. Bart-Delabesse, E., J. Sarfati, J. P. Debeaupuis, W. van Leeuwen, A. van Belkum, S. Bretagne, and J. P. Latge. 2001. Comparison of restriction fragment length polymorphism, microsatellite length polymorphism, and random amplification of polymorphic DNA analyses for fingerprinting *Aspergillus fumigatus* isolates. J Clin Microbiol 39:2683-6.
16. Bensch, S., and M. Akesson. 2005. Ten years of AFLP in ecology and evolution: Why so few animals? Mol Ecol 14:2899-2914.
17. Bertout, S., F. Renaud, R. Barton, F. Symoens, J. Burnod, M. A. Piens, B. Lebeau, M. A. Viviani, F. Chapuis, J. M. Bastide, R. Grillot, and M. Mallie. 2001. Genetic polymorphism of *Aspergillus fumigatus* in clinical samples from patients with invasive aspergillosis: investigation using multiple typing methods. J Clin Microbiol 39:1731-7.
18. Brenier, M. P., B. Lebeau, G. Devouassoux, P. Mondon, C. Pison, P. Ambroise-Thomas, and R. Grillot. 1998. *Aspergillus* and lung transplant recipients: A mycologic and molecular epidemiologic study. J Heart Lung Transplant 17:972-9.
19. Buffington, J., R. Reporter, B. A. Lasker, M. M. McNeil, J. M. Lanson, L. A. Ross, L. Mascola, and W. R. Jarvis. 1994. Investigation of an epidemic of invasive aspergillosis: Utility of molecular typing with the use of random amplified polymorphic DNA probes. Pediatr Infect Dis J 13:386-93.
20. Campbell, G., H. H. Garcia, M. Nakao, A. Ito, and P. S. Craig. 2006. Genetic variation in Taenia solium. Parasitol Int 55 Suppl:S121-6.
21. Chazalet, V., J. P. Debeaupuis, J. Sarfati, J. Lortholary, P. Ribaud, P. Shah, M. Cornet, H. Vu Thien, E. Gluckman, G. Brucker, and J. P. Latge. 1998. Molecular typing of environmental and patient isolates of *Aspergillus fumigatus* from various hospital settings. J Clin Microbiol 36:1494-500.
22. Cimon, B., F. Symoens, R. Zouhair, D. Chabasse, N. Nolard, A. Defontaine, and J. P. Bouchara. 2001. Molecular epidemiology of airway colonisation by *Aspergillus fumigatus* in cystic fibrosis patients. J Med Microbiol 50:367-74.
23. de Almeida, M. B., M. H. Bussamra, and J. C. Rodrigues. 2006. Allergic bronchopulmonary aspergillosis in paediatric cystic fibrosis patients. Paediatr Respir Rev 7:67-72.
24. de Ruiter, M. T., H. A. de Valk, J. F. Meis, and C. H. Klaassen. 2007. Retrotransposon insertion-site context (RISC) typing: a novel typing method for *Aspergillus fumigatus* and a convenient PCR alternative to restriction fragment length polymorphism analysis. J Microbiol Methods 70:528-34.
25. de Valk, H. A., J. F. G. M. Meis, I. M. Curfs, K. Muehlethaler, J. W. Mouton, and C. H. W. Klaassen. 2005. Use of a novel panel of nine short tandem repeats for exact and high-resolution fingerprinting of *Aspergillus fumigatus* isolates. J Clin Microbiol 43:4112-20.
26. de Valk, H. A., J. F. G. M. Meis, B. E. de Pauw, P. J. Donnelly, and C. H. W. Klaassen. 2007. Comparison of two highly discriminatory molecular fingerprinting assays for analysis of multiple *Aspergillus fumigatus* isolates from patients with invasive aspergillosis. J Clin Microbiol 45:1415-9.
27. de Valk, H. A., J. F. G. M. Meis, and C. H. W. Klaassen. 2007. Microsatellite based typing of *Aspergillus fumigatus*: Strengths, pitfalls and solutions. J Microbiol Methods 69:268-72.

28. de Vries, R. P., J. C. Frisvad, P. J. van de Vondervoort, K. Burgers, A. F. Kuijpers, R. A. Samson, and J. Visser. 2005. *Aspergillus vadensis*, a new species of the group of black Aspergilli. Antonie Van Leeuwenhoek 87:195-203.
29. Debeaupuis, J. P., J. Sarfati, V. Chazalet, and J. P. Latge. 1997. Genetic diversity among clinical and environmental isolates of *Aspergillus fumigatus*. Infect Immun 65:3080-5.
30. Denning, D. W., K. V. Clemons, L. H. Hanson, and D. A. Stevens. 1990. Restriction endonuclease analysis of total cellular DNA of *Aspergillus fumigatus* isolates of geographically and epidemiologically diverse origin. J Infect Dis 162:1151-8.
31. Diaz-Guerra, T. M., E. Mellado, M. Cuenca-Estrella, L. Gaztelurrutia, J. I. Navarro, and J. L. Rodriquez-Tudela. 2000. Genetic similarity among one *Aspergillus flavus* strain isolated from a patient who underwent heart surgery and two environmental strains obtained from the operating room. J Clin Microbiol 38:2419-22.
32. Esteban, A., M. L. Abarca, M. R. Bragulat, and F. J. Cabanes. 2006. Effect of water activity on ochratoxin A production by *Aspergillus niger* aggregate species. Int J Food Microbiol 108:188-95.
33. Esteban, A., S. L. Leong, and N. Tran-Dinh. 2005. Isolation and characterization of six polymorphic microsatellite loci in *Aspergillus niger*. Molecular ecology notes 5:375-7.
34. Fridkin, S. K., and W. R. Jarvis. 1996. Epidemiology of nosocomial fungal infections. Clin Microbiol Rev 9:499-511.
35. Girardin, H., J. P. Latge, T. Srikantha, B. Morrow, and D. R. Soll. 1993. Development of DNA probes for fingerprinting *Aspergillus fumigatus*. J Clin Microbiol 31:1547-54.
36. Girardin, H., J. Sarfati, H. Kobayashi, J. P. Bouchara, and J. P. Latge. 1994. Use of DNA moderately repetitive sequence to type *Aspergillus fumigatus* isolates from aspergilloma patients. J Infect Dis 169:683-5.
37. Girardin, H., J. Sarfati, F. Traore, J. Dupouy Camet, F. Derouin, and J. P. Latge. 1994. Molecular epidemiology of nosocomial invasive aspergillosis. J Clin Microbiol 32:684-90.
38. Gonzalez-Salgado, A., B. Patino, C. Vazquez, and M. T. Gonzalez-Jaen. 2005. Discrimination of *Aspergillus niger* and other *Aspergillus species* belonging to section Nigri by PCR assays. FEMS Microbiol Lett 245:353-61.
39. Gruteke, P., A. van Belkum, L. M. Schouls, W. D. Hendriks, F. A. Reubsaet, J. Dokter, H. Boxma, and H. A. Verbrugh. 1996. Outbreak of group A streptococci in a burn center: use of pheno- and genotypic procedures for strain tracking. J Clin Microbiol 34:114-8.
40. Guarro, J., M. Sole, R. Castany, J. Cano, A. Teixido, I. Pujol, J. Gene, A. Castro, and P. Sarda. 2005. Use of random amplified microsatellites to type isolates from an outbreak of nosocomial aspergillosis in a general medical ward. Med Mycol 43:365-71.
41. Healy, M., K. Reece, D. Walton, J. Huong, K. Shah, and D. P. Kontoyiannis. 2004. Identification to the species level and differentiation between strains of *Aspergillus* clinical isolates by automated repetitive-sequence-based PCR. J Clin Microbiol 42:4016-24.

42. Hosid, E., I. Grishkan, Z. Frenkel, S. P. Wasser, E. Nevo, and A. Korol. 2005. Microsatellite markers for assessing DNA polymorphism of *Emericella nidulans* in nature. Molecular ecology notes 5:647-9.
43. James, M. J., B. A. Lasker, M. M. McNeil, M. Shelton, D. W. Warnock, and E. Reiss. 2000. Use of a repetitive DNA probe to type clinical and environmental isolates of *Aspergillus flavus* from a cluster of cutaneous infections in a neonatal intensive care unit. J Clin Microbiol 38:3612-8.
44. Jolley, K. A., M. S. Chan, and M. C. Maiden. 2004. mlstdbNet - distributed multi-locus sequence typing (MLST) databases. BMC Bioinformatics 5:86.
45. Kevei, F., Z. Hamari, J. Varga, Z. Kozakiewicz, and J. H. Croft. 1996. Molecular polymorphism and phenotypic variation in *Aspergillus carbonarius*. Antonie Van Leeuwenhoek 70:59-66.
46. Kumeda, Y., and T. Asao. 1996. Single-strand conformation polymorphism analysis of PCR-amplified ribosomal DNA internal transcribed spacers to differentiate species of *Aspergillus* section Flavi. Appl Environ Microbiol 62:2947-52.
47. Lasker, B. A. 2002. Evaluation of performance of four genotypic methods for studying the genetic epidemiology of *Aspergillus fumigatus* isolates. J Clin Microbiol 40:2886-2892.
48. Lass-Florl, C., K. Grif, and D. P. Kontoyiannis. 2007. Molecular typing of *Aspergillus terreus* isolates collected in Houston, Texas, and Innsbruck, Austria: evidence of great genetic diversity. J Clin Microbiol 45:2686-90.
49. Lass-Florl, C., P. Rath, D. Niederwieser, G. Kofler, R. Wurzner, A. Krezy, and M. P. Dierich. 2000. *Aspergillus terreus* infections in haematological malignancies: molecular epidemiology suggests association with in-hospital plants. J Hosp Infect 46:31-5.
50. Latge, J.-P. 1999. *Aspergillus fumigatus* and aspergillosis. Clin Microbiol Rev 12:310-350.
51. Leenders, A. C., A. van Belkum, M. Behrendt, A. Luijendijk, and H. A. Verbrugh. 1999. Density and molecular epidemiology of *Aspergillus* in air and relationship to outbreaks of *Aspergillus* infection. J Clin Microbiol 37:1752-7.
52. Lin, D., P. F. Lehmann, B. H. Hamory, A. A. Padhye, E. Durry, R. W. Pinner, and B. A. Lasker. 1995. Comparison of three typing methods for clinical and environmental isolates of *Aspergillus fumigatus*. J Clin Microbiol 33:1596-601.
53. Lindstedt, B. A. 2005. Multiple-locus variable number tandem repeats analysis for genetic fingerprinting of pathogenic bacteria. Electrophoresis 26:2567-82.
54. Loudon, K. W., J. P. Burnie, A. P. Coke, and R. C. Matthews. 1993. Application of polymerase chain reaction to fingerprinting *Aspergillus fumigatus* by random amplification of polymorphic DNA. J Clin Microbiol 31:1117-21.
55. Verduyn-Lunel, F., L. Licciardello, S. Stefani, H. A. Verbrugh, W. J. G. Melchers, J. F. G. M. Meis, S. Scherer, and A. van Belkum. 1998. Lack of consistent short sequence repeat polymorphisms in genetically homologous colonizing and invasive *Candida albicans* strains. J Bacteriol 180:3771-3778.
56. McAlpin, C. E., and B. Mannarelli. 1995. Construction and characterization of a DNA probe for distinguishing strains of *Aspergillus flavus*. Appl Environ Microbiol 61:1068-72.

57. McEwen, J. G., J. W. Taylor, D. Carter, J. Xu, M. S. Felipe, R. Vilgalys, T. G. Mitchell, T. Kasuga, T. White, T. Bui, and C. M. Soares. 2000. Molecular typing of pathogenic fungi. Med Mycol 38 Suppl 1:189-97.

58. Megnegneau, B., F. Debets, and R. F. Hoekstra. 1993. Genetic variability and relatedness in the complex group of black Aspergilli based on random amplification of polymorphic DNA. Curr Genet 23:323-9.

59. Mellado, E., T. M. Diaz-Guerra, M. Cuenca-Estrella, V. Buendia, J. Aspa, E. Prieto, J. R. Villagrasa, and J. L. Rodriguez-Tudela. 2000. Characterization of a possible nosocomial aspergillosis outbreak. Clin Microbiol Infect 6:543-8.

60. Menotti, J., J. Waller, O. Meunier, V. Letscher-Bru, R. Herbrecht, and E. Candolfi. 2005. Epidemiological study of invasive pulmonary aspergillosis in a haematology unit by molecular typing of environmental and patient isolates of *Aspergillus fumigatus*. J Hosp Infect 60:61-8.

61. Meyer, W., E. Lieckfeldt, K. Kuhls, E. Z. Freedman, T. Borner, and T. G. Mitchell. 1993. DNA- and PCR-fingerprinting in fungi. Exs 67:311-20.

62. Mondon, P., M. P. Brenier, F. Symoens, E. Rodriguez, E. Coursange, F. Chaib, B. Lebeau, M. A. Piens, A. M. Tortorano, M. Mallie, F. Chapuis, A. Carlotti, J. Villard, M. A. Viviani, N. Nolard, J. M. Bastide, P. Ambroise-Thomas, and R. Grillot. 1997. Molecular typing of *Aspergillus fumigatus* strains by sequence-specific DNA primer (SSDP) analysis. FEMS Immunol Med Microbiol 17:95-102.

63. Montiel, D., M. J. Dickinson, H. A. Lee, P. S. Dyer, D. J. Jeenes, I. N. Roberts, S. James, L. J. Fuller, K. Matsuchima, and D. B. Archer. 2003. Genetic differentiation of the *Aspergillus* section Flavi complex using AFLP fingerprints. Mycol Res 107:1427-34.

64. Moody, S. F., and B. M. Tyler. 1990. Restriction enzyme analysis of mitochondrial DNA of the *Aspergillus flavus* group: *A. flavus*, *A. parasiticus*, and *A. nomius*. Appl Environ Microbiol 56:2441-52.

65. Neeleman, C., C. H. Klaassen, H. A. de Valk, M. T. de Ruiter, and J. W. Mouton. 2004. Amplified fragment length polymorphism fingerprinting is an effective technique to distinguish *Streptococcus pneumoniae* from other Streptococci and an efficient alternative to pulsed-field gel electrophoresis for molecular typing of Pneumococci. J Clin Microbiol 42:369-71.

66. Nelson, L. A., M. L. Callerame, and R. H. Schwartz. 1979. Aspergillosis and atopy in cystic fibrosis. Am Rev Respir Dis 120:863-73.

67. Neuveglise, C., J. Sarfati, J. P. Debeaupuis, H. Vu Thien, J. Just, G. Tournier, and J. P. Latge. 1997. Longitudinal study of *Aspergillus fumigatus* strains isolated from cystic fibrosis patients. Eur J Clin Microbiol Infect Dis 16:747-50.

68. Neuveglise, C., J. Sarfati, J. Latge, and S. Paris. 1996. Afut1, a retrotransposon-like element from *Aspergillus fumigatus*. Nucl Acids Res 24:1428-1434.

69. Paris, S., and J. P. Latge. 2001. Afut2, a new family of degenerate gypsy-like retrotransposon from *Aspergillus fumigatus*. Med Mycol 39:195-8.

70. Perrone, G., G. Mule, A. Susca, P. Battilani, A. Pietri, and A. Logrieco. 2006. Ochratoxin A production and amplified fragment length polymorphism analysis of *Aspergillus carbonarius*, *Aspergillus tubingensis*, and *Aspergillus niger* strains isolated from grapes in Italy. Appl Environ Microbiol 72:680-5.

71. Pitt, J. I. 2000. Toxigenic fungi: Which are important? Med Mycol 38 Suppl 1:17-22.

72. Puers, C., H. A. Hammond, L. Jin, C. T. Caskey, and J. W. Schumm. 1993. Identification of repeat sequence heterogeneity at the polymorphic short tandem repeat locus HUMTH01[AATG]n and reassignment of alleles in population analysis by using a locus-specific allelic ladder. Am J Hum Genet 53:953-8.

73. Raclavsky, V., J. Trtkova, L. Ruskova, V. Buchta, R. Bolehovska, M. Vackova, and P. Hamal. 2006. Primer R108 performs best in the RAPD strain typing of three *Aspergillus species* frequently isolated from patients. Folia Microbiol 51:136-0.

74. Radford, S. A., E. M. Johnson, J. P. Leeming, M. R. Millar, J. M. Cornish, A. B. Foot, and D. W. Warnock. 1998. Molecular epidemiological study of *Aspergillus fumigatus* in a bone marrow transplantation unit by PCR amplification of ribosomal intergenic spacer sequences. J Clin Microbiol 36:1294-9.

75. Rath, P. M., and R. Ansorg. 1997. Value of environmental sampling and molecular typing of aspergilli to assess nosocomial sources of aspergillosis. J Hosp Infect 37:47-53.

76. Rath, P. M., S. Kamphoff, and R. Ansorg. 1999. Value of different methods for the characterisation of *Aspergillus terreus* strains. J Med Microbiol 48:161-6.

77. Rath, P. M., K. Petermeier, P. E. Verweij, and R. Ansorg. 2002. Differentiation of *Aspergillus ustus* strains by random amplification of polymorphic DNA. J Clin Microbiol 40:2231-3.

78. Renders, N., Y. Romling, H. Verbrugh, and A. van Belkum. 1996. Comparative typing of *Pseudomonas aeruginosa* by random amplification of polymorphic DNA or pulsed-field gel electrophoresis of DNA macrorestriction fragments. J Clin Microbiol 34:3190-5.

79. Rinyu, E., J. Varga, and L. Ferenczy. 1995. Phenotypic and genotypic analysis of variability in *Aspergillus fumigatus*. J Clin Microbiol 33:2567-75.

80. Rodriguez, E., F. Symoens, P. Mondon, M. Mallie, M. A. Piens, B. Lebeau, A. M. Tortorano, F. Chaib, A. Carlotti, J. Villard, M. A. Viviani, F. Chapuis, N. Nolard, R. Grillot, and J. M. Bastide. 1999. Combination of three typing methods for the molecular epidemiology of *Aspergillus fumigatus* infections. European Research Group on Biotype and Genotype of *Aspergillus*. J Med Microbiol 48:181-94.

81. Savelkoul, P. H., H. J. Aarts, J. de Haas, L. Dijkshoorn, B. Duim, M. Otsen, J. L. Rademaker, L. Schouls, and J. A. Lenstra. 1999. Amplified-fragment length polymorphism analysis: The state of an art. J Clin Microbiol 37:3083-91.

82. Schmidt, H., M. Ehrmann, R. F. Vogel, M. H. Taniwaki, and L. Niessen. 2003. Molecular typing of *Aspergillus ochraceus* and construction of species specific SCAR-primers based on AFLP. Syst Appl Microbiol 26:138-46.

83. Schmidt, H., M. H. Taniwaki, R. F. Vogel, and L. Niessen. 2004. Utilization of AFLP markers for PCR-based identification of *Aspergillus carbonarius* and indication of its presence in green coffee samples. J Appl Microbiol 97:899-909.

84. Semighini, C. P., G. Delmas, S. Park, D. Amstrong, D. Perlin, and G. H. Goldman. 2001. New restriction fragment length polymorphism (RFLP) markers for *Aspergillus fumigatus*. FEMS Immunol Med Microbiol 31:15-9.

85. Singh, A., R. V. Goering, S. Simjee, S. L. Foley, and M. J. Zervos. 2006. Application of molecular techniques to the study of hospital infection. Clin Microbiol Rev 19:512-30.

86. Skowasch, K., P. Wiegand, and B. Brinkmann. 1992. pMCT 118 (D1S80): A new allelic ladder and an improved electrophoretic separation lead to the demonstration of 28 alleles. Int J Legal Med 105:165-8.

87. Soll, D. R. 2000. The ins and outs of DNA fingerprinting the infectious fungi. Clin Microbiol Rev 13:332-70.

88. Somashekar, D., E. R. Rati, and A. Chandrashekar. 2004. PCR-restriction fragment length analysis of aflR gene for differentiation and detection of *Aspergillus flavus* and *Aspergillus parasiticus* in maize. Int J Food Microbiol 93:101-7.

89. Symoens, F., S. Bertout, M. A. Piens, J. Burnod, F. Renaud, N. Nolard, F. Chapuis, and R. Grillot. 2001. A longitudinal study of lung transplant recipients infected with *Aspergillus*: Genetic polymorphism of *A. fumigatus*. J Heart Lung Transplant 20:970-8.

90. Symoens, F., J. P. Bouchara, S. Heinemann, and N. Nolard. 2000. Molecular typing of *Aspergillus terreus* isolates by random amplification of polymorphic DNA. J Hosp Infect 44:273-80.

91. Symoens, F., J. Burnod, B. Lebeau, M. A. Viviani, M. A. Piens, A. M. Tortorano, N. Nolard, F. Chapuis, and R. Grillot. 2002. Hospital-acquired *Aspergillus fumigatus* infection: Can molecular typing methods identify an environmental source? J Hosp Infect 52:60-7.

92. Tang, C. M., J. Cohen, A. J. Rees, and D. W. Holden. 1994. Molecular epidemiological study of invasive pulmonary aspergillosis in a renal transplantation unit. Eur J Clin Microbiol Infect Dis 13:318-21.

93. Taylor, J. W., and M. C. Fisher. 2003. Fungal multilocus sequence typing--it's not just for bacteria. Curr Opin Microbiol 6:351-6.

94. Tenover, F. C., R. D. Arbeit, R. V. Goering, P. A. Mickelsen, B. E. Murray, D. H. Persing, and B. Swaminathan. 1995. Interpreting chromosomal DNA restriction patterns produced by pulsed-field gel electrophoresis: criteria for bacterial strain typing. J Clin Microbiol 33:2233-9.

95. Thio, C. L., D. Smith, W. G. Merz, A. J. Streifel, G. Bova, L. Gay, C. B. Miller, and T. M. Perl. 2000. Refinements of environmental assessment during an outbreak investigation of invasive aspergillosis in a leukemia and bone marrow transplant unit. Infect Control Hosp Epidemiol 21:18-23.

96. Tran-Dinh, N., and D. Carter. 2000. Characterization of microsatellite loci in the aflatoxigenic fungi *Aspergillus flavus* and *Aspergillus parasiticus*. Mol Ecol 9:2170-2.

97. Urbanelli, S., V. Della Rosa, F. Punelli, D. Porretta, M. Reverberi, A. A. Fabbri, and C. Fanelli. 2007. DNA-fingerprinting (AFLP and RFLP) for genotypic identification in species of the *Pleurotus eryngii* complex. Appl Microbiol Biotechnol 74:592-600.

98. Urwin, R., and M. C. Maiden. 2003. Multi-locus sequence typing: a tool for global epidemiology. Trends Microbiol 11:479-87.

99. van Belkum, A., W. Melchers, C. Ijsseldijk, L. Nohlmans, H. Verbrugh, and J. Meis. 1997. Outbreak of amoxicillin-resistant *Haemophilus influenzae* type b: Variable number of tandem repeats as novel molecular markers. J Clin Microbiol 35:1517-1520.

100. van Belkum, A., W. G. Quint, B. E. de Pauw, W. J. Melchers, and J. F. Meis. 1993. Typing of *Aspergillus species* and *Aspergillus fumigatus* isolates by interrepeat polymerase chain reaction. J Clin Microbiol 31:2502-5.

101. van Leeuwen, W., M. Sijmons, J. Sluijs, H. Verbrugh, and A. van Belkum. 1996. On the nature and use of randomly amplified DNA from *Staphylococcus aureus*. J Clin Microbiol 34:2770-7.

102. Varga, J. 2006. Molecular typing of aspergilli: Recent developments and outcomes. Med Mycol 44 Suppl:149-61.

103. Varga, J., and J. H. Croft. 1994. Assignment of RFLP, RAPD and isoenzyme markers to *Aspergillus nidulans* chromosomes, using chromosome-substituted segregants of a hybrid of *A. nidulans* and *A. quadrilineatus*. Curr Genet 25:311-7.

104. Verweij, P. E., J. F. G. M. Meis, J. Sarfati, J. A. Hoogkamp-Korstanje, J. P. Latge, and W. J. Melchers. 1996. Genotypic characterization of sequential *Aspergillus fumigatus* isolates from patients with cystic fibrosis. J Clin Microbiol 34:2595-7.

105. Vonberg, R. P., and P. Gastmeier. 2006. Nosocomial aspergillosis in outbreak settings. J Hosp Infect 63:246-54.

106. Vos, P., R. Hogers, M. Bleeker, M. Reijans, T. van de Lee, M. Hornes, A. Frijters, J. Pot, J. Peleman, M. Kuiper, and et al. 1995. AFLP: A new technique for DNA fingerprinting. Nucl Acids Res 23:4407-14.

107. Warris, A., C. H. W. Klaassen, J. F. G. M. Meis, M. T. de Ruiter, H. A. de Valk, T. G. Abrahamsen, P. Gaustad, and P. E. Verweij. 2003. Molecular epidemiology of *Aspergillus fumigatus* isolates recovered from water, air, and patients shows two clusters of genetically distinct strains. J Clin Microbiol 41:4101-4106.

Outline of the thesis

Aspergillus fumigatus is by far the predominant cause of invasive fungal disease. Many different molecular methods have been developed to distinguish between individual *A. fumigatus* isolates. Some techniques showed to be highly discriminating, but they lack reproducibility. Other methods are highly reproducible, but fell short of discriminatory power. Therefore we developed a novel assay for *A. fumigatus* based on short tandem repeats, the so-called STR*Af* assay (Short Tandem Repeats for *A. fumigatus*) which was found to be reproducible and highly discriminating. This assay is described in detail in **Chapter 2**. The remaining of this thesis is focused on two main themes: Technical aspects of this new typing assay and clinical applications.

Part 1. Technical aspects of the STR*Af* assay

Firstly the STR*Af* assay was compared to other typing techniques. In **Chapter 3** this assay was compared with a high-resolution fingerprinting technique, the AFLP (Amplified Fragment Length Polymorphism) method. In **Chapter 4** the STR*Af* assay was tested against the RISC (Retrotransposon Insertion-Site Context typing) method, which is a PCR alternative to RFLP (Restriction Fragment Length Polymorphism), the so-called gold standard for *A. fumigatus* typing. To gain more insight in the genetic relationships between isolates from worldwide origin, it is important that fingerprinting methods are reproducible and transferable regardless of the place of analysis. We tested varying components of the PCR amplification mixtures in **Chapter 5** to evaluate the robustness of the STR*Af* assay. Although the STR*Af* assay proved to be an extremely robust assay, one has to be aware that different electrophoresis platforms need careful calibration to exchange STR*Af* data between laboratories. This has to be established with the use of allelic ladders. In **Chapter 6** we describe the development of these allelic ladders, which were subsequently tested in an international multicenter study.

In **Chapter 7** the extent of stability of the STR*Af* markers in successive generations of clonally expanded *A. fumigatus* isolates was investigated. This data is useful to interpret relations between isolates with minor differences in the STR*Af* typing results.

Part 2. Clinical applications

There are several clinical manifestations in which *A. fumigatus* plays an important role. One of these is invasive aspergillosis, which has emerged as a leading cause of morbidity and mortality in immunocompromised patients. To provide a better understanding of the epidemiology and pathogenesis of this disease, 55 isolates from 15 patients with proven IA were investigated in **Chapter 3**. STR*Af* typing was subsequently tested for its utility in outbreak settings where it is important to recognize genetically related clusters from genetically unrelated genotypes. Several epidemiologically linked *A. fumigatus* isolates from multiple outbreak investigations across Northern America were typed and described in **Chapter 8**. *A. fumigatus* can colonize the respiratory tract of patients with compromised airways. Whether colonization of the respiratory tract is due to one or several isolates or if frequent recolonization takes place can be evaluated by typing cultured isolates from sequential respiratory samples. In **Chapter 9** several *A. fumigatus* isolates from cystic fibrosis patients were typed and the different colonization patterns in the patients were linked to the clinical aspects. In **Chapter 10** the STR*Af* assay was used for detection and genotyping of *A. fumigatus* directly in clinical samples. Genotypes, obtained from cultured isolates, were compared with the STR*Af* results from formalin fixed, paraffin-embedded (FFPE) deep tissue samples and from corresponding patient sera.

Overall results and conclusions of the studies in this thesis are discussed and summarized in **Chapter 11**.

Chapter 2

Use of a novel panel of nine short tandem repeats for exact and high-resolution fingerprinting of *Aspergillus fumigatus* isolates

Hanneke A. de Valk, Jacques F.G.M. Meis, Ilse M. Curfs, Konrad Muehlethaler, Johan W. Mouton and Corné H.W. Klaassen

Journal of Clinical Microbiology 2005, 43;8: 4112-4120.

Abstract

In this chapter we describe a new panel of short tandem repeats (STRs) for a novel exact typing assay that can be used to discriminate between *Aspergillus fumigatus* isolates. A total of nine STR markers were selected from available genomic *A. fumigatus* sequences and were divided into three multicolor multiplex PCRs. Each multiplex reaction amplified three di-, tri-, or tetranucleotide repeats, respectively. All nine STR markers were used to analyze 100 presumably unrelated *A. fumigatus* isolates. For each marker, between 11 and 37 alleles were found in this population. One isolate proved to be a mixture of at least two different isolates. With the remaining 99 isolates, 96 different fingerprinting profiles were obtained. The Simpson's diversity index for the individual markers ranged from 0.77 to 0.97. The diversity index for the multiplex combination of di-, tri-, and tetranucleotide repeats ranged from 0.9784 to 0.9968. The combination of all nine markers yielded a Simpson's diversity index of 0.9994, indicative of the high discriminatory power of these new loci. In theory, this panel of markers is able to discriminate between no less than 27×10^9 different genotypes. The multicolor multiplex approach allows large numbers of markers to be tested in a short period of time. The exact nature of the assay combines high reproducibility with the easy exchange of results and makes it a very suitable tool for large-scale epidemiological studies.

Introduction

Aspergillus species are widely distributed fungi which release large amounts of conidia that are dispersed into the environment by air. Inhalation of *Aspergillus* conidia is a common event but rarely causes complications in immunocompetent hosts. In immunocompromised individuals, however, it can cause invasive aspergillosis (IA), often with fatal consequences. Because of the increase in the numbers of patients undergoing bone marrow or solid organ transplantation, the incidence of IA has increased dramatically in recent years. *Aspergillus fumigatus* is the most common species responsible for IA [9].

Molecular tools may provide a better understanding of the genetic and the epidemiological relationships between environmental and clinical isolates and thereby allow assessment of potential routes of transmission. Understanding of these routes may lead to improved strategies toward the prevention of *Aspergillus* infections. Several phenotypic and genotypic studies that can be used to distinguish between individual *A. fumigatus* isolates have been described. Phenotypic typing schemes include morphological and serological features as well as isoenzyme analysis. Genotypic methods include several techniques, such as random amplified polymorphic DNA (RAPD) analysis [1, 10, 11], restriction fragment length polymorphism (RFLP) analysis [13], amplified fragment length polymorphism (AFLP) analysis [18], and microsatellite length polymorphism [2]. The last technique is sometimes also called microsatellite polymorphism analysis [5] or polymorphic microsatellites marker analysis [8]. However, a major problem with pattern-based techniques, such as RAPD, RFLP, and AFLP analyses, is the poor interlaboratory reproducibility. The exchange of the results obtained by these techniques is therefore very difficult, if not impossible. Microsatellites, or short tandem repeats (STRs), are extensively being used for highresolution fingerprinting of the human genome, but they have also been shown to provide a high level of discrimination between different isolates of several bacterial pathogens [7, 14, 16] and yeast [17]. If they are properly performed, STR analyses yield highly reproducible, exact typing results. This would eliminate the need for repetitive analyses and would allow the easy exchange of data.

We describe in this chapter a new panel of nine STRs for the exact and high-resolution fingerprinting of *A. fumigatus*.

Materials and Methods

Tandem repeat locus identification. Preliminary sequence data were obtained from The Wellcome Trust Sanger Institute (www.sanger.ac.uk) and were analyzed for the presence of STRs by using the Tandem Repeats Finder software (http://c3.biomath.mssm.edu/trf.html) [4]. Since the *A. fumigatus* genome is estimated to contain about 35×10^6 bp, the available genomic sequences may represent nearly the entire genome. The minimum alignment score was set at 40, and the maximum period size (repeat unit) was set at 10 nucleotides. We selected three di-, tri-, and tetranucleotide repeats based on loci with the highest repeat numbers and counterselected on loci containing two or more repeat sequences within the boundaries of potential PCR primer regions. Interrupted repeats which may have a lower chance of displaying interstrain repeat number variation were not taken into consideration.

Isolates. Ninety-nine clinical *A. fumigatus* isolates and one reference strain (CBS 487.65) were used in this study. The isolates were assumed to be unrelated because they were all collected from different patients either at different hospitals, from different wards, or at different points in time. The isolates were collected at six different hospital centers; five are located in The Netherlands, and one is located in Bern, Switzerland. Fifteen isolates were collected from patients with IA over a period of 4 years obtained from the University Hospital Nijmegen (Nijmegen, The Netherlands; location A). Another 29 isolates were obtained from the Canisius Wilhelmina Hospital (Nijmegen, The Netherlands); of these 29 isolates, 16 were from patients on the Intensive Care Unit (location B) and 13 were from patients the Pulmonology Ward (location C). Eleven isolates were collected over a period of 2 years from a cystic fibrosis center (Dekkerswald, Nijmegen, The Netherlands; location D). Another 14 isolates were from the Maashospital (Boxmeer, The Netherlands; location E).

Eight isolates were from the University Hospital Maastricht (Maastricht, The Netherlands; location F), and 22 isolates were collected at the University Hospital Bern (Bern, Switzerland; location G). Isolates were stored at -80°C, according to standard procedures. Eleven *Aspergillus* species other than *A. fumigatus* were taken from the private collection at the Canisius Wilhelmina Hospital.

Identification of isolates. Fungal isolates were identified by their macroscopic and microscopic characteristics. Furthermore, all *A. fumigatus* isolates were tested for their ability to grow at 48°C.

DNA isolation. DNA was isolated by using the following culture and pretreatment conditions. Isolates were grown on Sabouraud agar plates at 30°C until sporulation. A prewetted cotton swab was saturated with conidia from a sporulating culture. Next, the spores were resuspended in a vial containing 350 µl lysis buffer and ceramic beads (Roche Diagnostics, Almere, The Netherlands) and subjected to mechanical lysis in a Magnalyzer instrument (Roche Diagnostics) for 30 s at 6,500 rpm. Following pretreatment, the DNA was further extracted and purified with a MagNA Pure LC instrument (Roche Diagnostics) in combination with a MagNA Pure DNA isolation kit III, according to the recommendations of the manufacturer. This DNA extraction protocol routinely yielded 5 to 20 µg DNA of excellent purity, as determined by UV absorbance measurements.

PCR amplification and genotyping. Three separate multiplex PCRs (M2, M3, and M4, respectively), each of which contained three different STRs, were developed. Multiplex PCR STR*Af* 2 (M2) amplified three dinucleotide loci. Multiplex PCR STR*Af* 3 (M3) amplified three trinucleotide loci, and multiplex PCR STR*Af* 4 (M4) contained three tetranucleotide loci. For all loci selected, PCR primers were designed with the use of the Primer3 software (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) [15].

For each of the three multiplex PCRs, the forward primers were labeled with carboxyfluorescein (FAM), hexachlorofluorescein (HEX), or tetrachlorofluorescein (TET) at the 5' end, respectively. An overview of the PCR primer sequences is shown in Table 1. Each PCR mixture contained 1 μM of all amplification primers, 0.2 mM deoxynucleoside triphosphates, 1 U of FastStart *Taq* DNA polymerase (Roche Diagnostics), and 1 ng of genomic DNA in 1 × reaction buffer. The optimal $MgCl_2$ concentrations were 1.5 mM for M2 and 3.0 mM for M3 and M4. Thermocycling was performed in a T1 thermocycler (Biometra, Göttingen, Germany) by using the following thermal protocol: 10 min of denaturation at 95°C, followed by 30 cycles of 30 s of denaturation at 95°C, 30 s of annealing at 60°C, and 1 min of extension at 72°C. Before the reaction mixtures were cooled to room temperature, an additional incubation for 10 min at 72°C was performed. All temperature transitions were performed with maximal heating and cooling settings (5°C/s). The fragments obtained were combined with the ET400-R size standard (GE Healthcare, Roosendaal, The Netherlands) and analyzed on a MegaBACE 500 automated DNA platform (GE Healthcare), according to the instructions of the manufacturer. In order to avoid PCR amplicon contamination, pre- and post-PCR procedures (DNA isolation, preparation of master mixes, amplicon analysis) were performed in physically separated facilities.

DNA sequence analysis. To determine the exact number of repeats in the obtained PCR products, a selected number of fragments (see Table 3) were inserted into the pGEM-T Easy Vector (Promega, Leiden, The Netherlands). After transformation of competent JM109 cells, individual colonies were selected and grown in liquid Luria-Bertani medium under selective pressure. Plasmid DNA was purified from the cultures that were obtained by using High Pure chemistry (Roche Diagnostics) and were sequenced with a universal M13 primer by using the MegaBACE DYEnamic ET Dye terminator kit, as suggested by the manufacturer (GE Healthcare).

The reaction products were purified by using SPRI chemistry (GC-Biotech, Schiedam, The Netherlands), eluted in distilled water, and analyzed on a MegaBACE 500 capillary DNA analysis platform (GE Healthcare) by using standard electrophoretic conditions.

Table 1. Overview of amplification primers for selected STR loci, details of repeat characteristics, and discriminatory indices

PCR and primer name	Forward primer sequence[a]	Reverse primer sequence[a]	Repeat unit	No. of repeats[b]			No. of alleles	D value[d]
				Min	Max	Ref[c]		
M2								
STR*Af* 2A	FAM-AAGGGTTATGGCCATTAGGG	GACCTCCAGGCAAAATGAGA	GA	10	28	26	14	0.88
STR*Af* 2B	HEX-TATTGGATCTGCTCCCAAGC	GAGATCATGCCCAAGGATGT	AG	9	29	18	16	0.88
STR*Af* 2C	TET-TCGGAGTAGTTGCAGGAAGG	AACGCGTCCTAGAATGTTGC	CA	8	33	18	17	0.88
M3								
STR*Af* 3A	FAM-GCTTCGTAGAGCGGAATCAC	GTACCGCTGCAAAGGACAGT	TCT	10	61	46	37	0.97
STR*Af* 3B	HEX-CAACTTGGTGTCAGCGAAGA	GAGGTACCACAACACAGCACA	AAG	8	73	20	14	0.84
STR*Af* 3C	TET-GGTTACATGGCTTGGAGCAT	GTACACAAAGGGTGGGATGG	TAG	5	51	23	32	0.86
M4								
STR*Af* 4A	FAM-TTGTTGGCCGCTTTTACTTC	GACCCAGCGCCTATAAATCA	TTCT	5	24	11	14	0.84
STR*Af* 4B	HEX-CGTAGTGACCTGAGCCTTCA	GGAAGGCTGTACCGTCAATCT	CTAT	5	26	10	11	0.80
STR*Af* 4C	TET-CATATTGGGAAACCCACTCG	ACCAACCCATCCAATTCGTAA	ATGT	5	45	8	15	0.77

[a] All primer sequences are given in the 5' to 3' direction.
[b] Number of repeats found in the reference population described in this study.
[c] Number of repeats in the reference strain AF293, upon which the genome sequences available from the *A. fumigatus* Sequencing Group at The Sanger Institute are based.
[d] The calculated D value according to Simpson's index of diversity.

Data analysis. The repeat numbers of the nine markers of all isolates were analyzed by using BioNumerics, version 3.5, software (Applied Maths, Kortrijk, Belgium) and the unweighted pair group method with arithmetic averages with the multistate categorical similarity coefficient. All markers were given an equal weight. In the resulting dendrogram, the indicated percentages reflect the number of corresponding markers. Accordingly, two strains with six of the nine corresponding markers are 66.7% identical.

Discriminatory power. The discriminatory power of a typing method can be defined mathematically as the probability that two unrelated isolates chosen at random from a test population will be different by that typing method and can be calculated by using the Simpson index of diversity (*D*):

$$D = 1 - \frac{1}{N(N-1)} \sum_{j=1}^{S} n_j (n_j - 1)$$

where N is the total number of isolates in the test population, s is the total number of types described, and n_j is the number of isolates belonging to the jth type [6]. A D value of 1.0 indicates that the typing method is able to discriminate between all isolates. A D value of 0.0 indicates that all isolates are identical.

Results

A selection of nine loci (three dinucleotide repeats, three trinucleotide repeats, and three tetranucleotide repeats) was made, and three multiplex PCRs (M2, M3, and M4) were developed and optimized. These three multiplex PCRs were used to analyze 100 *A. fumigatus* isolates from nonoutbreak situations. Typical amplification results are shown in figure 1.

The PCR amplification products of the tetranucleotide repeats consisted mainly of a single peak. However, the amplification products of the tri- and dinucleotide repeats usually contained more peaks, as they were one to three repeat units shorter. However, the longest peak was usually the most intense peak; this one was used for analysis (Fig. 1). Under some conditions (especially with high DNA concentrations), an extra peak 1 bp shorter than the main peak was observed; this is another established PCR artifact (Fig. 1).

In order to determine the exact repeat number of the repeats in all markers, a selected number of alleles throughout the entire range of alleles obtained were analyzed by DNA sequence analysis (Fig. 2). The repeat numbers of the remaining alleles (being at regularly spaced intervals) were determined by extrapolation, so that an exact repeat number could be specified for all nine loci.

The collection of 100 isolates was analyzed by using the complete panel of nine STR markers. One isolate displayed multiple peaks for all nine markers, which was probably the result of the fact that the isolate was a mixture of two different *A. fumigatus* isolates. With the remaining 99 isolates, a total of 96 different marker combinations were obtained. Three pairs of apparently unrelated isolates yielded the same combination. For all nine loci and combinations thereof, a D value was calculated (Tables 1 and 2).

The highest discriminatory power value for a single locus was obtained with STR*Af* 3A, with a *D* value of 0.97. With this marker no less than 37 different alleles were found in this population. An overview of all alleles obtained is shown in Table 3. The combination of all nine markers yielded a *D* value of 0.9994.

If the variation in length between the different alleles were the sole effect of variations in repeat number, then the sizes of all alleles would be expected to occur at regularly spaced intervals (i.e., a 2-bp interval for the dinucleotide repeats and 3- and 4-bp intervals for the tri- and tetranucleotide repeats, respectively). Incidentally, a peak was found at a position halfway to the next allele (i.e., in STR*Af* 4C between repeat numbers 7 and 8). DNA sequence analysis of this allele demonstrated that the fragment contained a 2-bp deletion. Analogous to the nomenclature for such alleles in the human situation, this allele was denominated 7.2 (the same size as 7 full repeats plus 2 bp). Other confirmed aberrations were named accordingly.

To test the stability of each locus, a random *A. fumigatus* isolate was subcultured a minimum of 30 times. DNA was isolated from every fifth subculture, and all DNA samples were tested by using all nine markers. The exact same number of repeats compared to the number in the original sample was obtained for all nine markers, indicating that the repeats are sufficiently stable to allow epidemiological surveys (data not shown).

Table 2: Diversity indices for all multiplex combinations

Combination of markers	No. of profiles[a]	D value[b]
M2	60	0.9879
M3	86	0.9968
M4	51	0.9784
M2+M3	94	0.9988
M2+M4	76	0.9928
M3+M4	95	0.9992
M2+M3+M4	96	0.9994

[a] The total number of isolates is 99.
[b] The calculated D value is based on Simpson's index of diversity.

In order to test the specificities of all markers for *A. fumigatus*, DNA from the following 11 other *Aspergillus* species were tested with all nine markers: *A. clavatus*, *A. chevalieri*, *A. flavus*, *A. glaucus*, *A. niger*, *A. nidulans*, *A. ochraceus*, *A. sodowii*, *A. terreus*, *A. ustus*, and *A. versicolor*. No amplification products were obtained with any of these species, indicating that the markers have a high specificity for *A. fumigatus*. Interestingly, one nonpigmented *A. fumigatus* variant from our collection yielded an amplification product with a single marker only, leaving the possibility of discrimination between cryptic *A. fumigatus* species.

Discussion

At this time sequencing and assembly of the entire *A. fumigatus* genome approaches completion. This provided the opportunity to search through already available genomic sequences for potential new microsatellite loci in silico by using software available in the public domain. We applied a number of criteria to select or deselect candidate loci. Obviously, a repeat sequence can be regarded as a repeat sequence only if the repeat number is equal to or greater than 2. Since the chance that interstrain variation will be displayed increases with the repeat number, we started by selecting potentially suitable loci at the high end. Only loci with perfect repeat sequences were included in our analysis; imperfect repeats containing point mutations and/or insertion or deletions were excluded. Another criterion used for the exclusion of loci is the presence of a second repeat sequence. Certain loci with high repeat numbers were not considered due to the presence of an additional repeat sequence within the boundaries of potential PCR primer binding sites. Different combinations of two repeat sequences in a single PCR amplicon may lead to the formation of fragments of the same length. This makes it impossible to determine the exact repeat number for each of the two repeats and compromises the exact nature of the STR assay. The final exclusion criterion involved the absence of flanking sequences. Obviously, in these cases no PCR primer sequences can be designed. For this reason, a number of potentially interesting hexanucleotide repeat sequences had to be excluded.

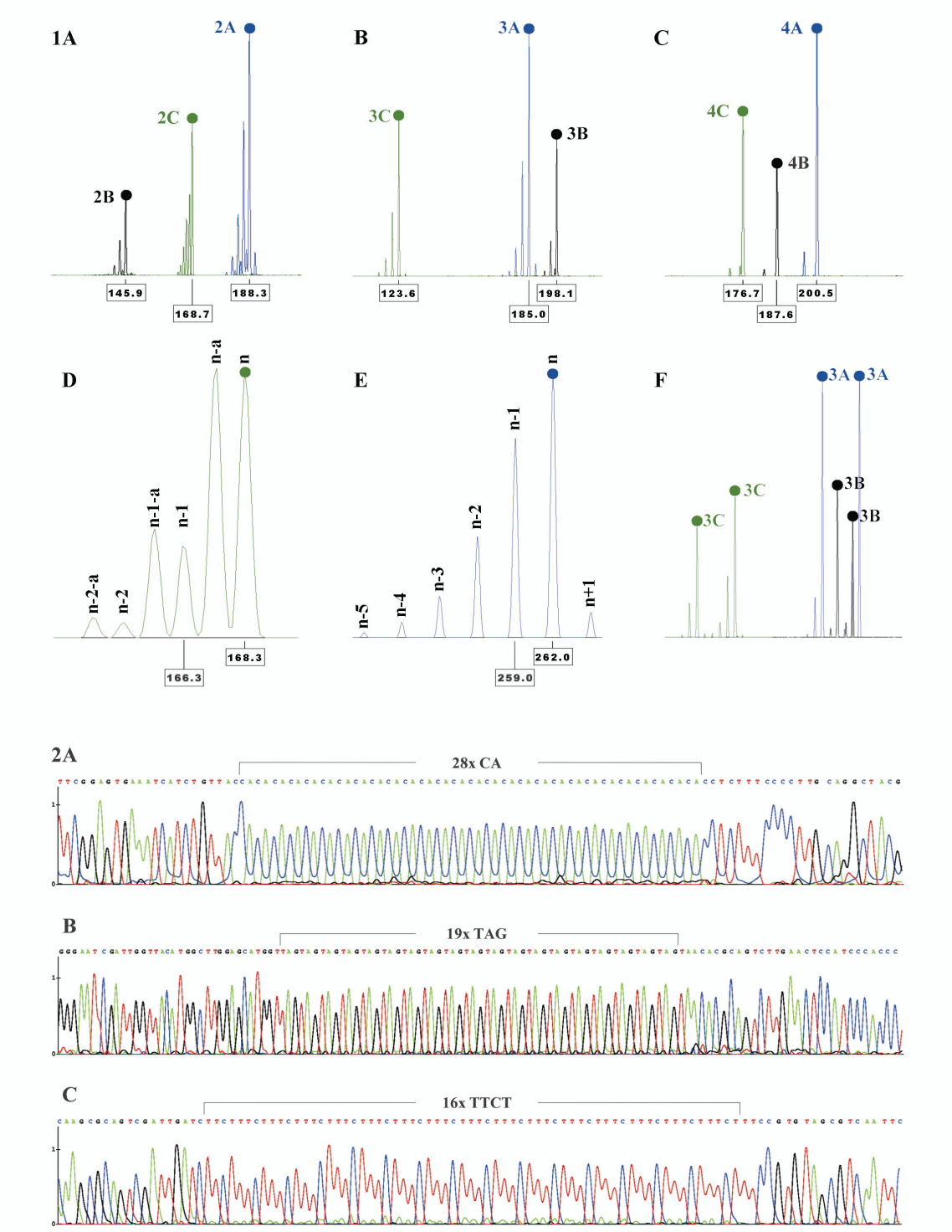


Figure 1. Examples of amplification results. The principle peak used for analysis is indicated with a colored dot. Blue traces represent the FAM label, black traces represent the HEX label, and green traces represent the TET label. Typical results obtained with the dinucleotide repeats (A), trinucleotide repeats (B), and tetranucleotide repeats (C) are shown. (D) Typical example of amplification artifacts obtained with a dinucleotide repeat. The principle peak is indicated with an "n." A second peak exactly 1 bp shorter is the result of incomplete A addition (hence, n - a). A stutter peak one repeat shorter is indicated with n - 1. Other artifact peaks are indicated accordingly. (E) Multiple stutter peaks with a trinucleotide repeat do not influence the ability to identify the principle peak in a sample. (F) A typical result obtained with a mixed sample.

Figure 2. Determination of repeat numbers by DNA sequencing. Examples are shown for a dinucleotide repeat (A), trinucleotide repeat (B), and tetranucleotide repeat (C).

Once the entire genome sequence of *A. fumigatus* has been assembled, these loci also could prove to be interesting candidates for analysis. We finally selected three dinucleotide loci, three trinucleotide loci, and three tetranucleotide loci for further analysis.

Analysis of 100 presumed unrelated *A. fumigatus* isolates yielded 96 different profiles; 3 profiles were found twice, and in one sample double bands were obtained for all nine markers. As mentioned above, all isolates were assumed to be unrelated because they were all collected from different patients either at different hospitals, from different wards, or at different points in time. However, our results may indicate that the three profiles found twice were actually from related isolates. Unfortunately, studies comparing different fingerprinting techniques for *A. fumigatus* show that, up to now, no single technique can be considered the "gold standard" [3, 8]. Additional fingerprinting of these isolates by AFLP analysis [18] also showed that these three pairs of isolates were indistinguishable from each other (results not shown). Since the genome of *Aspergillus* is haploid, double bands are typically a result of having started with an impure culture (i.e., a mixture of two different *A. fumigatus* isolates). In this case, the ratio between the heights and/or areas of these peaks will reflect the ratio between the two different isolates. The one isolate containing double bands for all nine markers was recultivated on Sabouraud agar plates. At the first occurrence of microcolonies, these were individually expanded on new agar plates and analyzed by using all nine markers. In these samples, two different profiles were obtained, each of which contained single peaks for all nine markers and each peak of which represented either of the two original peaks (not shown). The ability to easily identify the presence of multiple genotypes in a sample provides STR analysis with a unique advantage over other PCR fingerprinting methods like RAPD or AFLP analysis, by which such events usually remain unrecognized.

Table 3: Overview of the alleles obtained and their frequency distribution in this reference population

Allele	Fragment size (bp)	Repeat no.	Frequency (%)	Allele	Fragment size (bp)	Repeat no.	Frequency (%)
STR*Af* 2A	163.1	10[a]	9		200.7	30[a]	3
	164.9	11	1		203.5	31	5
	168.8	13	3		206.9	32	4
	170.8	14[a]	8		209.9	33	2
	176.6	17	1		212.3	34	6
	178.6	18	21		215.9	35	4
	180.5	19	4		218.7	36	3
	182.4	20	5		222.0	37	4
	184.3	21	6		225.2	38	2
	188.3	23	20		228.4	39	2
	190.2	24	3		230.7	40[a]	2
	192.2	25[a]	8		249.8	46[a]	1
	194.0	26	9		253.2	47	1
	198.1	28	1		255.5	48	2
					258.9	49	2
STR*Af* 2B	124.6	9	1		262.0	50[a]	2
	126.5	10[a]	1		284.1	57[a]	1
	130.3	12[a]	15		290.2	59	1
	134.2	14	2		296.4	61	1
	138.1	16[a]	13				
	140.2	17	1	STR*Af* 3B	158.0	8	1
	142.1	18	2		160.5	9	23
	144.0	19[a]	12		163.5	10	21
	146.0	20[a]	21		166.5	11	23
	147.8	21	7		169.3	12	7
	149.8	22	1		172.4	13	11
	151.6	23[a]	11		177.9	15	1
	153.7	24	2		183.6	17	4
	155.6	25	8		186.6	18	1
	161.4	28	1		198.0	22[a]	2
	163.6	29[a]	1		203.9	24	2
					226.9	32	1
STR*Af* 2C	154.8	8	23		232.0	34[a]	1
	156.5	9[a]	3		348.9	73	1
	158.7	10	12				
	160.5	11	13	STR*Af* 3C	77.5	5[a]	1
	162.8	12	4		80.1	6	3
	164.6	13[a]	2		82.9	7	33
	166.4	14[a]	3		86.2	8	1
	168.3	15	16		89.2	9[a]	1
	170.6	16[a]	7		92.2	10	6
	172.1	17	2		98.3	12	1
	174.5	18	2		101.2	13	3
	176.4	19[a]	6		104.6	14[a]	3
	184.3	23[a]	1		107.5	15	1
	189.9	26	1		110.9	16	1
	195.9	29[a]	2		114.0	17	2
	198.0	30	1		117.2	18	1
	203.9	33[a]	1		120.5	19[a]	1
					123.4	20	11
STR*Af* 3A	138.1	10	5		126.4	21	12
	141.3	11[a]	1		129.5	22	1
	147.3	13	1		135.7	24[a]	1
	150.5	14	1		138.9	25	2
	153.8	15	3		144.9	27	1
	156.5	16	2		157.4	31	1
	159.7	17	2		160.4	32	1
	163.3	18	2		163.7	33	1
	166.5	19[a]	1		166.9	34	1
	172.1	21	1		185.2	40[a]	1
	175.3	22[a]	2		190.8	42	1
	178.6	23	2		200.5	45	1
	182.2	24	1		203.5	46	1
	184.9	25	6		206.9	47[a]	1
	187.8	26[a]	6		212.5	49[a]	2
	191.3	27	7		215.8	50	1
	194.4	28	3		218.8	51	1
	197.3	29	5				

Continued on facing page

Table 3-*Continued*

Allele	Fragment size (bp)	Repeat no.	Frequency (%)	Allele	Fragment size (bp)	Repeat no.	Frequency (%)
STR*Af* 4A	167.8	5[a]	2		196.0	12[a]	1
	176.2	7	9		236.9	22	1
	180.6	8	30		240.7	23	1
	185.0	9	10		249.5	25	1
	188.9	10[a]	21		252.9	26	9
	197.4	12	2				
	200.6	12.3[a]	5	STR*Af* 4C	164.0	5[a]	41
	201.4	13	9		168.0	6	4
	209.6	15[a]	3		172.5	7	4
	216.7	16.3	1		174.8	7.2[a]	1
	221.1	17.3	1		176.4	8	23
	226.3	19[a]	1		185.1	10	5
	233.8	21[a]	4		189.1	11	1
	246.3	24	1		193.3	12	2
					201.6	14[a]	2
STR*Af* 4B	166.7	5[a]	9		213.7	17[a]	2
	175.3	7	1		225.8	20	9
	179.4	8	7		246.8	25[a]	1
	183.0	9	27		266.9	30[a]	2
	187.6	10[a]	32		295.8	37	1
	191.6	11	10		328.1	45	1

[a] The repeat was sequenced to determine the exact number of repeats; the repeat numbers of the remaining alleles were determined by extrapolation.

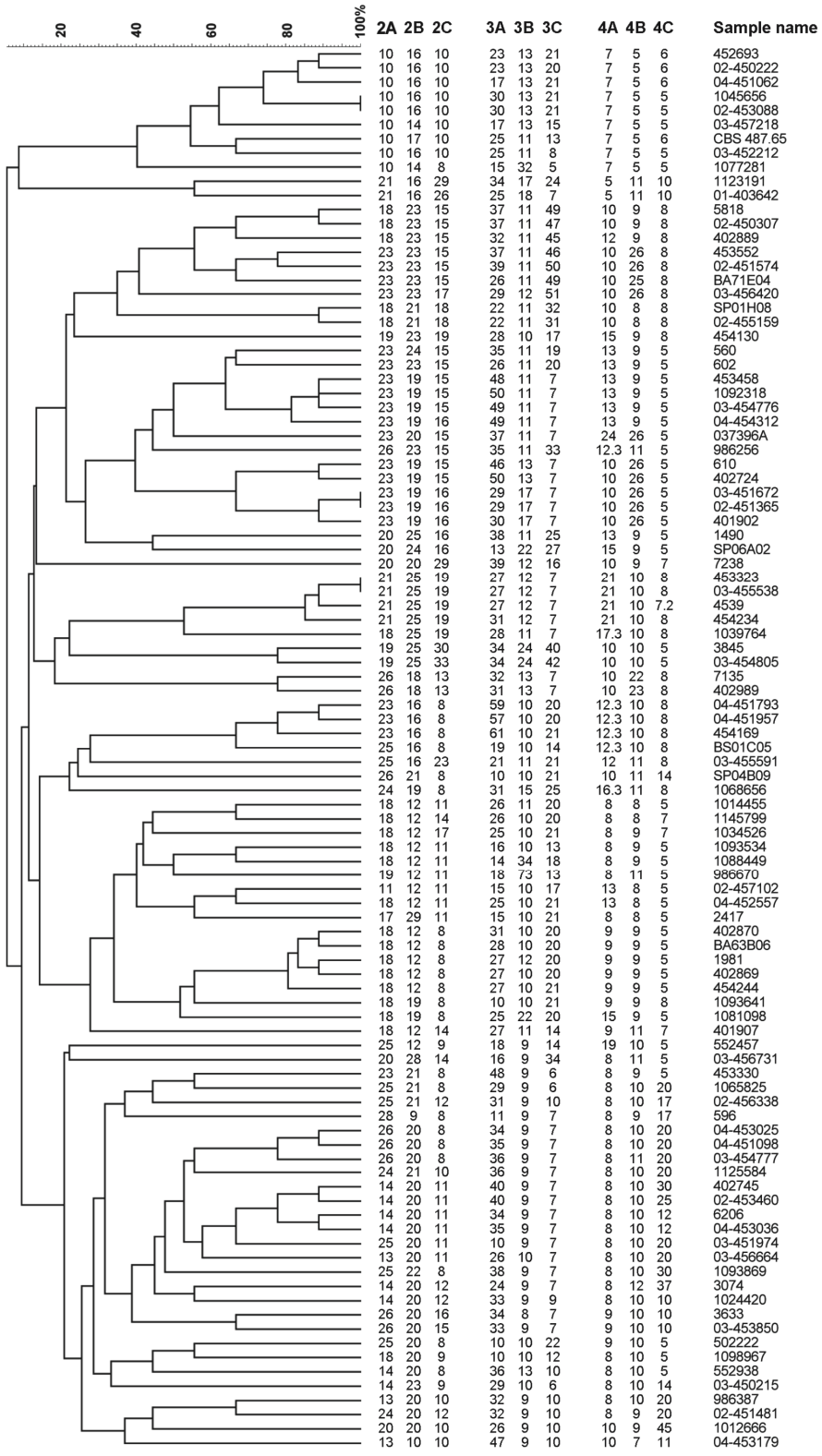
Unlike what might be expected from a fingerprinting technique that covers large parts of an organism's genome, this novel assay based on nine STR markers is probably not suitable for determination of the geographical origins of isolates. Indeed, there were no obvious subclusters of isolates from any of the hospitals (Fig. 3). This, however, must be substantiated by analyzing many more isolates of diverse geographical origins.

STR analysis is prone to several PCR artifacts that may influence the results. One of them is slippage of the *Taq* polymerase during amplification, a process that leads to the formation of so-called stutter peaks. There is a clear inverse relation between the size of the repeat unit and the formation of stutter peaks. With shorter repeat units, higher proportions of stutter peaks are generated [12]. This is also clearly observed in our results, where stutter peaks are most prominently seen with the dinucleotide repeats, fewer stutter peaks are seen with trinucleotide repeats, and stutter peaks are almost absent with the tetranucleotide repeats. The occurrence of too many stutter peaks may complicate identification of the principle peak in a sample. For this reason, mononucleotide repeats, which often yield as many as 10 to 15 stutter peaks, were not taken into consideration.

It has been suggested that addition of dimethyl sulfoxide to the amplification reactions or replacement of *Taq* DNA polymerase by other thermostable polymerases with higher processivities might reduce the formation of stutter peaks [12]. Such adaptations were also evaluated, but none of them yielded improved results (data not shown).

A basic understanding of potential PCR artifacts upon amplification and analysis of the STR sequences (as illustrated in Fig. 1D and E) is desired, but we conclude that the presence of stutter peaks will not influence the ability to identify the major peak in a sample when repeat units of three and above are used (i.e., at least trinucleotide repeats). This is a clear advantage over previously reported STR assays for *A. fumigatus*, where only dinucleotide repeats were analyzed [2]. Another wellknown PCR artifact is the addition of an extra A residue to the 3' end of the PCR product by *Taq* DNA polymerase (known as template-independent polymerase activity or extendase activity). This activity is most pronounced when the ultimate residue at the 3' end of the PCR product is a pyrimidine; the presence of a purine leads to incomplete A-residue addition and to the formation of additional unwanted peaks. To promote maximal addition of this A residue, all reverse amplification primers were designed to begin with a purine.

The number of different alleles found in our reference population with each of the nine markers is given in Table 1. This means that in theory no less than 27×10^9 different combinations can be discriminated with this panel of nine markers. This is an improvement of close to 10^6-fold over that obtained with the previously reported STR panel [2]. If we assume that all intermittent alleles (not present in our reference population) also exist, this number increases to an overwhelming 3×10^{13} possible combinations. However, since there is no known sexual reproduction of *A. fumigatus*, all nine markers do not inherit independently from parent to daughter. It is therefore unlikely that all possible combinations indeed occur. As a natural consequence, certain (combinations of) alleles will occur more frequently than others, a phenomenon that was also observed in our reference population. Each combination of these nine markers should therefore be considered a single haplotype.



2A	2B	2C	3A	3B	3C	4A	4B	4C	Sample name
10	16	10	23	13	21	7	5	6	452693
10	16	10	23	13	20	7	5	6	02-450222
10	16	10	17	13	21	7	5	6	04-451062
10	16	10	30	13	21	7	5	5	1045656
10	16	10	30	13	21	7	5	5	02-453088
10	14	10	17	13	15	7	5	5	03-457218
10	17	10	25	11	13	7	5	6	CBS 487.65
10	16	10	25	11	8	7	5	5	03-452212
10	14	8	15	32	5	7	5	5	1077281
21	16	29	34	17	24	5	11	10	1123191
21	16	26	25	18	7	5	11	10	01-403642
18	23	15	37	11	49	10	9	8	5818
18	23	15	37	11	47	10	9	8	02-450307
18	23	15	32	11	45	12	9	8	402889
23	23	15	37	11	46	10	26	8	453552
23	23	15	39	11	50	10	26	8	02-451574
23	23	15	26	11	49	10	25	8	BA71E04
23	23	17	29	12	51	10	26	8	03-456420
18	21	18	22	11	32	10	8	8	SP01H08
18	21	18	22	11	31	10	8	8	02-455159
19	23	19	28	10	17	15	9	8	454130
23	24	15	35	11	19	13	9	5	560
23	23	15	26	11	20	13	9	5	602
23	19	15	48	11	7	13	9	5	453458
23	19	15	50	11	7	13	9	5	1092318
23	19	15	49	11	7	13	9	5	03-454776
23	19	16	49	11	7	13	9	5	04-454312
23	20	15	37	11	7	24	26	5	037396A
26	23	15	35	11	33	12.3	11	5	986256
23	19	15	46	13	7	10	26	5	610
23	19	15	50	13	7	10	26	5	402724
23	19	16	29	17	7	10	26	5	03-451672
23	19	16	29	17	7	10	26	5	02-451365
23	19	16	30	17	7	10	26	5	401902
20	25	16	38	11	25	13	9	5	1490
20	24	16	13	22	27	15	9	5	SP06A02
20	20	29	39	12	16	10	9	7	7238
21	25	19	27	12	7	21	10	8	453323
21	25	19	27	12	7	21	10	8	03-455538
21	25	19	27	12	7	21	10	7.2	4539
21	25	19	31	12	7	21	10	8	454234
18	25	19	28	11	7	17.3	10	8	1039764
19	25	30	34	24	40	10	10	5	3845
19	25	33	34	24	42	10	10	5	03-454805
26	18	13	32	13	7	10	22	8	7135
26	18	13	31	13	7	10	23	8	402989
23	16	8	59	10	20	12.3	10	8	04-451793
23	16	8	57	10	20	12.3	10	8	04-451957
23	16	8	61	10	21	12.3	10	8	454169
25	16	8	19	10	14	12.3	10	8	BS01C05
25	16	23	21	11	21	12	11	8	03-455591
26	21	8	10	10	21	10	11	14	SP04B09
24	19	8	31	15	25	16.3	11	8	1068656
18	12	11	26	11	20	8	8	5	1014455
18	12	14	26	10	20	8	8	7	1145799
18	12	17	25	10	21	8	9	7	1034526
18	12	11	16	10	13	8	9	5	1093534
18	12	11	14	34	18	8	9	5	1088449
19	12	11	18	73	13	8	11	5	986670
11	12	11	15	10	17	13	8	5	02-457102
18	12	11	25	10	21	13	8	5	04-452557
17	29	11	15	10	21	8	8	5	2417
18	12	8	31	10	20	9	9	5	402870
18	12	8	28	10	20	9	9	5	BA63B06
18	12	8	27	12	20	9	9	5	1981
18	12	8	27	10	20	9	9	5	402869
18	12	8	27	10	21	9	9	5	454244
18	19	8	10	10	21	9	9	8	1093641
18	19	8	25	22	20	15	9	5	1081098
18	12	14	27	11	14	9	11	7	401907
25	12	9	18	9	14	19	10	5	552457
20	28	14	16	9	34	8	11	5	03-456731
23	21	8	48	9	6	8	9	5	453330
25	21	8	29	9	6	8	10	20	1065825
25	21	12	31	9	10	8	10	17	02-456338
28	9	8	11	9	7	8	9	17	596
26	20	8	34	9	7	8	10	20	04-453025
26	20	8	35	9	7	8	10	20	04-451098
26	20	8	36	9	7	8	11	20	03-454777
24	21	10	36	9	7	8	10	20	1125584
14	20	11	40	9	7	8	10	30	402745
14	20	11	40	9	7	8	10	25	02-453460
14	20	11	34	9	7	8	10	12	6206
14	20	11	35	9	7	8	10	12	04-453036
25	20	11	10	9	7	8	10	20	03-451974
13	20	11	26	10	7	8	10	20	03-456664
25	22	8	38	9	7	8	10	30	1093869
14	20	12	24	9	7	8	12	37	3074
14	20	12	33	9	9	8	10	10	1024420
26	20	16	34	8	7	9	10	10	3633
26	20	15	33	9	7	9	10	10	03-453850
25	20	8	10	10	22	9	10	5	502222
18	20	9	10	10	12	8	10	5	1098967
14	20	8	36	13	10	8	10	5	552938
14	23	9	29	10	6	8	10	14	03-450215
13	20	10	32	9	10	8	10	20	986387
24	20	12	32	9	10	8	9	20	02-451481
20	20	10	26	9	10	10	9	45	1012666
13	10	10	47	9	10	10	7	11	04-453179

Figure 3. Dendrogram based on profiles of nine STR*Af* markers from 99 presumably unrelated *A. fumigatus* isolates. The dendrogram was created by using BioNumerics, version 3.5, software (Applied Maths) and the unweighted pair group method with arithmetic averages method with the multistate categorical similarity coefficient. See the text for further details.

The total number of different haplotypes can be determined only experimentally, but since we already found 96 different haplotypes in a collection of 99 presumably unrelated isolates, this indicates that this number must be quite large.

This novel combination of nine STR markers for fingerprinting of *A. fumigatus* with the potential to resolve 3×10^{13} combinations of alleles may appear to be somewhat overdone. Since each of the three multiplex PCRs already has a very high discriminatory power, it may not always be necessary to analyze all nine markers in order to discriminate between certain isolates. One could consider starting with just the M3 combination (since this one has the highest discriminatory power, which is even higher than that of M2 and M4 combined) and to include M4 or M2 only when it is necessary. The additional value of including the third combination appears to be rather limited, but it could always serve as an additional backup.

In order to make full use of the potential of STR analysis, high-resolution analysis of the fragments obtained is a prerequisite. Standard agarose gel electrophoresis equipment usually does not yield sufficient resolution to discriminate between fragments that differ by as little as 2 bp, but at best, it could just suffice for the analysis of tri- and tetranucleotide repeats. Use of agarose gels also means that each marker must be analyzed individually since the different loci in our multiplex analysis overlap each other.
We chose to make multiplex combinations using a multicolor approach, since this allows testing for multiple loci in one run, which greatly improves throughput capabilities and allows rapid analysis of large numbers of markers. Since high-resolution equipment like capillary-based or acrylamide-based electrophoresis platforms are increasingly finding their way into the laboratories, this seems to be the most logical way to go. Use of high-resolution equipment also allows even 1-bp insertions or deletions to be identified reliably. If this is not an option and one would still prefer to use agarose-based systems, it would make more sense to restrict STR analysis to repeat units of 10 and beyond. Such repeats have not been analyzed in this work.

In this chapter we describe a new, highly discriminatory PCR fingerprinting assay for *A. fumigatus* with a novel panel of nine STRs. The multicolor multiplex approach allows large numbers of markers to be tested in a short period of time. The exact nature of the assay combines high reproducibility with the easy exchange of results.

Acknowledgments

Preliminary sequence data were obtained from The Wellcome Trust Sanger Institute (www.sanger.ac.uk). The *Aspergillus fumigatus* Sequencing Consortium was funded by the National Institute of Allergy and Infectious Diseases, The Wellcome Trust, and the Fondo de Investicagiones Sanitarias.

References

1. Aufauvre-Brown, A., J. Cohen, and D. W. Holden. 1992. Use of randomly amplified polymorphic DNA markers to distinguish isolates of *Aspergillus fumigatus*. J Clin Microbiol 30:2991-3.
2. Bart-Delabesse, E., J.-F. Humbert, E. Delabesse, and S. Bretagne. 1998. Microsatellite markers for typing *Aspergillus fumigatus* isolates. J Clin Microbiol 36:2413-2418.
3. Bart-Delabesse, E., J. Sarfati, J. P. Debeaupuis, W. van Leeuwen, A. van Belkum, S. Bretagne, and J. P. Latge. 2001. Comparison of restriction fragment length polymorphism, microsatellite length polymorphism, and random amplification of polymorphic DNA analyses for fingerprinting *Aspergillus fumigatus* isolates. J Clin Microbiol 39:2683-6.
4. Benson, G. 1999. Tandem repeats finder: A program to analyze DNA sequences. Nucl Acids Res 27:573-580.
5. Bertout, S., F. Renaud, R. Barton, F. Symoens, J. Burnod, M.-A. Piens, B. Lebeau, M.-A. Viviani, F. Chapuis, J.-M. Bastide, R. Grillot, M. Mallie, and THE EUROPEAN RESEARCH GROUP ON BIOTYPE AND GENOTYPE OF ASPERGILLUS. 2001. Genetic polymorphism of *Aspergillus fumigatus* in clinical samples from patients with invasive aspergillosis: Investigation using multiple typing methods. J Clin Microbiol 39:1731-1737.
6. Hunter, P. R., and M. A. Gaston. 1988. Numerical index of the discriminatory ability of typing systems: An application of Simpson's index of diversity. J Clin Microbiol 26:2465-6.
7. Klevytska, A. M., L. B. Price, J. M. Schupp, P. L. Worsham, J. Wong, and P. Keim. 2001. Identification and characterization of variable-number tandem repeats in the *Yersinia pestis* genome. J Clin Microbiol 39:3179-3185.
8. Lasker, B. A. 2002. Evaluation of performance of four genotypic methods for studying the genetic epidemiology of *Aspergillus fumigatus* isolates. J Clin Microbiol 40:2886-2892.
9. Latge, J.-P. 1999. *Aspergillus fumigatus* and aspergillosis. Clin Microbiol Rev 12:310-350.
10. Leenders, A., A. van Belkum, S. Janssen, S. de Marie, J. Kluytmans, J. Wielenga, B. Lowenberg, and H. Verbrugh. 1996. Molecular epidemiology of apparent outbreak of invasive aspergillosis in a hematology ward. J Clin Microbiol 34:345-351.
11. Loudon, K. W., J. P. Burnie, A. P. Coke, and R. C. Matthews. 1993. Application of polymerase chain reaction to fingerprinting *Aspergillus fumigatus* by random amplification of polymorphic DNA. J Clin Microbiol 31:1117-21.
12. Murray, V., C. Monchawin, and P. R. England. 1993. The determination of the sequences present in the shadow bands of a dinucleotide repeat PCR. Nucl Acids Res 21:2395-8.
13. Neuveglise, C., J. Sarfati, J. Latge, and S. Paris. 1996. Afut1, a retrotransposon-like element from *Aspergillus fumigatus*. Nucl Acids Res 24:1428-1434.

14. Onteniente, L., S. Brisse, P. T. Tassios, and G. Vergnaud. 2003. Evaluation of the polymorphisms associated with tandem repeats for *Pseudomonas aeruginosa* strain typing. J Clin Microbiol 41:4991-4997.
15. Rozen, S., and H. Skaletsky. 2000. Primer3 on the WWW for general users and for biologist programmers. Methods Mol Biol 132:365-86.
16. van Belkum, A., W. Melchers, C. Ijsseldijk, L. Nohlmans, H. Verbrugh, and J. Meis. 1997. Outbreak of amoxicillin-resistant *Haemophilus influenzae* type b: Variable number of tandem repeats as novel molecular markers. J Clin Microbiol 35:1517-1520.
17. Verduyn-Lunel, F., L. Licciardello, S. Stefani, H. A. Verbrugh, W. J. G. Melchers, J. F. G. M. Meis, S. Scherer, and A. van Belkum. 1998. Lack of consistent short sequence repeat polymorphisms in genetically homologous colonizing and invasive *Candida albicans* strains. J Bacteriol 180:3771-3778.
18. Warris, A., C. H. W. Klaassen, J. F. G. M. Meis, M. T. de Ruiter, H. A. de Valk, T. G. Abrahamsen, P. Gaustad, and P. E. Verweij. 2003. Molecular epidemiology of *Aspergillus fumigatus* isolates recovered from water, air, and patients shows two clusters of genetically distinct strains. J Clin Microbiol 41:4101-4106.

Chapter 3

Comparison of two highly discriminatory molecular fingerprinting assays for analysis of multiple *Aspergillus fumigatus* isolates from patients with invasive aspergillosis

Hanneke A. de Valk, Jacques F.G.M. Meis, Ben E. de Pauw, Peter J. Donnelly and Corné H.W. Klaassen

Journal of Clinical Microbiology 2007, 45;5: 1415-1419.

Abstract

Two highly discriminatory fingerprinting assays, short tandem repeat typing and amplified fragment length polymorphism (AFLP), were compared to determine the genetic relatedness between 55 isolates of *Aspergillus fumigatus* obtained from 15 different patients suffering from proven invasive aspergillosis. Both techniques showed that interpatient isolates belonged to different genotypes and that intrapatient isolates from deep sites were all of the same genotype. By contrast, multiple genotypes were found among isolates originating from respiratory samples. Both techniques have specific advantages and disadvantages. AFLP is more universally applicable, but short tandem repeat analysis offers better discriminatory power and should be the preferred method for standardizing typing of clinical isolates of *Aspergillus fumigatus*.

Introduction

Invasive aspergillosis (IA) is a life-threatening disease of patients who are immunocompromised by hematopoietic stem cell or solid organ transplantation, intensive chemotherapy, or treatment with corticosteroids [11]. *Aspergillus fumigatus* is most frequently involved in causing this life-threatening disease. IA is a growing problem in hospitals because of the inexorable rise in the number of severely immunocompromised patients [12, 14]. A better understanding of the epidemiology of this disease requires molecular techniques to investigate interstrain relatedness and strain dissemination [12].

Fingerprinting methods with high discriminatory power have to be applied to discriminate between unrelated isolates because of the wide genetic variability of *A. fumigatus*, [5] and several typing techniques have been described previously. Pattern-based techniques, such as random amplified polymorphic DNA (RAPD) analysis [2, 13], restriction fragment length polymorphism (RFLP) analysis [12, 19], and amplified fragment length polymorphism (AFLP) [20], have been employed but suffer from poor interlaboratory reproducibility and subjective interpretation of the fingerprinting data. Typing methods based on short tandem repeats (STRs), such as microsatellite length polymorphism [3] and STR*Af* typing [7], yield unambiguous typing data, as do those based on sequence-based typing schemes, such as multilocus sequence typing. These methods may be preferable to other typing methods, provided that they possess sufficient discriminatory power, and may have the characteristics necessary for the standardization of *A. fumigatus* typing.

STR*Af* typing is a recently described highly discriminatory and potentially reproducible fingerprinting assay for *A. fumigatus* [7, 8], but its utility in epidemiological surveys has not yet been established. In this study, we compared AFLP and STR*Af* typing to study the genetic relatedness between isolates of *A. fumigatus* from respiratory and tissue samples that had been obtained from 15 patients with proven IA [1]. Previous studies have shown that the genotypes of isolates from intrapatient samples of deep sites were identical [12], so these samples could be considered internal controls.

We therefore set out to evaluate the advantages and disadvantages of both techniques in terms of their discriminatory power and applicability as well as technical aspects and computerized data processing.

Materials and Methods

Isolates. Fifty-five isolates of *A. fumigatus* were obtained from ante- and postmortem specimens of 15 patients with proven IA who had been nursed in the hematology ward of the Radboud University Nijmegen Medical Center in the period from 1992 to 1998 (Table 1). Isolates were stored as spore suspensions in regular microbial freezing broth containing 12.5% (vol/vol) glycerol at -80°C. The isolates were revived by scraping off part of the sample, plating on Sabouraud's agar, and cultivation at 30°C. Fungal isolates were identified at the time of collection and again after revival by their macroscopic or microscopic appearance and their ability to grow at 48°C. Every isolate displayed normal growth characteristics after revival.

DNA isolation. Isolates were grown on Sabouraud's agar plates at 30°C until sporulation occurred. Spores were first mechanically lysed using the following procedures. A prewetted cotton swab was saturated with conidia from the sporulating culture. These spores were then suspended in a vial of MagNA Lyser green beads containing 350 µl lysis buffer and 50 µl proteinase K (all from Roche Diagnostics, Almere, The Netherlands). Lysis was performed in a MagNA Lyser instrument (Roche Diagnostics) for 30 s at 6,500 rpm. Next, the DNA was extracted and purified using a MagNA Pure LC instrument (Roche Diagnostics) in combination with MagNA Pure DNA isolation kit III according to the manufacturer's instructions. DNA yield and purity were estimated by UV absorbance measurements.

STR*Af* typing. Nine STR markers specific for *A. fumigatus* were analyzed using the STR*Af* primers and reaction conditions as previously described [7]. Briefly, three multiplex PCRs each amplified three STR markers.

The obtained PCR products were separated by size and detected on a MegaBACE 500 platform equipped with a 48-capillary array (GE Healthcare, Diegem, Belgium). The assignment of repeat numbers in each marker was performed using Fragment Profiler 1.2 software (GE Healthcare).

AFLP. Approximately 50 ng of genomic DNA was subjected to a combined restriction-ligation procedure containing 50 pmol of HpyCH4 IV adapter, 50 pmol Mse I adapter, 2 U of HpyCH4 IV (New England Biolabs, Beverly, MA), 2 U of Mse I (New England Biolabs), and 1 U of T4 DNAligase (Promega, Leiden, The Netherlands) in a total volume of 20 µl of 1 × reaction buffer for 1 h at 20°C. Next, the mixture was diluted five times with 10 mM Tris-HCl (pH 8.3) buffer. Adapters were made by mixing equimolar amounts of complementary oligonucleotides (5'-CTCGTAGACTGCGTACC-3' and 5'-CGGGTACGCAGTC-3' for HpyCH4 IV; 5'-GACGATGAGTCCTGAC-3' and 5'-TAGTCAGGACTCAT-3' for Mse I) and heating to 95°C, subsequently cooling slowly to ambient temperature. One microliter of the diluted restriction-ligation mixture was used for amplification in a volume of 25 µl under the following conditions: 1 µM HpyCH4 IV primer with one selective residue (underlined) (5'-Flu-GTAGACT GCGTACCCGTC-3'), 1 µM MseI primer with four selective residues (underlined) (5'-GATGAGTCCTGACTAATGAA-3'), 0.2 mM of each deoxynucleoside triphosphate, and 1 U of *Taq* DNA polymerase (Roche Diagnostics) in 1× reaction buffer containing 1.5 mM $MgCl_2$. Amplification was done as follows. After an initial denaturation step for 4 min at 94°C in the first 20 cycles, a touchdown procedure was applied: 15 s of denaturation at 94°C, 15 s of annealing at 66°C, with the temperature for each successive cycle lowered by 0.5°C, and 1 min of extension at 72°C. Cycling was then continued for a further 30 cycles with an annealing temperature of 56°C. After completion of the cycles, an incubation at 72°C for 10 min was performed before the reaction mixtures were cooled to room temperature. The amplicons were then combined with the ET400-R size standard (GE Healthcare) and analyzed on a MegaBACE 500 automated DNA platform (GE Healthcare) according to the manufacturer's instructions.

Data analysis. All data were analyzed with the BioNumerics 4.5 unweighted-pair group method using average linkage clustering (Applied Maths, Sint-Martens- Latem, Belgium), employing the multistate categorical similarity coefficient for STR data and the Pearson correlation coefficient for DNA fragments between 60 and 250 bases for AFLP analysis.

Results

All AFLP fingerprints obtained with Mse I and HpyCH4 IV contained multiple bands in the range of 50 to 250 bp (Fig. 1), and strong and weak bands were visible. When all samples were compared to each other, the fingerprints consisted of some 30 invariable bands present in all samples and around 20 variable bands which were present only in certain samples. Based on the variable bands, the different fingerprints can be distinguished from each other. In total, 20 visually recognizable different patterns were obtained. The calculated similarity for visually identical patterns ranges from 97 to 99%. The calculated similarity for patterns with visually recognizable differences ranges from 89 to 99%. Originally, the collection consisted of 56 isolates. However, one isolate yielded a fingerprint without resemblance to any of the other isolates. The reidentification of this isolate by internal transcribed spacer sequencing showed that this particular isolate proved to be *Neosartorya fischeri* (results not shown). This isolate was excluded from further analysis.

Using STR*Af* typing, all *A. fumigatus* isolates yielded a numerical typing result (Fig. 1). A total of 20 different genotypes were obtained with this technique. The *N. fischeri* isolate did not yield PCR products with the STR*Af* assay.

Table 1: Origin of *A. fumigatus* isolates from patients with IA

Patient	Origin	Date of isolation (day-mo-yr)	AFLP type	STR*Af* type	Sample
A	Bronchial secretion	22-07-1992	1	1	A1
	Tissue	25-07-1992	1	1	A2
B	Bronchial secretion	26-08-1992	2	2	B1
	Bronchial secretion	28-08-1992	2	2	B2
	Pleural abscess	31-08-1992	2	2	B3
C	Bronchial secretion	15-01-1993	3	3	C1
	Lung biopsy sample	19-01-1993	3	3	C2
	BAL fluid	19-01-1993	3	3	C3
D	Left lung	28-12-1993	4	4	D1
	Right kidney	28-12-1993	4	4	D2
	Left kidney	28-12-1993	4	4	D3
E	Feces	01-06-1994	5	5	E1
	Pharynx swab	06-06-1994	5	5	E2
	Feces	09-06-1994	5	5	E3
	Pharynx swab	13-06-1994	5	5	E4
	Sputum	20-06-1994	5	5	E5
	Lung	13-07-1994	5	5	E6
F	Pharynx swab	04-08-1994	6	6	F1
	Right lung	18-08-1994	6	6	F2
	Left kidney	18-08-1994	6	6	F3
	Left lung	18-08-1994	6	6	F4
G	BAL fluid	28-10-1994	7	7	G1
	Lung	04-11-1994	8	8	G2
H	Sputum	18-04-1995	9	9	H1
	Left lung	02-05-1995	10	10	H2
I	Pus	29-08-1995	11	11	I1
	Feces	31-08-1995	11	11	I2
	Pharynx swab	04-09-1995	11	11	I3
	Feces	14-09-1995	11	11	I4
J	BAL fluid	16-10-1995	12	12	J1
	Right lung	25-10-1995	12	12	J2
	Left lung	25-10-1995	12	12	J3
K	Feces	29-03-1996	13	13	K1
	Left lung	01-04-1996	13	13	K2
	Right kidney	01-04-1996	13	13	K3
	Heart	01-04-1996	13	13	K4
	Left kidney	01-04-1996	13	13	K5
	Right lung	01-04-1996	13	13	K6
	Spleen	01-04-1996	13	13	K7
	Liver	01-04-1996	13	13	K8
L	Bronchial secretion	02-01-1997	14	14	L1
	Bronchial secretion	03-01-1997	14	14	L2
	Bronchial secretion	09-01-1997	14	14	L3
	Right lung	13-01-1997	14	14	L4
M	Lung biopsy sample	26-03-1997	15	15	M1
	BAL fluid	26-03-1997	16	16	M2
	BAL fluid	26-03-1997	17	17	M3
	Lung biopsy sample	26-03-1997	18	15	M4
N	Pharynx swab	11-12-1997	13	18	N1
	Left lung	17-12-1997	13	18	N2
	Spleen	17-12-1997	13	18	N3
	Liver	17-12-1997	13	18	N4
O	BAL fluid	19-01-1998	19	19	O1
	BAL fluid	19-01-1998	20	20	O2
	Left lung	23-01-1998	19	19	O3

In general, there is an excellent agreement between the two typing methods, with only two tissue isolates (M1 and M4) of patient M that were differentiated by AFLP but not by STR*Af* typing, which yielded the same genotype. AFLP yielded the same genotype for all isolates of patients K and N, but these could be clearly distinguished from each other using STR*Af* typing. When all intrapatient isolates were compared to each other, samples of nonrespiratory origin belonged to the same genotype. By contrast, multiple genotypes were detected in isolates originating from respiratory samples, such as sputum and bronchoalveolar lavage (BAL) fluid samples, from the same patient. For instance, all eight isolates from the lung, kidney, heart, spleen, and liver of patient K shared the same STR*Af* genotype. An identical unique genotype was found in isolates from BAL fluid and lung samples obtained from patient O, whereas another BAL fluid sample yielded a different genotype. A similar observation was made for patients G, H, and M, as interpatient isolates belonged to different genotypes as determined by STR*Af* typing.

Discussion

We compared two high-resolution PCR fingerprinting assays for analyzing the epidemiological relationships between multiple *A. fumigatus* isolates from patients with proven IA. Both AFLP analysis and STR*Af* analysis provided high-resolution data allowing discrimination between clinical isolates from different patients and between different genotypes in multiple samples from a patient. In general, interpatient isolates belonged to different genotypes whereas intrapatient isolates were of the same genotype. Cross-infections of respiratory origin or common-source infections were highly unlikely since the patients were nursed at different times in the ward.

The use of molecular epidemiological tools such as RAPD, multilocus enzyme electrophoresis, and sequence-specific DNA primer analyses [10] has led to suggestions that *A. fumigatus* isolates from several IA patients nursed in the same ward may originate from a single environmental source.

However, these techniques lack discriminatory power and are not considered by us to be sufficiently reproducible to employ for this study. Rather, our results confirm the results reported by Girardin *et al.* [12] and Denning *et al.* [9], who investigated the genetic diversity of patients with IA by using RFLP and REA analyses, respectively. Our results are consistent with a scenario in which the respiratory tract may be colonized by several different genotypes, as inhalation of fungal conidia is assumed to be the primary means of acquiring IA [11], but a single genotype gains supremacy and invades the surrounding tissue, subsequently disseminating to the lung tissue or other organs. Thus, an isolate obtained from respiratory samples may not necessarily represent the same genotype as that found in deep sites, as appeared to be the case for patients G, H, M, and O. This also confirms that individuals may well be constantly exposed to a large variety of different *Aspergillus* genotypes from the environment [5, 15].

Both AFLP and the STR*Af* assay are seen as high-resolution and robust molecular fingerprinting assays for *A. fumigatus* isolates [7, 8, 20]. As shown here, both techniques are suitable for unraveling the genetic relationship between clinical *A. fumigatus* isolates, with good correlation between the results of the two assays. Yet, compared to each other, both assays have their advantages and disadvantages. A clear advantage of the STR*Af* assay over AFLP is its ability to identify mixtures of strains. Mixed genotypes are recognized instantaneously by the presence of multiple peaks in each of the nine markers analyzed [7]. Mixed AFLP genotypes are extremely difficult, if not impossible, to detect. Thus, when employing AFLP analysis for epidemiological analysis, it is important that pure cultures be obtained and analyzed. Assuming that individuals are, in fact, likely to be colonized by more than one genotype, it is important to subculture and analyze each colony separately when several are obtained from a respiratory sample in order to prevent false conclusions from being drawn [4, 6].

DNA fragments are amplified in AFLP from random locations throughout the entire genome and, although in a single AFLP experiment only a very small fraction of the entire genome is analyzed (<0.1%), the amount analyzed by the STR*Af* assay is even smaller. In addition, unlike the AFLP assay, the STR*Af* approach is highly biased toward nine arbitrarily selected loci, so any genomic change outside these loci will remain undetected, as was the case for isolates M1 and M4. The AFLP technique is basically applicable to any organism's genome without the need for prior sequence information. However, depending on the genome size and GC content, there may be a need for optimizing the choice of restriction enzymes and selective residues. By contrast, the STR*Af* assay is specific for *A. fumigatus* and requires specific sequence information for the detection of STR loci and flanking sequences to be used as primer binding sites. However, both techniques do permit improperly identified isolates to be recognized. The nonvariable bands that are obtained with *A. fumigatus* isolates may represent species-specific markers. Upon analysis of isolates from species other than *A. fumigatus*, most of these invariable bands will not show up and a banding pattern will be obtained that is usually less than 30% similar to the fingerprint of *A. fumigatus* isolates (as was the case for the *N. fischeri* isolate). Likewise, using the STR*Af* approach, improperly identified isolates can be recognized by the absence of typical amplification products, provided that technical issues that may lead to PCR failure can be ruled out.

In order to compare the different AFLP fingerprints to each other and to determine the genetic relatedness between multiple isolates, one has to rely on specialized computer software. AFLP fingerprints are usually analyzed using a pattern-based algorithm. However, repeat analyses may show small differences in the AFLP fingerprint due to small variations occurring during restriction/ligation, PCR amplification, and fragment analysis that may affect the final peak intensity. For the same reasons, the long-term reproducibility and interlaboratory comparisons of AFLP fingerprints may be quite challenging. Thus, identical samples are seldom identical using a patternbased approach, although they may appear to be so by visual examination. In the pattern-based dendrogram shown in figure 1, visually identical fingerprints were calculated to be 97 to 99% similar (i.e., all samples of patient K).

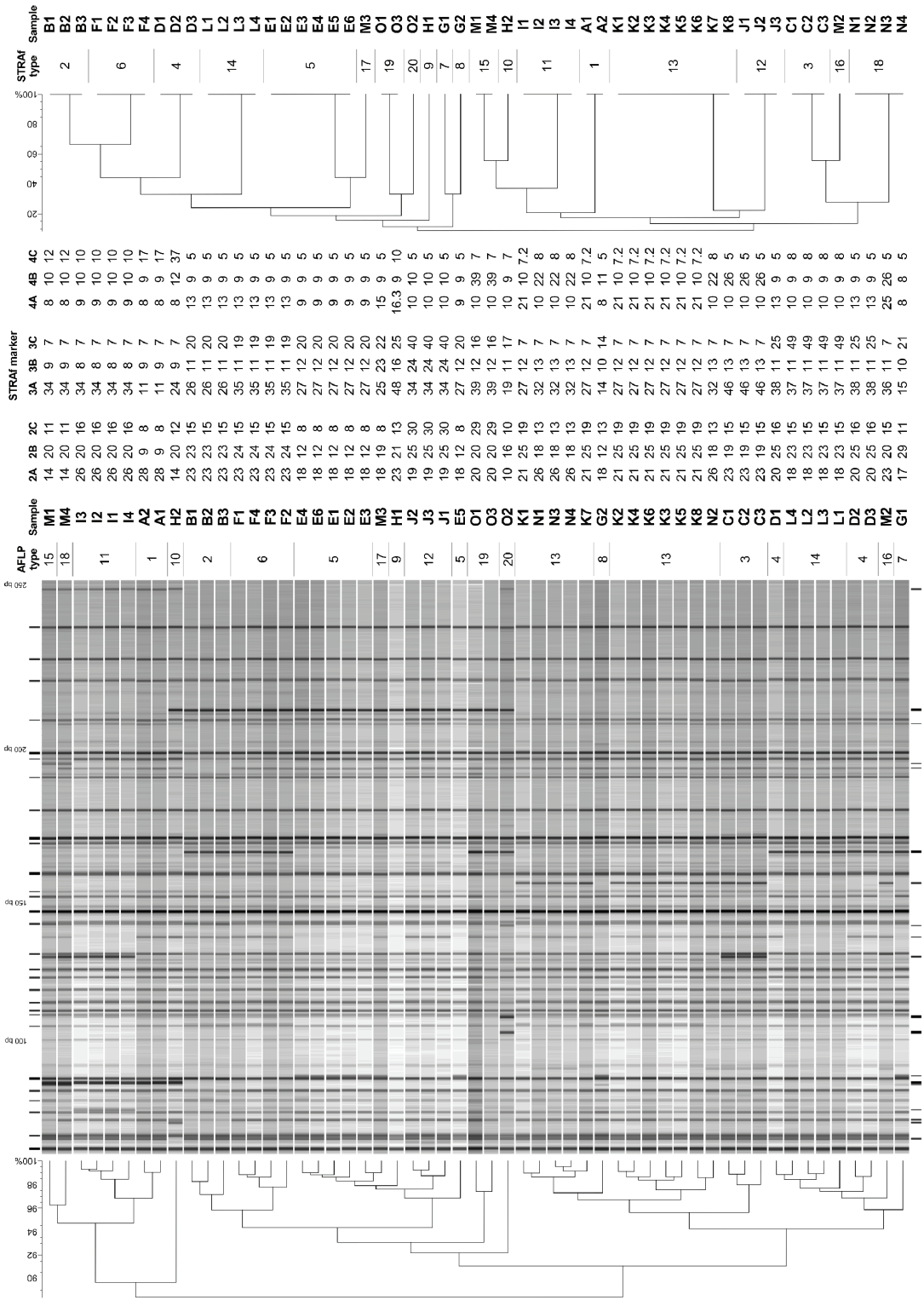


Figure.1. Results of both AFLP and STR*Af* typing on a collection of 55 clinical *A. fumigatus* isolates. The dendrogram on the left is based on the AFLP fingerprints. The indicator lines above the traces represent invariable bands, whereas the indicator lines below the traces represent variable bands in the AFLP fingerprints. After each fingerprint are the typing results for the nine markers of the STR*Af* panel. The dendrogram on the right is based on the STR*Af* results. Types were assigned arbitrarily, starting with the oldest isolate. The scale bars indicate the percentages of similarity. Sample numbers correspond to those in Table 1.

However, in the same analysis, samples with visually recognizable differences were also calculated to be up to 99% similar (i.e., samples D1 and L4) (Fig. 1). Consequently, a gray zone was obtained during interpretation of the results of a pattern-based approach in which the wrong conclusions may be drawn when relying solely on the computer-based dendrogram. This requires a visual inspection of the fingerprints to be included in the final interpretation. Similar limitations apply to computer-based analysis of other pattern-based fingerprinting methods, such as RAPD and RFLP analyses. By contrast, due to the more exact nature of STR*Af* typing, intralaboratory reproducibility is virtually 100% and the typing result is unambiguous.

Due to the large number of available restriction enzymes, an almost indefinite number of restriction enzyme combinations can be used to generate an AFLP fingerprint. The success of a particular combination is dictated primarily by the size and composition (i.e., GC content and the presence of multicopy elements) of the genome to be investigated. The number of fragments to be obtained can be modulated by changing the number of selective residues. In previous studies, other restrictionenzyme combinations such as EcoR I and Mse I [16, 20] and EcoR I and Bfa I [17] have been used to generate AFLP fingerprints for *Aspergillus* spp. To rule out the possibility that the results of this study may have been influenced by the specific use of the combination of HpyCH4 IV and Mse I, all isolates were also tested with the combination of EcoR I and Mse I. Although obviously completely different fingerprints were obtained, the same interstrain relatedness was observed (data not shown).

A problem with any typing method is the subjective interpretation of a "genotype." The assignment of a genotype is user dependent using AFLP, whereas the genotype resulting from STR*Af* typing is unambiguous. Nonetheless, two different but highly similar fingerprints could originate from two closely related isolates. Unfortunately, at present there are no established guidelines for defining a genotype for these methods (such as the criteria of Tenover *et al.* for the interpretation of pulsed-field gel electrophoresis fingerprints [18]).

However, since there is an excellent correlation between the groupings of genotypes by using AFLP and STR*Af* typing, we believe that the differences between the fingerprints of the isolates in this study, although minor, are substantial enough to validate our assignment of genotypes. For instance, isolates E3 and M3 differed by only one band in the AFLP fingerprint but by five markers in the STR*Af* assay. This means that even a single band difference between two AFLP fingerprints could be interpreted as constituting different genotypes. Likewise, with the STR*Af* assay, any two different genotypes differ by a minimum of three markers. For instance, isolates from patients B and F differed by one repeat in marker 2B, nine repeats in marker 3A, and one repeat in marker 3C. In the AFLP fingerprint, there were at least two visually recognizable differences between these isolates. At present, it is unclear whether fewer than three differences in an STR*Af* genotype should also be interpreted as different genotypes, so more closely related isolates need to be analyzed.

In conclusion, both AFLP and STR*Af* typing are excellent techniques for analyzing the interstrain relatedness of *A. fumigatus* isolates. Each of the two techniques has its specific advantages and disadvantages. Therefore, the choice should be based primarily on the specific goal for analyzing collections of isolates. STR*Af* typing offers the most benefits for the standardization of typing *A. fumigatus* isolates.

References

1. Ascioglu, S., J. H. Rex, B. de Pauw, J. E. Bennett, J. Bille, F. Crokaert, D. W. Denning, J. P. Donnelly, J. E. Edwards, Z. Erjavec, D. Fiere, O. Lortholary, J. Maertens, J. F. Meis, T. F. Patterson, J. Ritter, D. Selleslag, P. M. Shah, D. A. Stevens, and T. J. Walsh. 2002. Defining opportunistic invasive fungal infections in immunocompromised patients with cancer and hematopoietic stem cell transplants: An international consensus. Clin Infect Dis 34:7-14.
2. Aufauvre-Brown, A., J. Cohen, and D. W. Holden. 1992. Use of randomly amplified polymorphic DNA markers to distinguish isolates of *Aspergillus fumigatus*. J Clin Microbiol 30:2991-3.
3. Bart-Delabesse, E., J.-F. Humbert, E. Delabesse, and S. Bretagne. 1998. Microsatellite markers for typing *Aspergillus fumigatus* isolates. J Clin Microbiol 36:2413-2418.
4. Bertout, S., F. Renaud, R. Barton, F. Symoens, J. Burnod, M. A. Piens, B. Lebeau, M. A. Viviani, F. Chapuis, J. M. Bastide, R. Grillot, and M. Mallie. 2001. Genetic polymorphism of *Aspergillus fumigatus* in clinical samples from patients with invasive aspergillosis: Investigation using multiple typing methods. J Clin Microbiol 39:1731-7.
5. Chazalet, V., J. P. Debeaupuis, J. Sarfati, J. Lortholary, P. Ribaud, P. Shah, M. Cornet, H. Vu Thien, E. Gluckman, G. Brucker, and J. P. Latge. 1998. Molecular typing of environmental and patient isolates of *Aspergillus fumigatus* from various hospital settings. J Clin Microbiol 36:1494-500.
6. Cimon, B., F. Symoens, R. Zouhair, D. Chabasse, N. Nolard, A. Defontaine, and J. P. Bouchara. 2001. Molecular epidemiology of airway colonisation by *Aspergillus fumigatus* in cystic fibrosis patients. J Med Microbiol 50:367-74.
7. de Valk, H. A., J. F. Meis, I. M. Curfs, K. Muehlethaler, J. W. Mouton, and C. H. Klaassen. 2005. Use of a novel panel of nine short tandem repeats for exact and high-resolution fingerprinting of *Aspergillus fumigatus* isolates. J Clin Microbiol 43:4112-20.
8. de Valk, H. A., J. F. Meis, and C. H. Klaassen. 2007. Microsatellite based typing of *Aspergillus fumigatus*: strengths, pitfalls and solutions. J Microbiol Methods 69:268-72.
9. Denning, D. W., K. V. Clemons, L. H. Hanson, and D. A. Stevens. 1990. Restriction endonuclease analysis of total cellular DNA of *Aspergillus fumigatus* isolates of geographically and epidemiologically diverse origin. J Infect Dis 162:1151-8.
10. Ellis, M., M. Richardson, and B. de Pauw. 2000. Epidemiology. Hosp Med 61:605-9.
11. Fridkin, S. K., and W. R. Jarvis. 1996. Epidemiology of nosocomial fungal infections. Clin Microbiol Rev 9:499-511.
12. Girardin, H., J. Sarfati, F. Traore, J. Dupouy Camet, F. Derouin, and J. P. Latge. 1994. Molecular epidemiology of nosocomial invasive aspergillosis. J Clin Microbiol 32:684-90.
13. Leenders, A., A. van Belkum, S. Janssen, S. de Marie, J. Kluytmans, J. Wielenga, B. Lowenberg, and H. Verbrugh. 1996. Molecular epidemiology of apparent outbreak of invasive aspergillosis in a hematology ward. J Clin Microbiol 34:345-351.

14. Loudon, K. W., J. P. Burnie, A. P. Coke, and R. C. Matthews. 1993. Application of polymerase chain reaction to fingerprinting *Aspergillus fumigatus* by random amplification of polymorphic DNA. J Clin Microbiol 31:1117-21.
15. Menotti, J., J. Waller, O. Meunier, V. Letscher-Bru, R. Herbrecht, and E. Candolfi. 2005. Epidemiological study of invasive pulmonary aspergillosis in a haematology unit by molecular typing of environmental and patient isolates of *Aspergillus fumigatus*. J Hosp Infect 60:61-8.
16. Perrone, G., G. Mule, A. Susca, P. Battilani, A. Pietri, and A. Logrieco. 2006. Ochratoxin A production and amplified fragment length polymorphism analysis of *Aspergillus carbonarius*, *Aspergillus tubingensis*, and *Aspergillus niger* strains isolated from grapes in Italy. Appl Environ Microbiol 72:680-5.
17. Schmidt, H., M. Ehrmann, R. F. Vogel, M. H. Taniwaki, and L. Niessen. 2003. Molecular typing of *Aspergillus ochraceus* and construction of species specific SCAR-primers based on AFLP. Syst Appl Microbiol 26:138-46.
18. Tenover, F. C., R. D. Arbeit, R. V. Goering, P. A. Mickelsen, B. E. Murray, D. H. Persing, and B. Swaminathan. 1995. Interpreting chromosomal DNA restriction patterns produced by pulsed-field gel electrophoresis: criteria for bacterial strain typing. J Clin Microbiol 33:2233-9.
19. Verweij, P. E., J. F. Meis, J. Sarfati, J. A. Hoogkamp-Korstanje, J. P. Latge, and W. J. Melchers. 1996. Genotypic characterization of sequential *Aspergillus fumigatus* isolates from patients with cystic fibrosis. J Clin Microbiol 34:2595-7.
20. Warris, A., C. H. W. Klaassen, J. F. G. M. Meis, M. T. de Ruiter, H. A. de Valk, T. G. Abrahamsen, P. Gaustad, and P. E. Verweij. 2003. Molecular epidemiology of *Aspergillus fumigatus* isolates recovered from water, air, and patients shows two clusters of genetically distinct strains. J Clin Microbiol 41:4101-4106.

Chapter 4

Retrotransposon insertion-site context (RISC) typing: a novel typing method for *Aspergillus fumigatus* and a convenient PCR alternative to restriction fragment length polymorphism analysis

Maaike T. de Ruiter, Hanneke A. de Valk, Jacques F.G.M. Meis and
Corné H.W. Klaassen

Journal of Microbiological Methods 2007, 70: 528-534.

Abstract

Retrotransposon(-like) sequences in *Aspergillus fumigatus* have been used as typing targets through restriction fragment length polymorphism (RFLP)/Southern blotting approaches. Differences in fingerprints between unrelated isolates are the result of variations in copy-number and differences in the regions flanking the retrotransposon elements. Here, we present retrotransposon insertion-site context (RISC) typing as a novel and convenient PCR-based typing alternative to the RFLP approach. RISC typing aims at amplifying the sequences flanking the retrotransposonlike sequences in *A. fumigatus* and allows large numbers of isolates to be analyzed in a timely fashion with excellent discriminatory power.

Introduction

Aspergillus fumigatus is a common inhabitant of damp environments such as soil, decaying vegetation and organic debris. It releases high numbers of conidia into the air. Inhalation of these conidia by immunocompromised hosts can lead to respiratory diseases such as allergic bronchopulmonary aspergillosis, aspergilloma and invasive aspergillosis (IA). The systemic form of the latter disease often has fatal consequences. The incidence of IA has grown dramatically in recent years due to the increase in number of patients undergoing bone marrow or solid organ transplantation.

Molecular biological typing methods can be used to obtain a better understanding of the uniqueness of each clinical isolate and its dissemination route. Various techniques for genotyping *A. fumigatus* isolates have been reported such as random amplified polymorphic DNA (RAPD) analysis [1], restriction fragment length polymorphism (RFLP) analysis followed by Southern blot hybridization [2, 5, 6], amplified fragment length polymorphism (AFLP) analysis [4, 12] and short tandem repeats (STR) [3]. RFLP analysis has been shown to be an excellent typing method for *A. fumigatus* isolates [2, 5, 6].

When performing RFLP analysis, genomic DNA is digested with a restriction enzyme and the obtained fragments are separated according to size using agarose gel electrophoresis and subsequently transferred to a membrane. After that, a specific DNA probe is used to detect certain multicopy elements. In the case of *A. fumigatus*, this probe is part of a degenerate retrotransposon-like element (Afut1) from the gypsy family [7]. Analysis of the hybridization patterns is performed by eye or specialized software. It has been shown that the use of retrotransposons as typing target for *A. fumigatus*, yields very stable fingerprints [6]. Differences in patterns between unrelated isolates can be expected because of differences in copy-number and/or flanking sequences of the target regions.

Unfortunately, compared to PCR-based assays, RFLP analysis is a time-consuming and laborious method and requires relatively large amounts of high molecular weight genomic DNA.

Table 1: Overview of *A. fumigatus* isolates used in this study

Patient	Origin	Date of isolation (day-mo-yr)	Sample
A	Bronchial secretion	22-07-1992	A1
	Tissue	25-07-1992	A2
B	Bronchial secretion	26-08-1992	B1
	Bronchial secretion	28-08-1992	B2
	Pleural abscess	31-08-1992	B3
C	Bronchial secretion	15-01-1993	C1
	Lung biopsy sample	19-01-1993	C2
	BAL fluid	19-01-1993	C3
D	Left lung	28-12-1993	D1
	Right kidney	28-12-1993	D2
	Left kidney	28-12-1993	D3
E	Feces	01-06-1994	E1
	Pharynx swab	06-06-1994	E2
	Feces	09-06-1994	E3
	Pharynx swab	13-06-1994	E4
	Sputum	20-06-1994	E5
	Lung	13-07-1994	E6
F	Pharynx swab	04-08-1994	F1
	Right lung	18-08-1994	F2
	Left kidney	18-08-1994	F3
	Left lung	18-08-1994	F4
G	BAL fluid	28-10-1994	G1
	Lung	04-11-1994	G2
H	Sputum	18-04-1995	H1
	Left lung	02-05-1995	H2
I	Pus	29-08-1995	I1
	Feces	31-08-1995	I2
	Pharynx swab	04-09-1995	I3
	Feces	14-09-1995	I4
J	BAL fluid	16-10-1995	J1
	Right lung	25-10-1995	J2
	Left lung	25-10-1995	J3
K	Feces	29-03-1996	K1
	Left lung	01-04-1996	K2
	Right kidney	01-04-1996	K3
	Heart	01-04-1996	K4
	Left kidney	01-04-1996	K5
	Right lung	01-04-1996	K6
	Spleen	01-04-1996	K7
	Liver	01-04-1996	K8
L	Bronchial secretion	02-01-1997	L1
	Bronchial secretion	03-01-1997	L2
	Bronchial secretion	09-01-1997	L3
	Right lung	13-01-1997	L4
M	Lung biopsy sample	26-03-1997	M1
	BAL fluid	26-03-1997	M2
	BAL fluid	26-03-1997	M3
	Lung biopsy sample	26-03-1997	M4
N	Pharynx swab	11-12-1997	N1
	Left lung	17-12-1997	N2
	Spleen	17-12-1997	N3
	Liver	17-12-1997	N4
O	BAL fluid	19-01-1998	O1
	BAL fluid	19-01-1998	O2
	Left lung	23-01-1998	O3

In this study, retrotransposon insertion-site context (RISC) typing is described as a convenient PCR-based alternative for the RFLP procedure. RISC typing aims at amplifying the flanking sequences of the retrotransposon elements by using an outward oriented, fluorescently labeled internal primer targeting the 5′ long terminal repeat (LTR) of the Afut 1 element. A second primer binding site is provided by ligation of an adapter to cohesive ends generated by restriction enzyme digestion. Thus, RISC typing is a hemi-nested ligation dependent PCR amplification assay. Fluorescent amplification products are analyzed using a high-resolution electrophoresis platform. Like in RFLP analysis, differences in target copy-number and/or flanking sequences will result in different DNA fingerprints.

Materials and methods

Isolates. A collection of 55 clinical *A. fumigatus* isolates was used. These isolates were collected ante- and postmortem over a period of 6 years from 15 patients with established invasive aspergillosis (IA) at the Academic Medical Center Nijmegen St. Radboud (Nijmegen, the Netherlands) (Table 1). The genetic relatedness of these isolates was previously established by AFLP and STR*Af* typing as described elsewhere[4] and in chapter 3.

DNA isolation. Isolates were grown on Sabouraud agar plates at 30°C until sporulation. A prewetted cotton swab, saturated with conidia from a sporulating culture, was resuspended in a vial of MagNA Lyser Green Beads containing 350 µl Lysis buffer and 50 µl of proteinase K (Roche Diagnostics, Almere, the Netherlands). Next, the suspension was subjected to mechanical lysis in a MagNA Lyser instrument (Roche Diagnostics) for 30 s at 6500 rpm. Afterwards, DNA was further extracted and purified using a MagNA Pure LC instrument (Roche Diagnostics) in combination with the MagNA Pure DNA isolation kit III according to the instructions supplied by the manufacturer.

Adapter ligation. Following isolation of the DNA, a combined restriction–ligation procedure was used in which approximately 50 ng of genomic DNAwas incubated with 50 pmol of either Bfa I, Mse I or Msp I adapter, 2 U of the corresponding restriction enzyme (New England Biolabs, Beverly,MA, USA), and 1 U of T4 DNA Ligase (Promega, Leiden, The Netherlands) in a total volume of 20 µl of 1 × reaction buffer for 1 h at ambient temperature, after which the mixture was diluted five times with 10 mM Tris–HCl pH 8.3. (Sigma, Zwijndrecht, the Netherlands). Adapters were made by mixing equimolar amounts of complementary oligonucleotides (a and b, Table 2) followed by heating the mixture to 95°C, with subsequent slow cooling to ambient temperature. Adapters were designed to interrupt the restriction site upon ligation.

Table 2. Overview of oligonucleotides used in this study

Adapter primers		
Mse I	(a)	5'-GACGATGAGTCCTGAC-3'
	(b)	5'-TAGTCAGGACTCAT-3'
Msp I	(a)	5'-GACGATGAGTCCTGAG-3'
	(b)	5'-CGCTCAGGACTCAT-3'
Bfa I	(a)	same as Msp I (a)
	(b)	5'-TACTCAGGACTCAT-3'
Amplification primers		
Mse I	5'-GATGAGTCCTGACTAA-3'	
Msp I	5'-GATGAGTCCTGAGCGG-3'	
Bfa I	5'-GATGAGTCCTGAGTAG-3'	
RISC5	5'-Flu-GCTTAYRTTRTTCYRAGCATGTGA-3'	

Amplification. One microliter of the diluted restriction–ligation mixture was used for amplification in a volume of 25 µl using the following conditions: 1 µM of 5′ fluorescein labeled internal primer, 1 µM adapter primer (unlabeled), 0.2 mM each deoxynucleoside triphosphate, 1 × reaction buffer containing $MgCl_2$ and 1 U of Taq DNA polymerase (Roche Diagnostics). The following amplification protocol was performed in a T1 thermocycler (Biometra, Göttingen, Germany): denaturation at 95°C for 4 min, followed by 40 cycles of 15 s denaturation at 95°C, 30 s annealing at 52°C and 1 min extension at 72°C, continued with a 10 minute incubation at 72°C before the reactions were cooled to room temperature. All temperature transitions were performed at maximum heating and cooling settings (5°C/s).

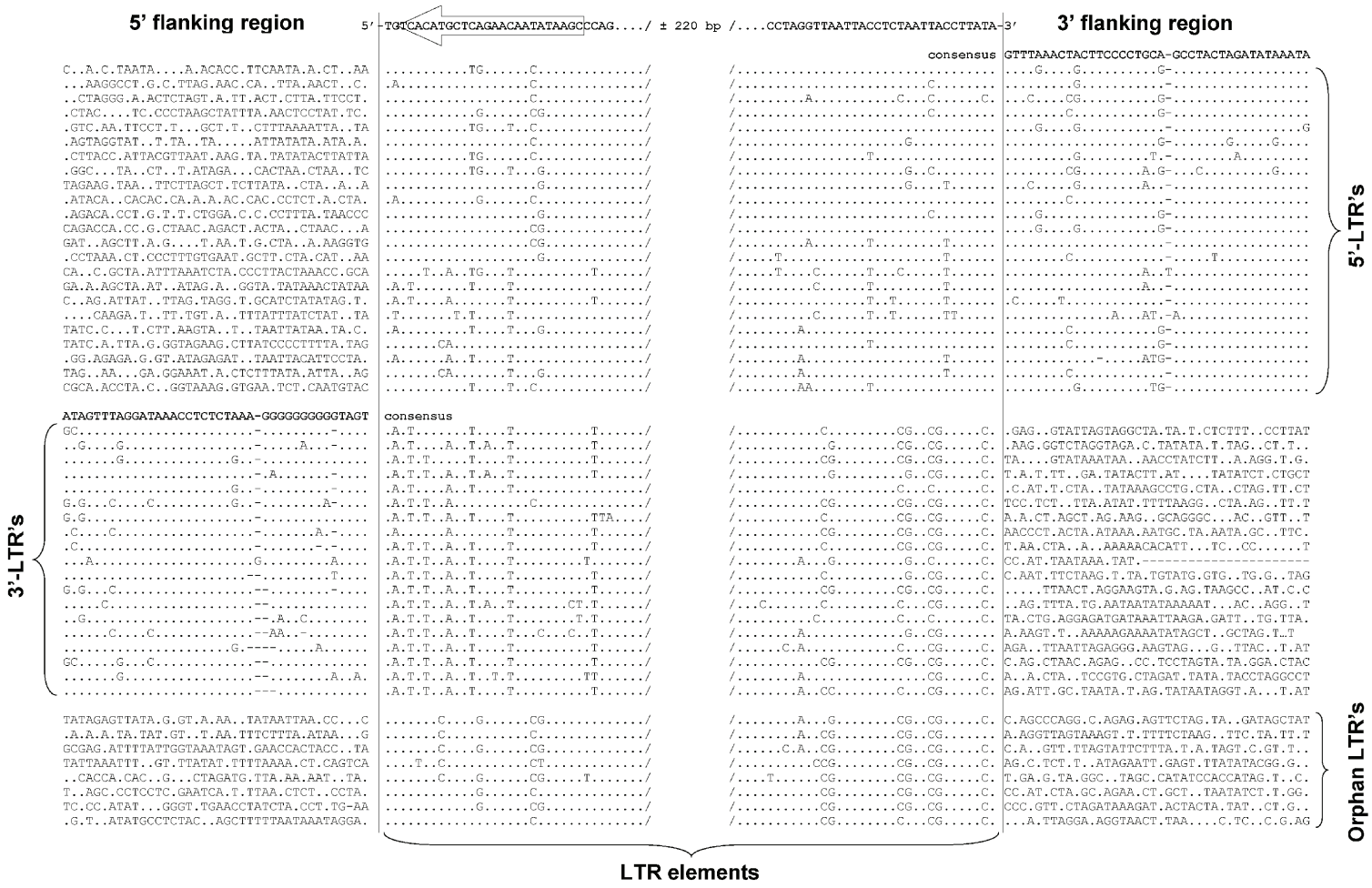


Figure 1. Alignment of 50 LTR sequences (including 40 bp of flanking sequences on both 5′ and 3′ end) from the *A. fumigatus* 293 genome sequence. In 23 cases, the 3′ flanking sequences are conserved, indicating that these LTR's represent the 5′-LTR of the retrotransposon elements. In 19 cases, the 5′ flanking sequences are conserved, indicative of representing 3′ LTR elements. In 8 cases there are no conserved sequences on either side. These LTR's are referred to as 'orphan LTR's'. The reference sequence on top represents the consensus sequence of the 5'LTR elements. Dots indicate residues identical to the 5′-LTR consensus sequences and to the consensus flanking sequences (in bold). Gaps are indicated with a dash. The open arrow indicates the position of the internal amplification primer.

Fragment analysis. The PCR fragments were combined with the ET550-R size standard (GE Healthcare, Diegem, Belgium) and analyzed on a MegaBACE 500 automated DNA analysis platform (GE Healthcare) equipped with a 48 capillary array, according to the recommendations of the manufacturer.

Data analysis. Obtained data was analyzed with BioNumerics version 4.6 software (Applied Maths, Sint-Martens-Latem, Belgium) and the unweighted pair group method using arithmetic averages clustering and the Pearson correlation coefficient and is expressed as percent similarity. DNA fragments smaller than 60 bp or larger than 300 bp were excluded from the analysis.

Results

Principle and design of the assay. Individual *A. fumigatus* isolates may contain up to 25 copies of the Afut1 element [6]. Each retrotransposon element is characterized by the presence of two LTR's encompassing 3 coding regions. A BLAST analysis was performed using the 5′-LTR sequence from clone 4.11 [7] to search for other LTR copies in the preliminary genome sequences of *A. fumigatus* (Af293) [9]. Since the 5′- and 3′-LTR of each retrotransposon element share some 90% nucleotide identity [7], this BLAST search will likely retrieve both LTR's. In total, more than 50 hits were obtained. The sequences of the 50 best hits including 40 bp of flanking sequences were aligned using ClustalX [11]. The result is shown in figure 1. The flanking sequences of 19 hits are highly conserved at the 5′ end, indicating that these hits correspond to the 3′-LTR of the retrotransposon elements. In 23 hits, the flanking sequences of the LTR's are conserved at the 3′ end, indicating that these correspond to the 5′-LTR elements. In the remaining 8 hits, the flanking sequences were not conserved at either side. Conserved sequence differences between the 5′- and 3′-LTR were used to formulate an internal primer specifically recognizing the 5′-LTR and aimed at amplifying the 5′ flanking sequences of each retrotransposon element (Table 2).

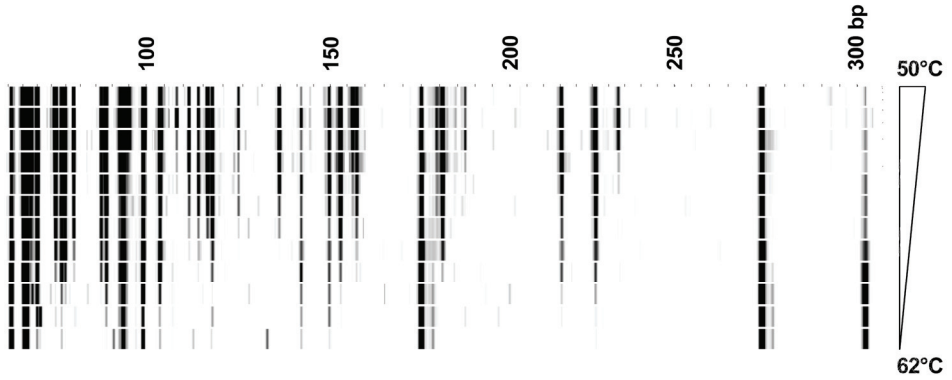


Figure 2. Influence of an increasing annealing temperature on RISC typing. Since one of the amplification primers contains multiple degenerate positions, at the higher annealing temperature of 62 °C, only the primers with the most G+C bases will participate. At the lower annealing temperature of 50°C, all targets are allowed to participate. At the right, the applied temperature gradient is indicated.

Assay optimization. At first, the choice for an appropriate annealing temperature was evaluated. Since considerable sequence variation exists in the individual copies of the 5′-LTR's, the internal primer contained a number of degenerate positions to allow a perfect match with almost all of the individual copies of the LTR elements. The effect of the annealing temperature was determined experimentally. At a relatively low annealing temperature of 50°C, some 25 bands appeared. Upon increasing the annealing temperature up to 62°C, the number of bands gradually decreased to 10 (Fig. 2). From preliminary results with more isolates, 52°C was arbitrarily chosen as the annealing temperature for further experiments (results not shown). Secondly, the optimal restriction enzyme was determined. The average fragment size that will be obtained with this approach will depend on the prevalence of restriction enzyme recognition sites in the flanking regions surrounding the retrotransposon elements. Three restriction enzymes were evaluated, Mse I, Msp I and Bfa I since these enzymes have either an AT-rich (TTAA), GC-rich (CCGG) or AT/GC balanced (CTAG) recognition site, respectively. With the use of Mse I, multiple fragments were obtained of which the majority ranged in size from 50–150 bp (not shown).With Msp I, few fragments were obtained and these were all in the upper region of the size marker or beyond (> 500 bp, not shown). Since on average some 20 fragments were found in the range of 60–300 bp using Bfa I, this enzyme was chosen for further experiments.

Results with clinical isolates. A collection of 55 *A. fumigatus* isolates from 15 patients with invasive aspergillosis was analyzed using RISC typing. All isolates were obtained from either respiratory samples or infected organs. On average, some 20 bands were obtained within the analyzed range of 60–300 bp (Fig. 3). When multiple isolates from different organs within a patient were analyzed, the obtained fingerprints were virtually identical to each other and visually clearly different from fingerprints derived from other patients' isolates or when they were compared to respiratory isolates. Even though the fingerprints contain both multiple strong as well as weak bands, all bands could be reproducibly amplified.

Discussion

We report RISC typing as a convenient PCR-based alternative technique for RFLP/Southern blot analysis. In the design of this assay, the following aspects were taken into consideration: first, the internal primer should be specific for either the 5′-LTR or 3′-LTR and should be oriented outward. If not adhered to this prerequisite, a situation may arise in which primarily internal fragments are amplified that may only yield a poor discriminatory power. Second, for a similar reason, the internal primer should be located close to the termini of the retrotransposon element. Third, restriction enzymes with a 4-base recognition sequence were used to generate relatively short DNA fragments (100–500 bp) that are easily amplified by PCR. This is in marked contrast to the RFLP procedure where 6-base recognition sequences are used, generating large DNA fragments (1000–10,000 bp). Fourth, by using a fluorescently labeled internal primer, amplification products are easily detected on a high-resolution DNA analysis platform such as a capillary DNA sequencer.

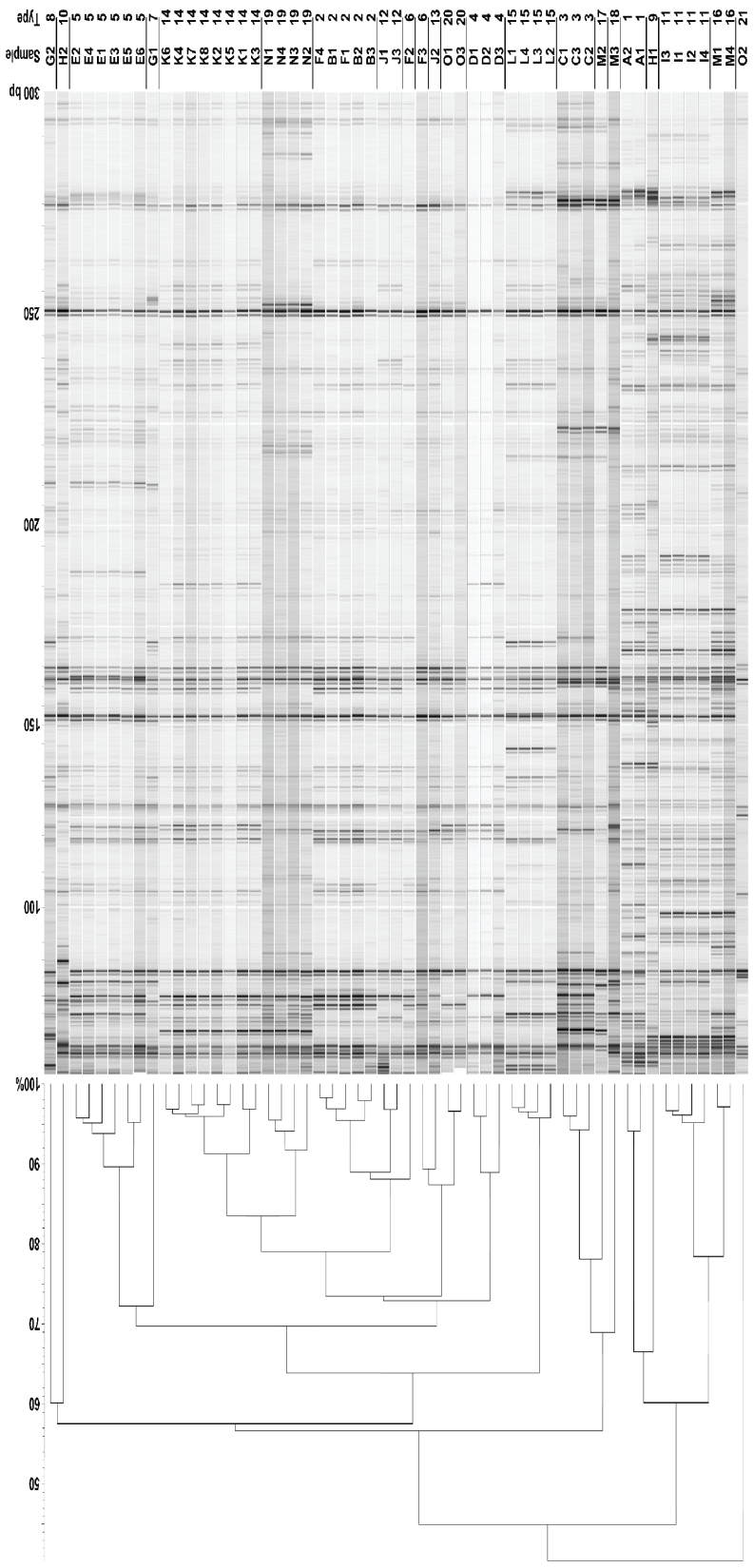


Figure 3. Result of RISC typing on a collection of 55 *A. fumigatus* isolates from 15 patients with invasive aspergillosis. The scale bar indicates the percentage identity. Sample numbers correspond to Table 1. Types were assigned arbitrarily based on the dendrogram as well as on visual inspection of the fingerprints, starting with the oldest isolate. The dendrogram was calculated using UPGMA clustering as described in the text.

DNA sequence alignment of the 50 highest scoring LTR sequences obtained using BLAST analysis and including 40 bp of flanking sequences, identified 23 5′-LTR elements and 19 3′-LTR elements. At the 5′ end of the 5′-LTR's, at least several bases are present that allow design of an internal primer specifically targeting most of the 5′-LTR's. Therefore, in the design of this assay, the 5′-LTR was chosen as the target for the internal primer. Interestingly, an additional 8 LTR's were detected with no conserved flanking sequences on either 5′ or 3′ side. These LTR's carry both 5′ and 3′ signature bases and hence are probably derived from recombination events between the 5′- and 3′-LTR's from an individual copy of the retrotransposon element. Since these LTR elements are no longer associated with a retrotransposon like element, we refer to them as orphan LTR's.

Unfortunately, there is considerable sequence variation in the LTR regions. The internal primer was designed to match the 5′-LTR consensus sequence and contained a number of degenerate positions to cover most of the variations in this region. This perfectly explains the results that are illustrated in figure 2 where a temperature gradient was applied during the annealing phase of the PCR. As expected, at a relatively high annealing temperature, less DNA fragments were amplified than at a relatively low annealing temperature. This can be explained by assuming that at the higher annealing temperature, primarily the part of the primers containing the most G+C bases will be able to anneal to their target whereas at the lower annealing temperature also the primers with the A+T bases will participate. Interestingly, the intensity of some bands increases with increasing temperature. We believe this to be a competition effect as the result of relatively more resources being available for fewer bands to be amplified. Based on these results, 52°C was arbitrarily chosen as annealing temperature for all subsequent experiments.

With the aim of generating relatively short PCR amplifiable DNA fragments, 3 restriction enzymes with a 4-base recognition sequence were evaluated. A selection of enzymes was made with recognition sequences that are either GC rich (Msp I), GC neutral (Bfa I) or AT rich (Mse I). The genome of *A. fumigatus* is reported to have a GC-content of 49.9% [9].

Theoretically, because of this perfectly balanced composition, all three restriction enzymes have an equal chance of cutting anywhere in the genomic DNA (i.e. on average once in every 256 bp). The average length of the DNA fragments to be obtained using RISC typing was therefore expected to be around 256 bp. However, the use of Mse I resulted in many short DNA fragments (< 100 bp) whereas the use of Msp I resulted in relatively few large DNA fragments (> 300 bp). The use of Bfa I yielded both short and medium to long DNA fragments and this enzyme was chosen for further analyses. Because of these unexpected results, we analyzed the AT-content of the flanking region of the retrotransposon elements. The average AT-content of 200 bp of flanking region proved to be 70%, much higher than the average AT-content of the entire genome. This explains our observations when using different restriction enzymes. This also illustrates that the Afut1 element has a preference for inserting into AT-rich sequences. The underlying mechanism responsible for this preference is yet unknown.

Upon employing RISC typing to study the genetic relationships between clinical *A. fumigatus* isolates, a clear picture emerges. Isolates from deep sites within individual patients were of the same genotype whereas isolates from respiratory samples often were of a different genotype. Identical genotypes were not found in isolates from different patients. Only few exceptions to these findings were observed. These results are in excellent agreement with other highly discriminatory molecular fingerprinting assays such as AFLP and/or STR*Af* typing [4] but small differences were observed. For instance: all isolates from patients K and N yielded identical fingerprints using AFLP analysis. Using STR*Af* typing, all isolates from patient K were clearly different from the isolates from patient N. RISC typing also yielded clearly different fingerprints for the isolates from these patients. Vice versa, isolates M1and M4were identical using both RISC- and STR*Af* typing but yielded a different fingerprint using AFLP analysis [4]. Different AFLP types can be distinguished based on the differential presence or absence of only a single band between the corresponding AFLP fingerprints. Using RISC typing, discrimination between interpatient isolates is based on multiple different bands in the fingerprint. Thus, RISC typing appears to be more discriminatory than AFLP analysis.

Differences between the results of these fingerprinting techniques are not entirely unexpected since each of the three methods targets different parts of the fungal genome. Small changes may occur at certain locations in the genome that may show up using either of these methods, but may remain unnoticed using others.

RISC typing employs a generic principle that can be adapted to other micro-organisms as well. The basic requirement is the presence of a multicopy element that is distributed throughout the genome (rather than being clustered in a particular region). Retroelements have also been described in other *Aspergillus species* such as *A. flavus* [10] and *A. nidulans* [8]. These all qualify as targets for a similar approach as described here.

In conclusion, RISC analysis provides a more rapid and user-friendly PCR alternative for the classical RFLP analysis and may be a welcome addition to existing molecular fingerprinting methods for studying the genetic relationships between *A. fumigatus* isolates.

References

1. Aufauvre-Brown, A., J. Cohen, and D. W. Holden. 1992. Use of randomly amplified polymorphic DNA markers to distinguish isolates of *Aspergillus fumigatus*. J Clin Microbiol 30:2991-3.
2. Chazalet, V., J. P. Debeaupuis, J. Sarfati, J. Lortholary, P. Ribaud, P. Shah, M. Cornet, H. Vu Thien, E. Gluckman, G. Brucker, and J. P. Latge. 1998. Molecular typing of environmental and patient isolates of *Aspergillus fumigatus* from various hospital settings. J Clin Microbiol 36:1494-500.
3. de Valk, H. A., J. F. G. M. Meis, I. M. Curfs, K. Muehlethaler, J. W. Mouton, and C. H. W. Klaassen. 2005. Use of a novel panel of nine short tandem repeats for exact and high-resolution fingerprinting of *Aspergillus fumigatus* isolates. J Clin Microbiol 43:4112-20.
4. de Valk, H. A., J. F. G. M. Meis, B. E. de Pauw, P. J. Donnelly, and C. H. W. Klaassen. 2007. Comparison of two highly discriminatory molecular fingerprinting assays for analysis of multiple *Aspergillus fumigatus* isolates from patients with invasive aspergillosis. J Clin Microbiol 45:1415-9.
5. Debeaupuis, J. P., J. Sarfati, V. Chazalet, and J. P. Latge. 1997. Genetic diversity among clinical and environmental isolates of *Aspergillus fumigatus*. Infect Immun 65:3080-5.
6. Girardin, H., J. P. Latge, T. Srikantha, B. Morrow, and D. R. Soll. 1993. Development of DNA probes for fingerprinting *Aspergillus fumigatus*. J Clin Microbiol 31:1547-54.
7. Neuveglise, C., J. Sarfati, J. Latge, and S. Paris. 1996. Afut1, a retrotransposon-like element from *Aspergillus fumigatus*. Nucl Acids Res 24:1428-1434.
8. Nielsen, M. L., T. D. Hermansen, and A. Aleksenko. 2001. A family of DNA repeats in *Aspergillus nidulans* has assimilated degenerated retrotransposons. Mol Genet Genomics 265:883-7.
9. Nierman, W. C., A. Pain, M. J. Anderson, J. R. Wortman, H. S. Kim, J. Arroyo *et al.* 2005. Genomic sequence of the pathogenic and allergenic filamentous fungus *Aspergillus fumigatus*. Nature 438:1151-6.
10. Okubara, P. A., B. K. Tibbot, A. S. Tarun, C. E. McAlpin, and S. S. Hua. 2003. Partial retrotransposon-like DNA sequence in the genomic clone of *Aspergillus flavus*, pAF28. Mycol Res 107:841-6.
11. Thompson, J. D., T. J. Gibson, F. Plewniak, F. Jeanmougin, and D. G. Higgins. 1997. The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucl Acids Res 25:4876-82.
12. Warris, A., C. H. W. Klaassen, J. F. G. M. Meis, M. T. de Ruiter, H. A. de Valk, T. G. Abrahamsen, P. Gaustad, and P. E. Verweij. 2003. Molecular epidemiology of *Aspergillus fumigatus* isolates recovered from water, air, and patients shows two clusters of genetically distinct strains. J Clin Microbiol 41:4101-4106.

Chapter 5

Microsatellite based typing of *Aspergillus fumigatus*: Strengths, pitfalls and solutions

Hanneke A. de Valk, Jacques F.G.M. Meis and Corné H.W. Klaassen

Journal of Microbiological Methods 2007, 69: 268-272.

Abstract

Microsatellites, or short tandem repeats (STR's), are popular tools to discriminate between microbial isolates. In this chapter, we report on the robustness of a microsatellite panel for discrimination of *Aspergillus fumigatus* isolates. Two major PCR artefacts (stutter peaks and minus-A peaks) can complicate correct interpretation of STR data. We investigated the effect of alterations to the various components of the PCR amplification mixtures on these PCR artefacts and on the reproducibility of this assay. Some extreme conditions led to a loss of signal, but, under all conditions where a signal was obtained, identical typing results were produced. Furthermore, pitfalls with the exchange of results between labs are discussed. These pitfalls are primarily associated with sizing of the obtained PCR fragments.

Introduction

Microsatellites, or short tandem repeats (STR's) are short repeated stretches of DNA, up to ten basepairs long, which are abundantly present in the genome of most higher organisms and which are sufficiently polymorph to serve as effective genetic markers [8]. STR's are also increasingly being used for the purpose of strain identification and discrimination in a wide variety of microorganisms [1, 2, 7, 9]. Among the main advantages of microsatellites as typing targets are their high discriminatory power, high throughput and the portability and exchange of typing data. An STR analysis is usually performed in two steps: i amplification of STR loci by PCR and ii detection and sizing of amplification products followed by the assignment of repeat numbers. The first step is routinely being done in a standard PCR thermocycler. Because of the required resolution, the second step is usually performed either by capillary electrophoresis or by polyacrylamide based slab gel electrophoresis, both in combination with fluorescent detection capabilities. Assignment of repeat numbers is established by comparing the relative electrophoretic mobility of the fragments to the mobility of reference fragments with established repeat numbers.

Previously, we developed a panel of 9 STR markers for discrimination between *Aspergillus fumigatus* isolates [2]. These 9 markers are divided over 3 multicolor multiplex PCR reactions aimed at amplifying 3 dinucleotide repeat markers (further referred to as the STR*Af*2 assay), 3 trinucleotide repeat markers (STR*Af*3) and 3 tetranucleotide repeat markers (STR*Af*4). The ability to study multiple STR markers in parallel with multicolor fluorescence detection technologies has greatly improved the amount of information that can be collected in a timely fashion. Results obtained so far on a collection of close to 1000 *A. fumigatus* isolates with this panel of 9 markers displayed excellent discriminatory power and showed 100% typeability (unpublished results). Therefore, these panels could be excellent candidates for standardization of *A. fumigatus* typing using STR's. However, a typical problem associated with multiplex PCR reactions is the reproducibility of the assay in different settings. Thorough evaluation and validation of new multiplex PCR assays is essential in order to be successfully transferred to other labs.

In this chapter, we report on the robustness of the STR*Af* assay under different amplification conditions. We deliberately and systematically altered various components of the PCR reaction and thermocycling parameters to investigate their effect on the outcome of this assay. Furthermore, some pitfalls associated with interpretation of the data and the exchanges of results between laboratories are discussed. These pitfalls are primarily associated with sizing of the DNA fragments that can be obtained under different electrophoretic conditions or on different electrophoretic platforms.

Materials and methods

Standard PCR protocol. PCR primers for the STR*Af*2, − 3 and − 4 assay were exactly as described before [2] and in chapter 2. In each multiplex reaction, different fluorescent labels (FAM, HEX and TET, respectively) were used for the 3 markers in each panel in order to distinguish the different amplification products from each other. PCR reactions were performed in a final volume of 25 µl containing 1 ng of genomic DNA, 1 µM of all amplification primers, 0.2 mM of each dNTP, 3 mM $MgCl_2$ (1.5 mM for the STR*Af*2 panel), 1 U of FastStart Taq DNA polymerase (Roche diagnostics, Almere, The Netherlands) in 1 × reaction buffer (Roche diagnostics). Thermocycling was performed in a T1 thermocycler (Biometra, Göttingen, Germany) using the following protocol: 10 min of initial denaturation at 95°C, followed by 30 cycles of 30 s of denaturation at 95°C, 30 s of annealing at 60°C, and 1 min of extension at 72°C. Before the reaction mixtures were cooled to room temperature, an additional incubation for 10 min at 72°C was performed. All temperature transitions were performed with maximum heating and cooling settings (5°C/s). Alternative to the use of FastStart Taq DNA polymerase, regular Taq DNA polymerase (Roche diagnostics) or KOD DNA polymerase (Omnilabo, Breda, The Netherlands) was used. Changes to the experimental conditions were made as described in the text.

Separation and detection of amplification products. PCR reactions were diluted 30-fold with distilled water. One µl of diluted PCR products were combined with 8.75 µl of distilled water and 0.25 µl of ET-ROX 400 marker (GE Healthcare, Diegem, Belgium). Following denaturation of the samples for 1 min at 95°C and rapid cooling to 4°C, they were injected onto a MegaBACE 500 equipped with a 48 capillary array. Injection and running parameters were according to the recommendations of the manufacturer (GE Healthcare). Alternatively, the ILS600 size marker (Promega, Leiden, The Netherlands) was used.

Results

Standard protocol. Using the standard amplification protocol, all three multiplex PCR reactions yielded three clearly identifiable peaks of different colors, representative for each of the 3 fluorescently labeled markers. In the STR*Af*2 reaction, a significant portion of the amplification products consisted of stutter peaks and of minus-A peaks [2]. These, however, did not influence the ability to identify the principal peak in each sample. Stutter peaks as well as minus-A peaks were less abundant in the STR*Af*3 reaction and were virtually absent in the STR*Af*4 reaction.

Magnesiumchloride. Concentrations from 1 to 4 mM were tested. For all magnesium chloride concentrations good interpretable peaks were found for all three multiplex PCR reactions (Table 1). The STR*Af*3 and −4 reactions were surprisingly insensitive to altered $MgCl_2$ concentrations although at the relative low concentration of 1 mM, minus-A peaks appeared in the STR*Af*4 reaction. Increasing the $MgCl_2$ concentration led to a reduction of the fraction of minus-A peaks in the STR*Af*2 reaction.

Table 1. Results of altered reaction conditions on the intensity of the principal peak (plus-A peak) in a sample and of the relative intensity of the principal peaks compared to the artefactual minus-A peaks

		STR*Af* 2		STR*Af* 3		STR*Af* 4	
		principal peak[a]	ratio[b]	principal peak[a]	ratio[b]	principal peak[a]	ratio[b]
Primer concentration (µM)	1.0	++	+/-	+++	++	+++	++
	0.5	++	+/-	+++	++	+++	++
	0.2	+	+	+++	++	+++	++
	0.1	+/-	++	++	++	+++	++
FastStart Taq DNA polymerase (units)	1	++	+/-	+++	++	+++	++
	0.5	+/-	-	++	++	++	+
	0.2	-	N.A.	-	N.A.	-	N.A.
	0.1	-	N.A.	-	N.A.	-	N.A.
Taq DNA polymerase (units)	1	+	--	+	++	+	-
	0.5	+	--	+	++	+	-
	0.2	+/-	--	-	N.A.	+/-	-
	0.1	-	N.A.	-	N.A.	-	N.A.
Genomic DNA (ng)	100	++	--	+++	+	+++	+
	30	++	--	+++	++	+++	++
	10	++	--	+++	++	+++	++
	3	++	-	+++	++	+++	++
	1	++	+/-	+++	++	+++	++
	0.3	++	+	++	++	+	++
	0.1	+	++	+	++	-	N.A.
Magnesium chloride (mM)	4	++	++	+++	++	+++	++
	3	++	++	+++	++	+++	++
	2	++	+	++	++	++	++
	1.5	++	+/-	++	++	++	++
	1	++	-	++	++	++	+

N.A.: not applicable.
[a]: Intensity of the principal peaks in a sample: +++ very strong signal; ++ strong signal; + good signal; +/- poor signal; - no signal.
[b]: Difference in intensity between the principal peaks (plus-A peaks) and the minus-A peaks in a sample: ++ plus-A >> minus-A; + plus-A > minus-A; +/- plus-A = minus-A; - minus-A > plus-A; --minus-A >> plus-A.

DNA concentration. The amount of input DNA for each of the 3 assays was varied from 0.1 to 100 ng per reaction (Table 1). A concentration of 1 ng of genomic DNA per reaction was used with the standard protocol. Lower concentrations led to a reduction of the fraction of minus-A peaks, but also resulted in a lower signal. Increasing the concentration did not yield higher signals but did result in increased formation of minus-A peaks, especially in the STR*Af*2 reaction. At the highest concentrations, the peaks mainly consisted of minus-A peaks, the plus-A peaks were almost completely eliminated (Fig. 1A). Again, the STR*Af*3 and −4 reactions were relatively insensitive to changes in the genomic DNA concentration.

Primer concentration. Equimolar primer concentrations of 0.1–1 μM each were tested (Table 1). The STR*Af*4 reaction remained virtually unaffected even by lowering the primer concentration 10-fold. However, for the STR*Af*2 and −3 reactions, the intensity of the peaks was reduced at lower primer concentrations. The ratio between the 3 markers remained stable at all tested concentrations. An a-specific product, sometimes observed in the STR*Af*3 assay, disappeared by lowering the primer concentration. Lowering the primer concentration also led to a reduction of the fraction of minus-A peaks in the STR*Af*2 reaction.

DNA polymerase. At present it is common practice to use so-called 'hot-start' procedures to minimize the formation of primer-dimers and other unwanted artefacts in PCR reactions. Especially in multiplex PCR reactions this may prove to be a problem. We tested the STR*Af* panels using FastStart Taq DNA polymerase (a hot-start formulation) and regular Taq DNA polymerase. Both enzymes were tested in concentrations ranging from 0.1–1 U per reaction (Table 1). For regular Taq DNA Polymerase an initial denaturation step of 4 min was performed instead of the 10 min used with FastStart Taq DNA polymerase. With both enzymes, the intensity of the peaks dropped to undetectable levels when 0.2 units or less was used. FastStart Taq DNA polymerase yielded more intense signals than regular Taq DNA polymerase. Reducing the enzyme concentration also led to increased formation of minus-A peaks. Both enzymes have intrinsic template independent polymerase activity responsible for the addition of the unencoded A residue to the newly synthesized strand. Changing the enzyme to a proofreading formulation lacking this activity completely eliminated the formation of plus-A peaks. Hence, the sizes of all principle and stutter peaks in the samples were effectively reduced by 1 bp (Fig. 1B).

Annealing temperature. The annealing temperature was varied from 55°C to 65°C. In the STR*Af*3 reaction an atypical peak containing the FAM label was sometimes observed. Increasing the annealing temperature up to 65°C reduced the intensity of this peak but hardly affected the intensity of the true STR*Af* peaks. Changing the annealing temperature also did not affect the balance between the markers (results not shown).

Ramping. Several modern thermocyclers allow fast ramping between the different steps of the PCR process (up to 5 °C/s) whereas the older generation cyclers usually are restricted to ramping speeds of 1 °C/s. Changing ramping speeds could adversely affect the performance of a PCR reaction. With the STR*Af* panels, however, no effect was seen when the ramping speed was reduced from 5 °C to 1 °C/s (results not shown).

Size marker. Two different size markers were compared for sizing of the fragments; the routinely used ET-ROX 400 and the ILS600 marker. The calculated sizes for the alleles in the STR*Af*3 panel using the different size markers differed by a minimum of 2.9 bp and up to 5.0 bp from each other (Fig. 2) for each of the 3 markers in the STR*Af*3 panel.

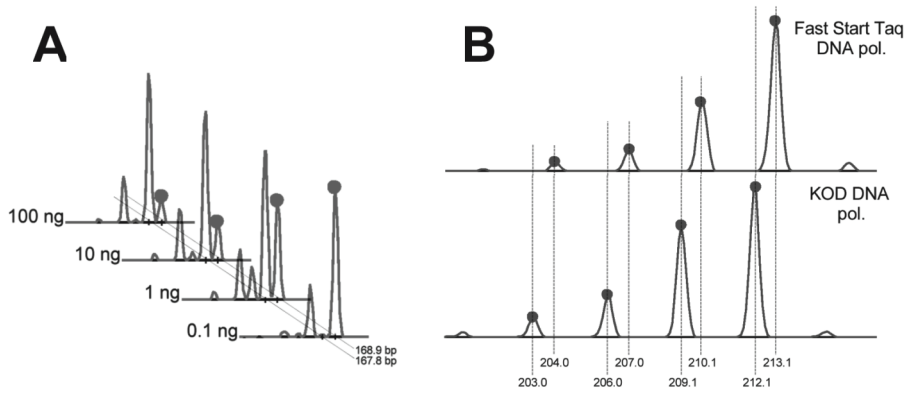


Figure 1 A: Illustration of the typical effect of the DNA concentration on the formation of minus-A peaks in a dinucleotide marker. Elevated DNA concentrations lead to increased formation of minus-A peaks (167.8 bp) and to a reduced intensity of the principal plus-A peaks (168.9 bp), potentially resulting in misassignment of the principal peak in a sample (indicated with a dot). Note that changes in the DNA concentration do not affect the formation of stutter peaks. B: Use of a proofreading enzyme (such as KOD DNA polymerase) results in the exclusive formation of minus-A peaks as shown for a trinucleotide marker.

Discussion

In order for any typing technique to be successfully used in another setting, the most important factor is reproducibility. Ideally, small pipetting errors and other minor changes to the reaction conditions should have no influence on the outcome of the assay. With this in mind, the robustness of the STR*Af* assay was investigated by deliberately and systematically changing various reaction components to suboptimal conditions and by varying thermocycling parameters. This way we mimicked unintentional altered reaction conditions. Certain extreme conditions, such as reducing the concentration of the DNA polymerase 5-fold or more, led to a loss of signal, but under all of the altered conditions that were investigated where a signal was obtained, identical typing results were produced. Thus, the STR*Af* assay proved to be an extremely robust assay. Some of the changes that we made to the protocol could be considered as standard parameters that need to be optimized upon developing a new assay. However, examples where the robustness of a high resolution microbial typing assay is so convincingly demonstrated are only scarce. In fact, it is well known that certain other PCR typing assays (such as RAPD) need to be performed using highly standardized reaction conditions in order to obtain stable and reproducible typing results. Based on our results, one could even predict how reaction conditions should be modified when suboptimal results are obtained upon development of a new STR panel. In the light of these results, we expect that introduction of this assay in another setting will not yield significant problems even when a major alteration (such as the use of a different enzyme or thermocycler — two factors that could drastically affect reaction performance) is being made to the protocol.

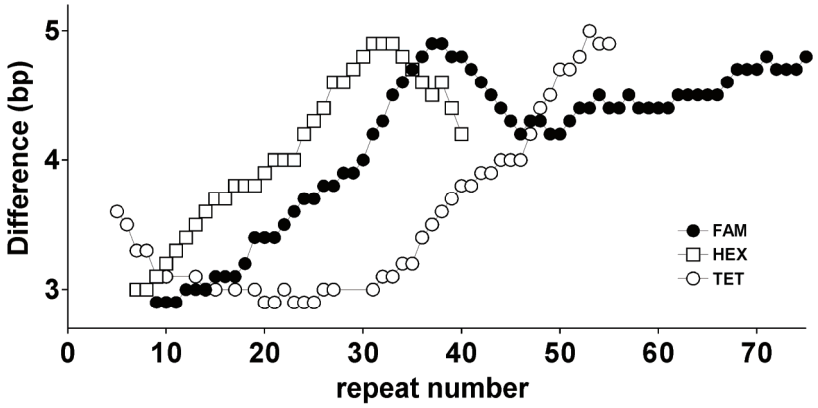


Figure 2 Difference in the calculated size (in bp) of the alleles in the STRAf3 panel as the result of the use of different size markers. The use of a different size marker may result in misassignment of repeat numbers by more than 1 repeat unit. Note that the size difference is not constant, but varies with repeat number. FAM, HEX and TET are the correspondingly labeled markers.

Apart from a high potential of generating reproducible PCR results, the other aspect that has to be taken into consideration is the interpretation of the electropherogram that will be obtained. Basically, interpretation of STR data can become complicated by two major PCR artefacts, i.e. minus-A peaks and stutter peaks. Minus-A peaks are the result of incomplete addition of an adenine to the 3′ end of the newly synthesized strand by Taq DNA polymerase. These minus-A peaks usually arise when too much DNA is used as input in the PCR reaction, when insufficient DNA polymerase activity is present (for instance as the result of suboptimal $MgCl_2$ concentrations), or when the last residue of the synthesized strand is not a pyrimidine (this should preferably be a cytosine). If one is unaware of these artefacts, excessive formation of minus-A peaks under suboptimal reaction conditions could result in the assignment of the wrong peak in an STR analysis and thus lead to a wrong typing result. As demonstrated here, the presence of minus-A peaks in certain markers is strongly influenced by the specific circumstances under which the markers are being amplified. However, as shown, the fraction of minus-A peaks can easily be minimized by adjusting the appropriate reaction conditions as can be deduced from Table 1. Alternatively, one could opt for elimination of the plus-A peaks by routinely using a DNA polymerase with proofreading activity. This, however, is usually associated with higher costs. The second artefact, formation of stutter peaks, is the result of strand slippage during synthesis of the complimentary strand by the DNA polymerase.

The intensity of stutter peaks in the amplification products is inversely related to the size of the repeat unit as well as to the repeat number. Hence they occur more frequently in the dinucleotide repeats (STR*Af*2) than in the tri- and tetranucleotide repeats (STR*Af*3 and −4). Likewise, their abundance is greater in repeat numbers of >20 than in fragments with repeat numbers of <10. The formation of stutter peaks remained virtually unaffected by the variations in reaction conditions that were tested here (not shown); hence they are notoriously difficult to get rid of. Although it appears that stutter peaks are only associated with negative aspects, in certain instances, stutter peaks also can be considered as a blessing in disguise. Incidentally, a-specific PCR fragments are amplified (i.e. when using a too low annealing temperature) as the result of a single primer product or as the result of mispriming if both primers are at amplifiable distance and orientation. If these fragments contain the fluorescent labeled primer, they may show up in the final electropherogram. However, since such a-specific fragments lack the short tandem repeat sequence, they consequently lack the characteristic associated stutter peaks.

If one is aware of the above complicating factors while interpreting STR electropherograms, usually it should be no problem to identify the principal peak in each marker. The next step in the process is assignment of the repeat number to this principal peak. This is routinely established by sizing of the DNA fragment and comparing it to the size of reference fragments with established repeat numbers. The major pitfall here is that DNA sizing data alone is not suitable for exchanging results between labs. It is often not realized that sizing DNA fragments by capillary electrophoresis only yields relative data. The calculated values could be multiple basepairs above or below the real value. Shortly after the introduction of capillary electrophoresis in DNA analyses, it has been well established that the electrophoretic mobility of a DNA fragment is not only influenced by the number of bases but also by other factors such as the exact base composition and sequence, the separation matrix, presence of denaturing compounds, temperature and fluorescent labels [5, 6]. As shown here, even on the same platform and under the same electrophoretic conditions, different fragment sizes are obtained when different size markers are being used.

Thus, different fragments could be mistaken as identical and identical fragments could mistakenly be called different when results obtained on different electrophoresis platforms, or obtained on the same platform but using different conditions, are compared to each other. Therefore, sizing data alone should not be used to compare results between labs. In order to be able to exchange STR data between labs, it is of utmost importance that a careful calibration of the different available electrophoresis platforms is performed. Such a calibration would consist of the establishment of reference sizing values for a number of well characterized alleles. We reasoned that the most straightforward method to achieve such a calibration would be the use of a series of allelic ladders. An allelic ladder is a mixture of the alleles that can be expected when testing isolates. Since the DNA fragments in the allelic ladders are identical to the DNA fragments that will be obtained upon testing isolates and since they carry the same fluorescent label, their electrophoretic mobility will also be exactly the same, provided they are analyzed using the same electrophoretic conditions. The major advantage of the use of allelic ladders is that there is no need to use identical electrophoretic conditions between labs: each lab is free to use their own routine running conditions as long as these conditions suffice to resolve the peaks in the allelic ladders. Such allelic ladders have been introduced as reference tools in human forensic applications in the early 1990's [3, 4] but, to the best of our knowledge, have not yet been described for calibration of microbial microsatellite based typing schemes. Experiments aimed at providing the 'proof of concept' for these allelic ladders are currently in progress in an international interlaboratory typing study for *A. fumigatus* using the STR*Af*.

References

1. Adair, D. M., P. L. Worsham, K. K. Hill, A. M. Klevytska, P. J. Jackson, A. M. Friedlander, and P. Keim. 2000. Diversity in a variable-number tandem repeat from *Yersinia pestis*. J Clin Microbiol 38:1516-9.
2. de Valk, H. A., J. F. G. M. Meis, I. M. Curfs, K. Muehlethaler, J. W. Mouton, and C. H. W. Klaassen. 2005. Use of a novel panel of nine short tandem repeats for exact and high-resolution fingerprinting of *Aspergillus fumigatus* isolates. J Clin Microbiol 43:4112-20.
3. Puers, C., H. A. Hammond, L. Jin, C. T. Caskey, and J. W. Schumm. 1993. Identification of repeat sequence heterogeneity at the polymorphic short tandem repeat locus HUMTH01[AATG]n and reassignment of alleles in population analysis by using a locus-specific allelic ladder. Am J Hum Genet 53:953-8.
4. Skowasch, K., P. Wiegand, and B. Brinkmann. 1992. pMCT 118 (D1S80): A new allelic ladder and an improved electrophoretic separation lead to the demonstration of 28 alleles. Int J Legal Med 105:165-8.
5. Tu, O., T. Knott, M. Marsh, K. Bechtol, D. Harris, D. Barker, and J. Bashkin. 1998. The influence of fluorescent dye structure on the electrophoretic mobility of end-labeled DNA. Nucl Acids Res 26:2797-802.
6. Vainer, M., S. Enad, V. Dolnik, D. Xu, J. Bashkin, M. Marsh, O. Tu, D. W. Harris, D. L. Barker, and E. S. Mansfield. 1997. Short tandem repeat typing by capillary array electrophoresis: comparison of sizing accuracy and precision using different buffer systems. Genomics 41:1-9.
7. van Belkum, A., W. Melchers, C. Ijsseldijk, L. Nohlmans, H. Verbrugh, and J. Meis. 1997. Outbreak of amoxicillin-resistant *Haemophilus influenzae* type b: Variable number of tandem repeats as novel molecular markers. J Clin Microbiol 35:1517-1520.
8. van Belkum, A., S. Scherer, L. van Alphen, and H. Verbrugh. 1998. Short-sequence DNA repeats in prokaryotic genomes. Microbiol Mol Biol Rev 62:275-93.
9. Verduyn-Lunel, F., L. Licciardello, S. Stefani, H. A. Verbrugh, W. J. G. Melchers, J. F. G. M. Meis, S. Scherer, and A. van Belkum. 1998. Lack of consistent short sequence repeat polymorphisms in genetically homologous colonizing and invasive *Candida albicans* strains. J Bacteriol 180:3771-3778.

Chapter 6

Interlaboratory reproducibility of a microsatellite based typing assay for *Aspergillus fumigatus* through the use of allelic ladders: Proof of concept

Hanneke A. de Valk, Jacques F.G.M. Meis, Stéphane Bretagne, Jean-Marc Costa, Brent A. Lasker, S. Arunmozhi Balajee, Alessandro C. Pasqualotto, Michael J. Anderson, Laura Alcázar-Fuoli, Emilia Mellado and Corné H.W. Klaassen

Clinical Microbiology and Infection, in press

Abstract

An interlaboratory study was performed aimed at investigating the reproducibility of a multiplex microbial microsatellite based typing assay in different settings using a variety of experimental and analytical conditions and with teams having variable prior microsatellite typing experience. In order to circumvent problems with exchange of sizing data, allelic ladders are introduced as a straightforward and universally applicable concept for standardization of such typing assays. Allelic ladders consist of mixtures of well-characterized reference fragments to act as reference points for the position in an electrophoretic trace of fragments with established repeat numbers. Five laboratories independently analyzed 6 microsatellite markers in 18 samples that were provided either as DNA or as *A. fumigatus* conidia. Allelic data were reported as repeat numbers and as sizes in nucleotides. Without the use of allelic ladders, size differences up to 6.7 nucleotides were observed resulting in interpretation errors up to 2 repeat units. Difficulties in interpretation were related to non-specific amplification products (which were resolved with explanation) and bleed-through of the different fluorescent labels. In contrast, standardization of sizing data by using allelic ladders enabled all participants to produce identical typing data. The use of allelic ladders as a routine part of molecular typing using microsatellite markers provides robust results suitable for interlaboratory comparisons and for deposition in a global typing database.

Introduction

Aspergillus fumigatus is the fungal species most frequently involved in human infections in some immunocompromized patient populations such as allogeneic stem cell transplant recipients. To gain more insight into the genetic and epidemiological relationships between isolates obtained from various origins, several molecular typing techniques have been developed [12, 15]. Ideally, typing results should be accurate, reproducible and easy to interpret. Importantly, methods should be transferable to other settings, so that individual results can be compared to each other irrespective of laboratory variables such as the use of different equipment, reagents and software. Unfortunately, many typing methods such as random amplified polymorphic DNA (RAPD) [1], restriction fragment length polymorphism (RFLP) [8] and amplified fragment length polymorphism (AFLP) [6], yield fingerprint profiles that consist of a complex banding pattern which are difficult to reproduce in different settings. Only a few fingerprinting methods have been developed for *A. fumigatus* that yield exact typing data that can be unambiguously and easily interpreted. One such format is multi-locus sequence typing (MLST) that offers only limited discriminatory power to distinguish between different *A. fumigatus* isolates [2]. Another exact and high-resolution typing format is based on microsatellite markers. Two such microsatellite based fingerprinting assays for *A. fumigatus* have been reported by Bart-Delabesse *et al.*, and de Valk *et al.* [3, 5]. Microsatellites, or short tandem repeats (STR's), provide high resolution analysis that is consistent with RFLP [4] and AFLP analyses [6]. The two methods primarily differ in the nature and number of markers that are analyzed. Amplification of microsatellite loci under high stringency conditions leads to reproducible amplifications that are hardly affected by minor experimental variables. Sizing of PCR fragments is done automatically using high-resolution electrophoresis platforms and such data can easily be converted into the corresponding number of repeats by comparison to reference fragments with established repeat numbers. Because of their compact and numerical output, STR typing is a very attractive system for exchanging results between laboratories in a digital format and to establish global typing databases.

However, the interlaboratory reproducibility and transferability of microbial STR typing systems have not yet been demonstrated. The complicating factor, which has been well documented, is that the electrophoretic mobility (and thus the calculated size) of a DNA fragment in capillary electrophoresis platforms is influenced by multiple factors. These include the exact base composition and sequence of the DNA, the separation matrix, presence of denaturing compounds, temperature and fluorescent labels [13, 14]. Additionally, even the size standard and the DNA polymerase that is used for amplification may affect the calculated size of an allele [7]. Thus, sizing values alone are not suitable for exchange unless a careful calibration of the different platforms has been established [7, 9]. Such a calibration could consist of the generation of calibration curves as recently demonstrated by Pasqualotto *et al.* [9]. Alternatively, a more straightforward and universally applicable method to achieve such a calibration is through the use of allelic ladders [10, 11]. An allelic ladder consists of a well-defined mixture of pre-amplified alleles with predetermined repeat numbers (by DNA sequencing), and can be used to create reference positions for the interpretation of typing results. Here, we report the results of an international multicenter study that provides the proof of concept for use of locus-specific allelic ladders in microsatellite-based microbial typing schemes.

Material and Methods

STR typing protocol. The STR markers used in this study is a subset of the panel described by de Valk *et al.* [5], and involves the M3 multiplex (containing the STR*Af*3A, -3B, -3C trinucleotide repeat markers) and M4 multiplex (containing the STR*Af*4A, -4B and -4C tetranucleotide repeat markers). In each multiplex reaction different fluorescent labels were used to discriminate between the individual markers. For economical reasons, the combination of FAM (carboxyfluorescein), HEX (hexachlorocarboxyfluorescein) and TET (tetrachlorocarboxyfluorescein) was chosen.

Construction of allelic ladders. Allelic ladders were constructed for each of the three trinucleotide markers in the STR*Af* 3 panel (STR*Af* 3A, -3B and -3C, respectively). Briefly, selected alleles were amplified in monoplex PCR reactions. The resulting fragments were cloned into the PGEM-T Easy Vector system (Promega, Leiden, The Netherlands). DNA from recombinant clones was isolated using High Pure chemistry (Roche diagnostics, Almere, the Netherlands) and quantified by UV measurements. Verification of the repeat number in each construct was performed by direct sequence analysis of the plasmid DNA preparations as described before [5]. A mixture of plasmid DNA preparations was made containing inserts with established repeat numbers. The mixture of plasmids was then amplified using monoplex PCR reactions and analyzed by capillary electrophoresis. Finally, the concentration of certain alleles was adjusted to allow unambiguous identification of each reference allele. Since PCR amplification of short tandem repeats leads to the formation of so called stutter peaks (e.g. additional fragments mostly containing fewer repeat numbers [5, 7]), it was not necessary to actually include all alleles in the amplification mixture. The missing alleles were generated automatically during the amplification process. As a result, although the sample used for amplication contained only 22 selected alleles, after amplification of this plasmid mixture the STR*Af* 3A allelic ladder contains 68 alleles with repeat numbers ranging from 9 to 76. Likewise, the STR*Af* 3B allelic ladder contains alleles with repeat numbers from 7 to 39 and the STR*Af* 3C allelic ladder from 5 to 53 as shown in figures 1A, 1B and 1C, respectively. An example of the use of these allelic ladders is illustrated with marker STRAf 3A in figure 2.

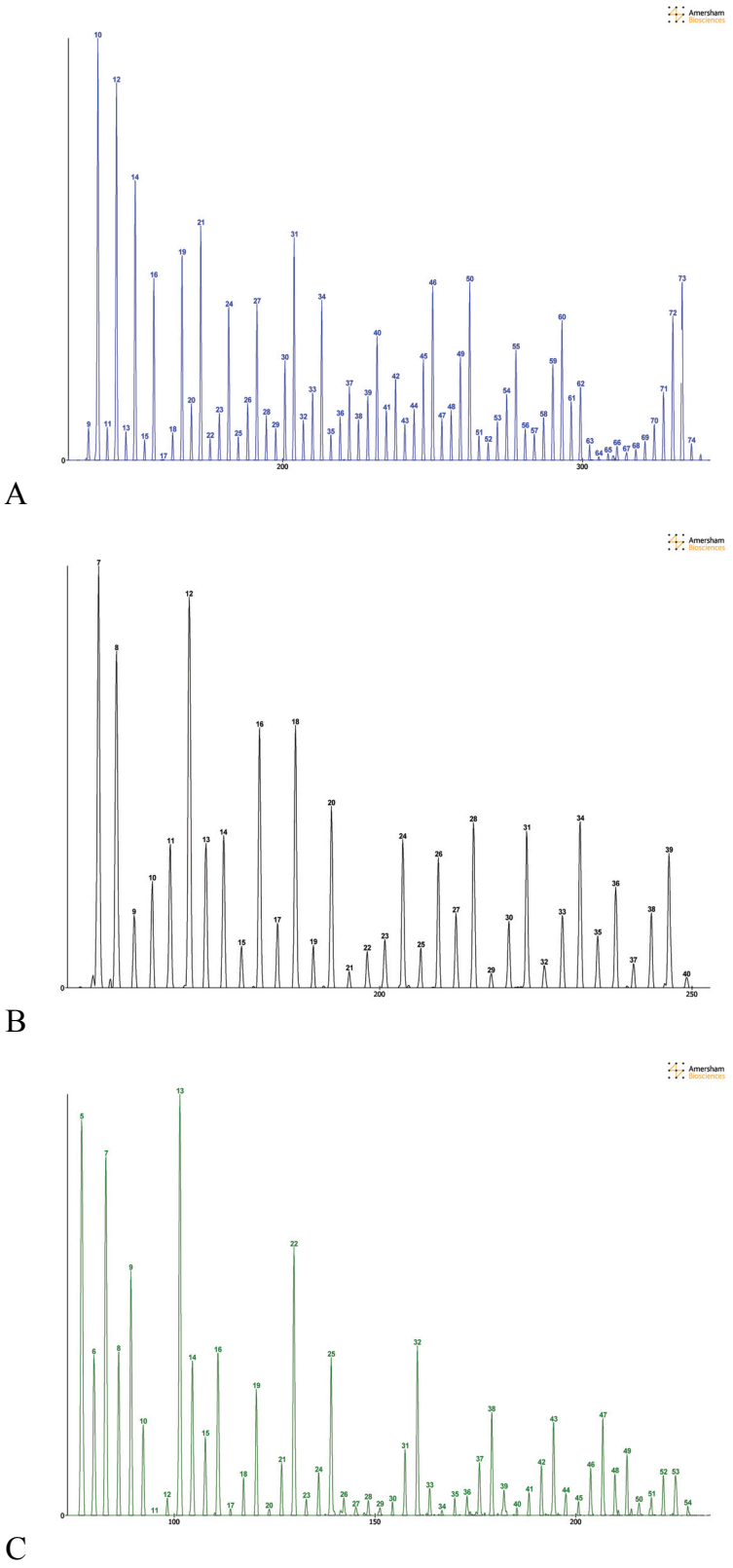


Figure 1. Illustration of the allelic ladders for the trinucleotide repeat markers. The numbers above the peaks correspond to the repeat numbers of each allele in these ladders. Boldface alleles in a larger font were actually included in the samples; the other alleles are the result of the formation of stutter peaks upon PCR amplification of the included alleles. A, B and C: the STR*Af*3A, -3B and -3C allelic ladder, respectively.

Study design and protocol. Five laboratories (denoted A-E throughout this manuscript) participated in the study. Each laboratory received 12 purified DNA samples (A1-A12), multiplex primermixes M3 and M4 (amplifying the 3 trinucleotide markers in the STR*Af*3 panel and the 3 tetranucleotide markers in the STR*Af*4 panel, respectively) [5], a size standard (ET400-R from GE Healthcare, Diegem, Belgium), allelic ladders for the STR*Af* 3 loci (including a graphical display of their appearance), and amplification instructions for the preparation of reaction mixes. Laboratories also received 6 *A. fumigatus* isolates (B1-B6, as conidia), that were included in order to examine the potential influence of the DNA quality on the outcome of the assay. Four out of these 6 isolates were also provided as DNA samples and should have yielded identical typing results. All participating laboratories had access (either in-house or through an external party) to a capillary electrophoresis platform with multicolor detection ability. Each participating laboratory was free to choose their DNA isolation procedure for samples B1-B6, preference of enzyme to be used for amplification (as long as it did not involve a proofreading formulation), and choice of thermocycler, size standard and capillary electrophoresis platform (including running conditions). Reference values for all samples were obtained independently in a blinded fashion by one of the participating laboratories.

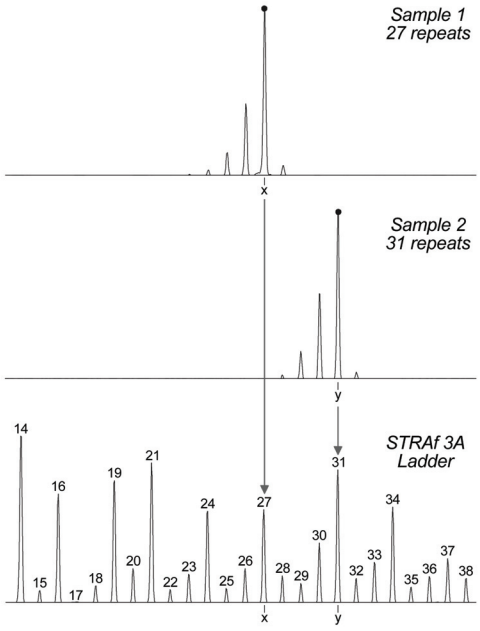


Figure 2. Application of allelic ladders for calibrating short tandem repeat based typing data between different laboratories. The example shown is for the STR*Af* 3A marker. Sample 1 yields a principal peak of size X. Size X corresponds to an allele with 27 repeats as can be deduced from the pre-amplified allelic ladder which is added to another capillary with the same size standard as used with regular samples. Sample 2 yields a principal peak of size Y which corresponds to 31 repeats. Although sizes X and Y can be very precisely reproduced within a laboratory (usually within 0.2 nt), they can be very different in another laboratory on another platform and/or using different electrophoretic conditions.

Data collection and analysis. Several data sets were reported by the participating laboratories. First, the sizes of the peaks in the allelic ladders were determined with a free choice of size standard. These values were to be used as reference values for the interpretation of the results of the STR*Af* 3 markers. In order to examine the influence of various size standards, the sizes of peaks in the allelic ladders were also determined using the ET400-R size standard. The results for the markers in the STR*Af*3 panel for all 18 samples were reported as repeat numbers. Second, the repeat numbers of the markers in the STR*Af* 4 panel were determined for all 18 samples using the ET400-R marker in combination with reference size values taken from the original publication [5]. For those alleles which did not exactly fit the reference positions in the allelic ladders or reference values from the literature, but which differed by one, two or three nucleotides, the notation n.1, n.2 or n.3 was used indicating the allele corresponded to a size of n repeats + 1, 2 or 3 nucleotides, respectively [5].

Results

The wide variety of experimental variables and equipment utilized by the participating laboratories is shown in Table 1. All the participating laboratories used a different capillary electrophoresis platform. In addition, 3 distinct thermocyclers, at least 3 distinct DNA isolation procedures and 4 different commercially available size standards were used.

First, the effect of the choice of the internal size standard on the calculated sizes of the alleles in the STR*Af*3 ladders was determined. Differences in sizing data for alleles in the allelic ladders are shown in figure 3. The sizes of the alleles in the allelic ladders were calculated using two size standards: the size standard that was routinely used in each participating laboratory (see Table 1) and the ET400-R size standard as supplied to all laboratories. The results show that identical alleles, analyzed on different electrophoretic platforms with different size standards may differ up to 6.7 nucleotides. Switching to the use of identical size standards still yielded size differences up to 5.4 nucleotides on the various platforms (results not shown).

Table 1. Overview of experimental variables from the participating laboratories.

	A	B	C	D	E
Prior STR experience	plenty	plenty	plenty	some	none
DNA isolation procedure	MagNA Lyser + MagNA Pure (Roche diagnostics)	MagNA Lyser (Roche diagnostics) + Qiagen (Qiagen)	Phenol/Chloroform + Qiagen (Qiagen)	FastDNA (Bio 101® systems) + DNeasy (Qiagen)	Phenol/Chloroform + Chromaspin (Clontech)
Enzyme	FastStart *Taq* (Roche diagnostics)	FastStart *Taq* (Roche diagnostics)	FastStart *Taq* (Roche diagnostics)	FastStart *Taq* (Roche diagnostics)	FastStart *Taq* (Roche diagnostics)
Thermocycler	T1 (BioMetra)	GeneAmp 9700 (Applied Biosystem)	GeneAmp 9700 (Applied Biosystems)	iCycler (BioRAD)	GeneAmp 9700 (Applied Biosystem)
Capillary platform	MegaBACE 500 (GE Healthcare)	ABI Prism 3100 (Applied Biosystems)	ABI Prism 310 (Applied Biosystems)	ABI Prism 3130 (Applied Biosystems)	ABI Prism 3700 (Applied Biosystems)
Location	In-house	In-house	In-house	External	External
Size standard	ET400-R (GE Healthcare)	400 HD Rox (Applied Biosystems)	GeneScan 500-TAMRA (Applied Biosystems)	GeneScan 500 LIZ (Applied Biosystems)	ET400-R (GE Healthcare)

The results for the determination of the repeat numbers in the trinucleotide markers in all 18 samples by the 5 participating laboratories are shown in Table 2. Assignment of alleles in the STR*Af* 3 panel for all samples was done by comparing the sizes of the fragments in the samples to the sizes of the fragments in the allelic ladders using the laboratory's routine size standard (as exemplified in figure 2). Two laboratories (A and B) yielded 100% identical results for all markers in all samples. Laboratory C made 1 interpretation error: sample B2 yielded a '9.2' allele where the expected result was a '10' allele. Examination of the corresponding electropherogram showed that this was the result of a reduced electrophoretic resolution. Under the experimental conditions as used, the platform (ABI Prism 310) was unable to adequately resolve minus-A peaks from plus-A peaks. This was apparent from the broad and round appearance of the peaks. Laboratory D made 3 interpretation errors. A '31' allele in STR*Af* 3A was reported as a '10.1' allele, a '15' allele in STR*Af* 3B was reported as a '10' allele and a '25' allele in STR*Af* 3C was reported as a '7' allele. Since all errors occurred in a single sample (A10), we believe this to be a sample handling issue. When the original electropherograms were re-examined in light of the expected results, low intensity peaks of the correct size were detected (31, 15 and 25 repeats, respectively).

Laboratory D also reported low fluorescent signals with samples A1-A12 that were markedly improved with use of a higher concentration of DNA in the PCR reactions. Repeating the A10 PCR with more DNA resulted in peaks of the expected sizes. Laboratory E made 8 interpretation errors. In all these cases, the reported alleles were reduced by 1 or 2 repeat units, probably because of the assignment of stutter peaks instead of the actual principle peaks. Interpretation of the results by lab E was also hampered by substantial bleed-through (a.k.a. cross-talk) of the different fluorescent labels on the ABI Prism 3700 which they were unable to resolve. With the exception of the above incidental interpretation errors, identical typing results were obtained for all samples that were provided both as isolates and DNA samples.

Table 2. Results of the trinucleotide repeat markers based on calibration with allelic ladders. Shaded results differ from the expected reference values.

	STR*Af* 3A					STR*Af* 3B					STR*Af* 3C				
	A	B	C	D	E	A	B	C	D	E	A	B	C	D	E
A1*	49	49	49	49	49	11	11	11	11	11	7	7	7	7	7
A2	48	48	48	48	46	11	11	11	11	11	7	7	7	7	7
A3	27	27	27	27	27	9	9	9	9	8	26	26	26	26	26
A4*	51	51	51	51	51	16	16	16	16	16	22	22	22	22	22
A5*	26	26	26	26	26	23	23	23	23	23	22	22	22	22	22
A6	25	25	25	25	25	8	8	8	8	8	10	10	10	10	10
A7	35	35	35	35	35	11	11	11	11	11	33	33	33	33	33
A8	36	36	36	36	36	11	11	11	11	11	7	7	7	7	7
A9*	18	18	18	18	18	9	9	9	9	9	6	6	6	6	6
A10	31	31	31	10.1	30	15	15	15	10	15	25	25	25	7	25
A11	34	34	34	34	34	24	24	24	24	24	40	40	40	40	39
A12	27	27	27	27	27	12	12	12	12	12	7	7	7	7	7
B1*	51	51	51	51	51	16	16	16	16	16	22	22	22	22	22
B2	10	10	10	10	10	10	10	9.2	10	10	10	10	10	10	10
B3*	26	26	26	26	26	23	23	23	23	21	22	22	22	22	22
B4*	49	49	49	49	49	11	11	11	11	11	7	7	7	7	7
B5*	18	18	18	18	17	9	9	9	9	9	6	6	6	6	5
B6	14	14	14	14	14	34	34	34	34	34	18	18	18	18	17

*Isolates B1, B3, B4 and B5 correspond to DNA samples A4, A5, A1 and A9, respectively

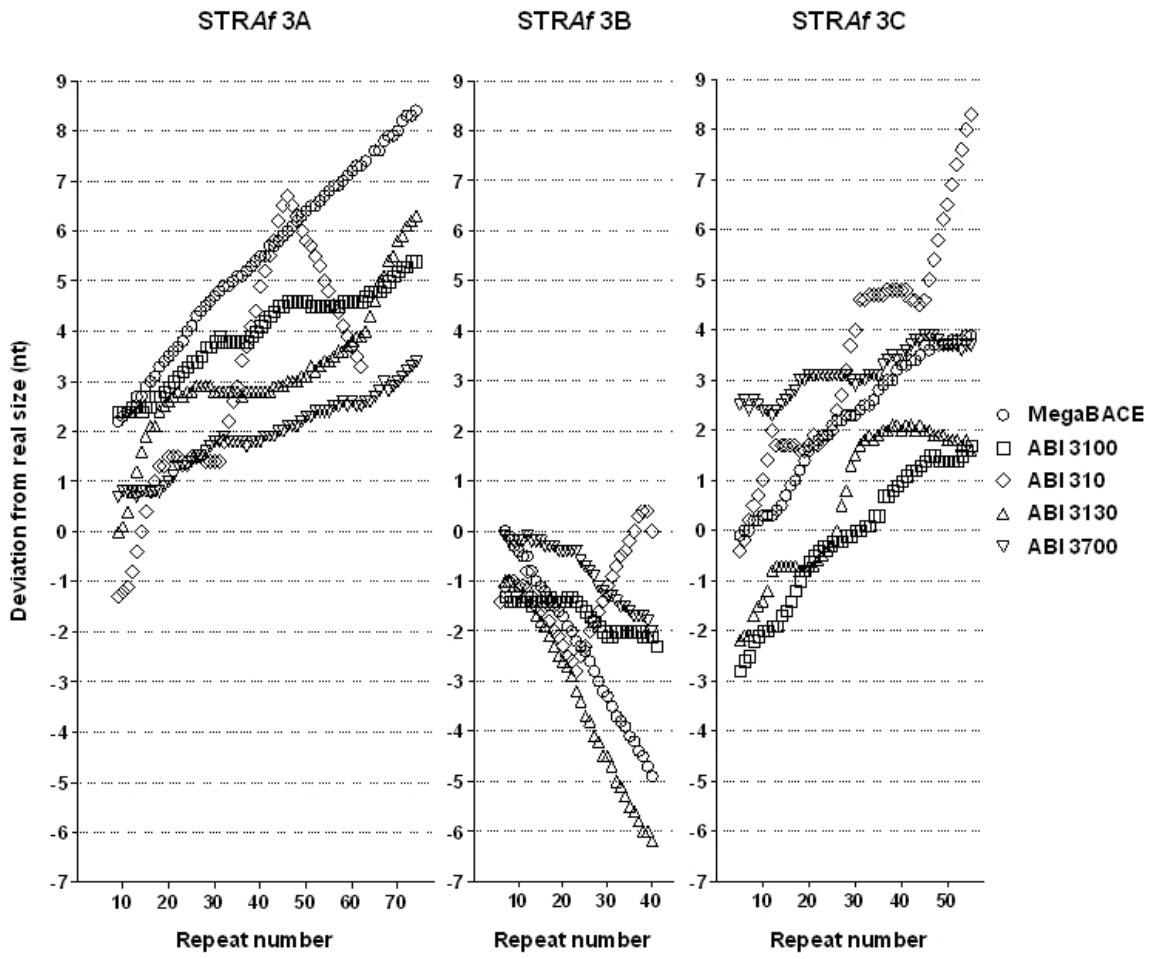


Figure 3. Graphical display of the size inaccuracy for each allele in the allelic ladders on different analytical platforms. The X-axes display the repeat number of the alleles and the Y-axes the difference between the calculated size and the actual size as determined by sequencing.

Discussion

A multicenter study was carried out to investigate the interlaboratory reproducibility and compatibility of a microsatellite-based typing assay in different settings. From a theoretical point of view, microsatellites are ideal targets for high resolution and exact fingerprinting of microbial isolates. The exchange of microsatellite typing data between laboratories is greatly hampered by the inability to accurately determine absolute fragment sizes by capillary electrophoresis (figure 3). Accurate sizing of identical DNA fragments using different high-resolution platforms is dependent on many experimental and environmental factors and often leads to differing results [7, 13, 14].

Intralaboratory sizing of alleles has been shown to be highly reproducible, yet, almost paradoxically; it is the sizing result itself that needs to be calibrated between laboratories. This is the first report of the use of allelic ladders to precisely and reproducibly determine repeat numbers of amplified alleles in an interlaboratory setting for typing *A. fumigatus* isolates. The use of allelic ladders for microsatellite analysis allows standardization of sizing results which in turn allows the direct comparison of microsatellite-based typing data between different laboratories and from different experimental settings. Allelic ladders consist of a mixture of common alleles that act as reference points for the position in an electrophoretic trace of fragments with specific numbers of repeats. Amplification of microsatellites by PCR leads to the formation of stutter peaks (e.g. additional fragments, mostly with fewer repeat numbers [5]). In the construction of our ladders we took advantage of the formation of these stutter peaks in order to fill in the gaps for those alleles that were not actually present in the mixture of cloned alleles, e.g. the -1 stutter peak that is formed upon amplification of the allele with 12 repeats will reveal the reference position of the allele with 11 repeats (figure 1). A circumstantial advantage of this approach is that it should be quite easy to unambiguously assign all alleles in the ladders since the included alleles will yield higher peaks than their associated stutter peak(s).

Some participants initially misassigned the repeat numbers in their chromatograms of the allelic ladders. As a consequence, the repeat numbers for all markers in the corresponding samples were effectively altered by 1 repeat unit. In retrospect, such misassignments could have been prevented by including the expected results for an additional reference DNA sample with known repeat numbers. In some cases a non-specific peak was assigned. Non-specific amplification products usually lack the stutter peaks that are associated with true alleles containing a short tandem repeat. Previously, we reported that the intensity of such non-specific amplification products is greatly influenced by the annealing temperature during amplification [7]. Hence, based on thermocycler specifications, some laboratories may experience more non-specific amplification products than others. After pointing this out, participants were granted the opportunity to reinterpret their results (Table 2).

The STR*Af* assay was developed as a multiplex assay in order to increase sample and marker throughput. This was easily established by using amplification primers with different fluorescent labels for each of the markers in the multiplex PCR reactions. For economical reasons, the combination of fluorescent labels. In this study five different capillary electrophoresis platforms were used. All platforms have multicolor detection capabilities either through the presence of optical (hardware) filters (MegaBACE) or through virtual (software) filters (ABI platforms). Theoretically, it should have been possible to calibrate each of these platforms to the combination of labels used in this study but this proved to be problematic for some of the ABI platforms. As a result, some participants were unable to properly calibrate their instrument and consequently suffered from substantial bleed-through of the different labels. This greatly complicated the interpretation of the results for lab E showing the most data analysis errors. Hence, being able to properly separate the different fluorescent labels seems to be crucial for successful interpretation of the data and implementation of this assay.

Previously, we showed that sizing identical alleles using different size standards on the same electrophoretic platform will result in sizing differences up to 5.0 nucleotides [7]. As shown in this study, these size differences can be as large as 6.7 nucleotides when different size standards are used on different electrophoretic platforms but can still amount up to 5.4 nucleotides when the same size standard is used on different analytical platforms. Furthermore, it is obvious from figure 3 that sizing differences are not constant across the range of alleles for a marker and that it changes with repeat number. In addition, individual markers behave differently on different analytical platforms. The examples clearly demonstrate that calculated allele sizes can not be used for the comparison of typing results between different laboratories (even when the same size standard is used) and that there is a clear need for size data to be calibrated in some manner. As demonstrated here, allelic ladders are ideally suited for such calibration purposes. All that needs to be done is to run the allelic ladders using the preferred running conditions to determine the relative size of all alleles using these conditions. Despite the substantial variations in sizing results, all laboratories were able to report identical typing data when the allelic ladders were used to calibrate the analysis.

Allelic ladders have already been in use in human forensics for more than a decade [10, 11] but up to now, have not been reported for use with microbial microsatellite based typing schemes.

No allelic ladders were provided for the markers in the STR*Af* 4 panel. Assignment of alleles in this panel for all samples was done by comparing the sizes of the fragments in the samples to the sizes of fragments in the original publication. In this case, all laboratories used the ET400-R marker. Identical alleles yielded sizing data differing up to 3.8 nucleotides. As a consequence, the reported repeat numbers for individual alleles in the STR*Af* 4 panel differed by up to 1 repeat unit from the expected values. Without the option to calibrate the results to an allelic ladder and when results were only interpreted based on the calculated (and relative) size of an allele, a total of 118 out of 264 reported alleles were incorrect (results not shown).

As might be expected, most interpretation errors were made by those participants with the least prior experience of microsatellite marker analysis. These were also the participants that left the actual electrophoretic running of the samples to an external party. Participants that produced identical typing results (laboratories A, B and C), had prior experience with this type of analyses and an in-house capillary electrophoresis platform (and software) that ensured correct detection and separation of the fluorescent labels.

In a previous study we demonstrated the robustness of the STR*Af* assay by deliberately and systematically changing various reaction components to suboptimal conditions and by varying thermocycling parameters [7]. In the current study all participants were allowed to use their regular DNA isolation procedure, DNA polymerase for performing PCR reactions, thermocycler, capillary electrophoresis platform and size standard. In practice, 3 distinct DNA isolation procedures, 3 different thermocyclers, 5 different capillary electrophoresis platforms and 4 size standards were used. The fact that all participating laboratories were able to produce identical typing data for all the STR*Af*3 markers in nearly all samples with this wide variety of experimental conditions again confirms the high robustness of a microsatellite-based typing assay and demonstrates the reproducibility and compatibility of results obtained with this assay in different settings.

Thus, microsatellite based typing assays are as suitable for interlaboratory comparisons as are sequence based typing assays such as multi-locus sequence typing. However, microsatellites offer a number of additional advantages in terms of speed, throughput, cost and discriminatory power. The present study also shows that although allelic ladders were shown to provide a straightforward way of standardizing microbial microsatellite-based typing assays, a certain level of technical experience is required in any participating laboratory to ensure correct data interpretation. However, for microsatellites this is no different than for any other molecular typing assay.

The use of allelic ladders allows a convenient means of standardizing microsatellite based typing assays between laboratories. This requires that such ladders should be made available to other interested laboratories. However, construction of an allelic ladder requires considerable effort and so therefore, at present and with the aim of demonstrating their usefulness, allelic ladders were constructed only for the trinucleotide repeat markers. If there is sufficient interest, allelic ladders for the dinucleotide and tetranucleotide markers can be constructed as well. In addition, this approach will only be successful if other laboratories are willing to commit to using the same primers and fluorescent labels as reported.

Acknowledgements

ACP received a grant from CAPES (Coordenação de Aperfeiçoamento de Pessoal de Nivel Superior)(Brazilian government). MJA was funded by a Wellcome Trust (UK) programme grant. We thank David Denning and Manuel Cuenca-Estrella for providing support for this work and for helpful comments and suggestions.

References

1. Aufauvre-Brown, A., J. Cohen, and D. W. Holden. 1992. Use of randomly amplified polymorphic DNA markers to distinguish isolates of *Aspergillus fumigatus*. J Clin Microbiol 30:2991-3.
2. Bain, J. M., A. Tavanti, A. D. Davidson, M. D. Jacobsen, D. Shaw, N. A. Gow, and F. C. Odds. 2007. Multilocus sequence typing of the pathogenic fungus *Aspergillus fumigatus*. J Clin Microbiol 45:1469-77.
3. Bart-Delabesse, E., J.-F. Humbert, E. Delabesse, and S. Bretagne. 1998. Microsatellite markers for typing *Aspergillus fumigatus* isolates. J Clin Microbiol 36:2413-2418.
4. Bart-Delabesse, E., J. Sarfati, J. P. Debeaupuis, W. van Leeuwen, A. van Belkum, S. Bretagne, and J. P. Latge. 2001. Comparison of restriction fragment length polymorphism, microsatellite length polymorphism, and random amplification of polymorphic DNA analyses for fingerprinting *Aspergillus fumigatus* isolates. J Clin Microbiol 39:2683-6.
5. de Valk, H. A., J. F. G. M. Meis, I. M. Curfs, K. Muehlethaler, J. W. Mouton, and C. H. W. Klaassen. 2005. Use of a novel panel of nine short tandem repeats for exact and high-resolution fingerprinting of *Aspergillus fumigatus* isolates. J Clin Microbiol 43:4112-20.
6. de Valk, H. A., J. F. G. M. Meis, B. E. de Pauw, P. J. Donnelly, and C. H. W. Klaassen. 2007. Comparison of two highly discriminatory molecular fingerprinting assays for analysis of multiple *Aspergillus fumigatus* isolates from patients with invasive aspergillosis. J Clin Microbiol 45:1415-9.
7. de Valk, H. A., J. F. G. M. Meis, and C. H. W. Klaassen. 2007. Microsatellite based typing of *Aspergillus fumigatus*: Strengths, pitfalls and solutions. J Microbiol Methods 69:268-72.
8. Girardin, H., J. P. Latge, T. Srikantha, B. Morrow, and D. R. Soll. 1993. Development of DNA probes for fingerprinting *Aspergillus fumigatus*. J Clin Microbiol 31:1547-54.
9. Pasqualotto, A. C., D. W. Denning, and M. J. Anderson. 2007. A cautionary tale: Lack of consistency in allele sizes between two laboratories for a published multilocus microsatellite typing system. J Clin Microbiol 45:522-8.
10. Puers, C., H. A. Hammond, L. Jin, C. T. Caskey, and J. W. Schumm. 1993. Identification of repeat sequence heterogeneity at the polymorphic short tandem repeat locus HUMTH01[AATG]n and reassignment of alleles in population analysis by using a locus-specific allelic ladder. Am J Hum Genet 53:953-8.
11. Skowasch, K., P. Wiegand, and B. Brinkmann. 1992. pMCT 118 (D1S80): A new allelic ladder and an improved electrophoretic separation lead to the demonstration of 28 alleles. Int J Legal Med 105:165-8.
12. Soll, D. R. 2000. The ins and outs of DNA fingerprinting the infectious fungi. Clin Microbiol Rev 13:332-70.
13. Tu, O., T. Knott, M. Marsh, K. Bechtol, D. Harris, D. Barker, and J. Bashkin. 1998. The influence of fluorescent dye structure on the electrophoretic mobility of end-labeled DNA. Nucl Acids Res 26:2797-802.

14. Vainer, M., S. Enad, V. Dolnik, D. Xu, J. Bashkin, M. Marsh, O. Tu, D. W. Harris, D. L. Barker, and E. S. Mansfield. 1997. Short tandem repeat typing by capillary array electrophoresis: comparison of sizing accuracy and precision using different buffer systems. Genomics 41:1-9.
15. Varga, J. 2006. Molecular typing of aspergilli: Recent developments and outcomes. Med Mycol 44 Suppl:149-61.

Chapter 7

Stability of microsatellite markers in *Aspergillus fumigatus*

Hanneke A. de Valk, Ilse M. Curfs, Maaike T. de Ruiter, Jacques F.G.M. Meis and Corné H.W. Klaassen

Submitted

Abstract

The stability of 9 highly polymorphic short tandem repeat markers for *Aspergillus fumigatus* was investigated in 100 successive generations of 5 clonally expanded isolates. In total, 473 generations were generated and analyzed. In five cases, a change in the number of repeat units was observed. All alterations involved a change in 1 repeat unit and all changes involved expansions. All changes were observed in the two out of three trinucleotide repeats, especially in isolates with high number of repeats. This suggests that the mutation rate increases with increasing repeat numbers. Between markers of the STR*Af* assay there were also differences concerning the stability. All these findings should be taken into account upon interpreting STR data.

Introduction

Short tandem repeats (STRs), also known as microsatellites, consist of tandemly repeated sequences loci up to ten basepaires (bp) long and are present in several prokaryotic and virtually every eukaryotic genome. Repeat copy number polymorphisms in STRs between different isolates are the result of expansions or contractions of one or more repeats units. The mechanisms behind these changes in repeat numbers are recombination and slipped-strand mispairing (SSM)[5-7, 14]. With the SSM model addition or contradiction of STRs depends on the strand orientation. When the nascent strand produces a loop during replication, one or more STRs will be added in the new DNA strand. Though, if the template strand produces a loop, one or more STRs can be deleted [9, 11]. Polymorphic STR markers can be predicted from the following characteristics: Number of repeated units, unit lengths and repeat purity [7]. The instability of STRs and the relative ease of detection of these polymorphisms via PCR amplification make them ideally suited for genotyping [5].

For the fungus *A. fumigatus* a robust typing method based on nine STRs was developed (STR*Af* assay) [this thesis, 2, 4]. *A. fumigatus* is the most common fungus, which can cause invasive aspergillosis in immunocompromised patients with often fatal consequences. Previously, the high discriminatory power of the STR*Af* assay was demonstrated by distinguishing 96 genotypes from 99 presumably unrelated isolates [2]. The utility in epidemiological surveys was also established. Firstly, by identifying identical isolates from different body sites of the same patient [3]. Secondly to establish epidemiological concordance among isolates from different outbreaks containing multiple isolates per outbreak [1]. In this last study, epidemiologic related isolates with differences of one repeat unit in a single STR marker were called microvariation. It is unknown how to deal with these variations, since no guidelines for the interpretation of STR data have been postulated.

In order to be able to properly interpret the relationship between isolates with differences in the STR markers, it is necessary to understand the dynamics of the individual markers. Unfortunately, little is known about the stability of STR markers in *A. fumigatus*. Therefore, we investigated the stability of 9 highly polymorphic microsatellite markers in 100 successive generations of 5 clonally expanded *A. fumigatus* isolates with randomly chosen genotypes.

Material and Methods

Generations: Four randomly chosen unrelated clinical *A. fumigatus* isolates and one reference strain (CBS 487.65) were clonally expanded up to 100 generations. All isolates were subcultured onto sabouraud agars and incubated at 30ºC until sporulation. Successive generations were generated from a single spore using the following procedure: A suspension of *A. fumigatus* spores was made in aqua dest. with 0.05% Tween 40 (Merck Nederland B.V., Amsterdam, The Netherlands). The transmission of this suspension was measured at a wavelength of 530 nm and adjusted to 80-82%, which corresponds to approximately 1.5 x 10^6 CFU/ml. This suspension was diluted to a concentration of 3 x 10^2 CFU/ml. About 100µl of this suspension was plated onto a Sabouraud agar and incubated at 30ºC and examined daily. A single non-sporulating colony was then picked and subcultured on a new Sabouraud agar and incubated at 30ºC until sporulation. Subsequent generations were made accordingly.

STR*Af* assay: DNA of all generations was extracted as previously described [2]. All DNA samples were analyzed using the 9 loci of the STR*Af* panel consisting of three di-, tri- and tetranucleotide repeats. PCR primers, fluorescent labels and amplification conditions were exactly as previous described [2]. Assignment of repeat numbers in each marker was performed using Fragment Profiler 1.2 software (GE Healthcare, Diegem, Belgium) and checked by visual interpretation of the electropherograms.

Results

Four unrelated randomly chosen *A. fumigatus* isolates and one reference isolate were clonally expanded. Four isolates yielded 100 generations and one slow-sporulating isolate yielded 73 generations. In total, 473 generations were generated and analyzed with the nine STR markers of the STR*Af* assay. This yielded a total of 4257 markergenerations. The results are shown in table 1.

In two of the five isolates (number 2 and 5) no change in any of the nine STR*Af* markers were observed. The number of repeats in the STR*Af* 3C marker changed from 20 to 21 in the 5th generation of isolate 1, this was the only change in this sample over 100 generations. Isolate 3 also showed a single alteration in the STR*Af* 3C marker, in this sample the repeat number changed from 50 to 51 in the 36th generation. Most changes were observed in sample 4. In this isolate, the STR*Af* 3A marker gradually changed from 53 repeat units to 56. The alterations were observed in the 5th, 48th and 66th generation.

In total, five times a change in the number of repeat units was observed. All alterations involved an increase in 1 repeat unit. All changes occurred in the trinucleotide repeat markers.

Table 1. Overview of the markergenerations of five randomly chosen isolates.

		STR*Af*			STR*Af*			STR*Af*		
Isolate	Generation	2A	2B	2C	3A	3B	3C	4A	4B	4C
1	1-5	18	12	8	27	12	20	9	9	5
	6-100	18	12	8	27	12	21	9	9	5
2	1-100	18	12	13	14	10	14	8	11	5
3	1-36	23	23	15	39	11	50	10	26	8
	37-100	23	23	15	39	11	51	10	26	8
4	1-5	23	19	15	53	11	7	13	9	5
	6-48	23	19	15	54	11	7	13	9	5
	49-66	23	19	15	55	11	7	13	9	5
	67-100	23	19	15	56	11	7	13	9	5
5	1-73	10	17	10	25	11	23	7	5	6

Discussion

A better understanding of the epidemiological relation between isolates can be provided by molecular fingerprinting methods. Isolates with differences in more than two STR*Af* markers are considered to be unrelated, as it is unlikely that two changes in the genome occur at the same time. The interpretation of the relationship between epidemiological related *A. fumigatus* isolates with minor variations between genotypes is unknown. To know how to interpret the relationships between these isolates, the stability of individual markers has to be taken into account. Therefore we studied the variation in repeat numbers in successively clonally expanded *A. fumigatus* isolates. Generations can be obtained by subculture multiple spores or by subculture a single spore. *A. fumigatus* isolates releases millions of spores. If a change in repeat numbers would occur in a part of these spores, this will not be recognized in the electropherogram. Thus, subcultures need to be obtained from a single spore. Totally 473 generations were generated from 5 randomly chosen isolates. All generations were analyzed with nine loci of the STR*Af* panel, consisting of three di-, tri- and tetranucleotide repeat markers. In these 4257 markergenerations 5 changes were observed. This means that the average marker in the STR*Af* panel is stable for approximately 1000 generations.

The number of copies within a marker seems to have great influence on the stability of that marker, since four out of 5 alterations were found in markers with 50 or more repeat numbers. This suggests that the mutation rate may increase with increasing repeat numbers. Several other studies have indeed shown that the number of repeats is a good predictor of the level of variability in other organisms, like *Escherichia coli, Saccharomyces cerevisiae* and maize (*Zea mays* supsp. *mays*) [7, 15, 16]. So, for *A. fumigatus* as well as for other organisms a higher copy number decreases the stability of STR markers. This means that expansion of one extra repeat unit to a repeat consisting of 10 units is less likely to occur in the same period of time than the expansion of a unit to a repeat that already contain 50 units. For the calculation of relations between isolates with these variations in a marker, this means that a difference of 10 to 11 repeats in a marker should be given more weight than a change from 50 to 51 repeat numbers.

All 5 mutations were found in the trinucleotide repeat markers, STR*Af* 3A and STR*Af* 3C, and none in the di- and tetranucleotide repeats. The unit length is thought to be one of the major parameters to predict the (in)stability [7] and that longer repeat units have higher mutations rate [13]. Weber and Wong tested both di- and tetranucleotide repeats in cell lines representing human pedigrees and found more mutations in tetranucleotide repeats than in dinucleotide repeats [17]. However, the opposite relationship has been observed in humans where dinucleotide repeat motifs mutated at a faster rate than those with larger repeat motifs and this was also found in some other organisms, including Drosphilia [13] and maize [15]. For our data, it could be a coincidence that most variations were found in the trinucleotide repeats since two out of the five randomly chosen isolates already had high copynumbers in the trinucleotide repeat markers. To confirm that the unit length has influence on the mutation rate in *A. fumigatus*, this experiment could be repeated using isolates with selected numbers of repetition for the different markers.

All five mutations were expansions of one repeat unit. It seems logical that more expansions are observed than contractions, otherwise the repeat would not have existed anymore. Vigouroux et al. analyzed the mutation in dinucleotide repeats in maize, they observed that there is a higher probability to change to a higher repeat number than to a lower one [15]. Noller et al. also observed that the majority of mutations in a hexanucleotide repeat for *E. coli* were single additions of repeat units [11]. Vogler et al. found a balance between expansions and contradictions in these loci for 8 *E. coli* isolates. One isolate also showed that the majority of repeats were insertions [16].

Another factor which could have an effect on the stability of markers is the location of repeat markers. All loci in the STR*Af* 2 panel are located in or nearby a gene. The most polymorphic marker of the STR*Af* panel, namely STR*Af* 3A, is located within the coding region of the Afu4g09070 gene [12], a putative ATP binding L-PSP endoribonuclease family protein. The remaining STR*Af* loci are not positioned nearby genes. The repeat number could be influenced in repeats located in active proteins by selection. Isolates tend to adjust to the environment. On the other hand, variation in repeat number may allow an isolate to evade a host defense.

In the Afu3g08990 gene [8] a change in the phenotype has been described, due to recombination events in their internal tandem repeats. In humans, the expansion of coding repeats is associated with various diseases, including Huntington's disease and fragile X syndrome [10]. For the selected STR*Af* loci it is not obvious if their stability was influenced by the location in genes.

Assays based on short tandem repeats have been shown to be a powerful tool for epidemiological studies of a wide range of organisms. The interpretation of data obtained with these assays requires proper understanding of the dynamics of repeat numbers in STR markers. High numbers of repeats in a marker seems to have the most influence on the variability in a marker. There are also differences in the stability between markers of the STR*Af* assay. All these findings should be taken into account in the interpretation of STR data.

References

1. Balajee, S. A., H. A. de Valk, B. A. Lasker, J. F. G. M. Meis, and C. H. W. Klaassen. 2008. Utility of a microsatellite assay for typing epidemiologically linked *Aspergillus fumigatus* isolates. J Microbiol Methods 73:252-6.
2. de Valk, H. A., J. F. G. M. Meis, I. M. Curfs, K. Muehlethaler, J. W. Mouton, and C. H. W. Klaassen. 2005. Use of a novel panel of nine short tandem repeats for exact and high-resolution fingerprinting of *Aspergillus fumigatus* isolates. J Clin Microbiol 43:4112-20.
3. de Valk, H. A., J. F. G. M. Meis, B. E. de Pauw, P. J. Donnelly, and C. H. W. Klaassen. 2007. Comparison of two highly discriminatory molecular fingerprinting assays for analysis of multiple *Aspergillus fumigatus* isolates from patients with invasive aspergillosis. J Clin Microbiol 45:1415-9.
4. de Valk, H. A., J. F. G. M. Meis, and C. H. W. Klaassen. 2007. Microsatellite based typing of *Aspergillus fumigatus*: Strengths, pitfalls and solutions. J Microbiol Methods 69:268-72.
5. Karaoglu, H., C. M. Lee, and W. Meyer. 2005. Survey of simple sequence repeats in completed fungal genomes. Mol Biol Evol 22:639-49.
6. Lai, Y., and F. Sun. 2003. The relationship between microsatellite slippage mutation rate and the number of repeat units. Mol Biol Evol 20:2123-31.
7. Legendre, M., N. Pochet, T. Pak, and K. J. Verstrepen. 2007. Sequence-based estimation of minisatellite and microsatellite repeat variability. Genome Res 17:1787-96.
8. Levdansky, E., J. Romano, Y. Shadkchan, H. Sharon, K. J. Verstrepen, G. R. Fink, and N. Osherov. 2007. Coding tandem repeats generate diversity in *Aspergillus fumigatus* genes. Eukaryot Cell 6:1380-91.
9. Lovett, S. T., and V. V. Feschenko. 1996. Stabilization of diverged tandem repeats by mismatch repair: evidence for deletion formation via a misaligned replication intermediate. Proc Natl Acad Sci USA 93:7120-4.
10. Mohmood, S., A. Sherwani, F. Khan, R. H. Khan, and A. Azfer. 2003. DNA trinucleotide repeat expansion in neuropsychiatric patients. Med Sci Monit 9:RA237-45.
11. Noller, A. C., M. C. McEllistrem, K. A. Shutt, and L. H. Harrison. 2006. Locus-specific mutational events in a multilocus variable-number tandem repeat analysis of *Escherichia coli* O157:H7. J Clin Microbiol 44:374-7.
12. Pasqualotto, A. C., D. W. Denning, and M. J. Anderson. 2007. A cautionary tale: Lack of consistency in allele sizes between two laboratories for a published multilocus microsatellite typing system. J Clin Microbiol 45:522-8.
13. Schug, M. D., C. M. Hutter, K. A. Wetterstrand, M. S. Gaudette, T. F. Mackay, and C. F. Aquadro. 1998. The mutation rates of di-, tri- and tetranucleotide repeats in *Drosophila melanogaster*. Mol Biol Evol 15:1751-60.

14. van Belkum, A. 2007. Tracing isolates of bacterial species by multilocus variable number of tandem repeat analysis (MLVA). FEMS Immunol Med Microbiol 49:22-7.
15. Vigouroux, Y., J. S. Jaqueth, Y. Matsuoka, O. S. Smith, W. D. Beavis, J. S. Smith, and J. Doebley. 2002. Rate and pattern of mutation at microsatellite loci in maize. Mol Biol Evol 19:1251-60.
16. Vogler, A. J., C. Keys, Y. Nemoto, R. E. Colman, Z. Jay, and P. Keim. 2006. Effect of repeat copy number on variable-number tandem repeat mutations in *Escherichia coli* O157:H7. J Bacteriol 188:4253-63.
17. Weber, J. L., and C. Wong. 1993. Mutation of human short tandem repeats. Hum Mol Genet 2:1123-8.

Chapter 8

Utility of a microsatellite assay for identifying clonally related isolates of *Aspergillus fumigatus*

S. Arunmozhi Balajee, Hanneke A. de Valk, Brent A. Lasker, Jacques F.G.M. Meis and Corné H.W. Klaassen

Journal of Microbiological Methods 2008, 73: 252-256

Abstract

A microsatellite assay based on short tandem repeats (STR*Af*) has been recently described as a discriminatory, high throughput assay for fingerprinting *Aspergillus fumigatus* isolates. However, the STR*Af* assay has not been tested for its utility in outbreak settings where it is critical to distinguish clonal clusters from genetically unrelated genotypes. In the present study, employing a panel of epidemiologically linked *A. fumigatus* isolates obtained from 6 different outbreaks of invasive aspergillosis (IA), we demonstrate that the STR*Af* assay can be a valuable molecular tool to support epidemiological investigations. We also report for the first time the detection of microvariation events in the *A. fumigatus* population studied.

Introduction

Invasive aspergillosis (IA) is a life-threatening disease in immunocompromised patient populations and evidence suggests that such infections may be acquired from hospital environments; accordingly, numerous outbreaks of IA, largely due to *Aspergillus fumigatus*, have been reported [2, 8, 9, 11]. A molecular typing method that can distinguish "clonally related" or genetically related isolates from unrelated isolates can be a powerful tool in outbreak investigations where the assumption is that isolates with identical genotypes (or "clonal" clusters) could indicate a common source of exposure, whereas epidemiologically unrelated isolates will have different genotypes [12]. Thus, genotyping methods can indicate the source and map the route of transmission of IA, thereby informing epidemiological investigations and ultimately guide prevention strategies.

Numerous molecular methods have been evaluated for subtyping *A. fumigatus* including microsatellite based fingerprinting assays. Recently, a robust microsatellite assay, the STR*Af* assay, based on nine, short tandem repeat loci has been described for high resolution fingerprinting of *A. fumigatus* [3, 5]. Previously, the STR*Af* assay was able to segregate 99 presumably unrelated *A. fumigatus* isolates into 96 unique genotypes demonstrating its very high discriminatory power [3], delineate unique genotypes amongst *A. fumigatus* isolates recovered from multiple different patients in a single hospital, and finally identified genetically related isolates from different body sites of the same patient [4]. Thus, the STR*Af* assay satisfies a critical measure of a good typing system, that is, it can provide distinctive fingerprints from genetically unrelated isolates and identical or highly similar fingerprints from closely related organisms. According to the guidelines of the European Study Group on Epidemiological markers (ESGEM), another important criterion of an ideal typing scheme is the system's capacity to establish epidemiological concordance among strains recovered from the same outbreak, which can be evaluated by testing sets of isolates from different outbreaks containing multiple isolates per outbreak [13]. However, it is not known whether the STR*Af* assay can fulfill this criterion, and the present study was designed to evaluate the STR*Af* assay for its utility as a molecular tool to support outbreak investigations of aspergillosis.

The Mycotic Diseases Branch at the Centers for Disease Control and Prevention has a collection of several *A. fumigatus* isolates from multiple outbreak investigations across Northern America [6, 10, 11]. These isolates have been subjected to several genotyping methods to assess their genetic relatedness to support epidemiological investigations including the Afut1 DNA hybridization profiles (Afut1 method) and a recently developed single locus sequencing typing method employing the putative cell surface protein gene Afu3g08990, designated as "CSP typing" method [1]. Given the availability of relevant epidemiological data for each outbreak investigation, this panel was uniquely suited and was therefore chosen to test the suitability of the STR*Af* method to type epidemiologically linked *A. fumigatus* isolates.

Materials and methods

Aspergillus isolates used in the study. A total of 55 *A. fumigatus* isolates obtained from six different outbreak investigations (denoted as OB1–OB6) were included in this study.

Isolates in cluster OB1 were obtained from a renal transplant unit in California. This outbreak was thought to be associated with construction related activities; however genotyping results using the Afut1 method showed that the isolates recovered from the environment were unrelated to the clinical isolates from patients [10]. Outbreak OB2 occurred among liver transplant recipients. Debridement of a patient's wound infected with *A. fumigatus* caused aerosolization of *Aspergillus* conidia resulting in patient-to-patient transmission and was implicated in a subsequent outbreak of pulmonary aspergillosis in the intensive care unit [11]. Here, analyses with the Afut1 assay demonstrated that isolates recovered from infected abdominal tissue of one patient, environmental isolates recovered from the immediate vicinity of the wound debridement procedure, and an isolate from another patient housed in the same unit had indistinguishable genotypes [11].

Cluster OB3 included isolates from an outbreak of IA in a cardiac transplant unit in New York [6]. Cluster OB4 isolates were obtained from a surgical unit in a Colorado hospital where patients had undergone prosthetic heart valve replacement surgery and subsequently had IA. OB5 isolates were recovered from a poultry farm outbreak investigation in which *A. fumigatus* was recovered from lungs of chickens and from chicken litter. Previous genotyping results with Afut1 method indicated that isolates from OB3, 4 and 5 were unique isolates representing multiple different genotypes (unpublished data).

Cluster OB6 included isolates obtained from a heart transplant unit in a Canadian hospital where genotypes of isolates collected from the ventilation system of rooms in which the patients were housed and clinical isolates from patient with documented IA were shown to be highly related by Afut1 analyses; here the source of the infection was considered to be the ventilation system in the room (unpublished data). All the isolates had been genotyped using the Afut1 assay and/or the CSP typing method [1].

STRAf assay. Fungal DNA was extracted and purified from all isolates as described previously [6]. PCR primers and fluorescent labels for the STR*Af*2, 3 and 4 panels were as previously described [3]. The obtained PCR products were diluted 30-fold with distilled water. One microliter of diluted PCR products was combined with 8.75 µl of distilled water and 0.25 µl of ET-ROX 400 marker (GE Healthcare, Diegem, Belgium). Following denaturation of the samples for 1 min at 95°C and rapid cooling to 4°C, they were injected onto a MegaBACE 500 equipped with a 48 capillary array. Injection and running parameters were according to the recommendations of the manufacturer (GE Healthcare).

Data analysis. Electropherograms were analyzed using Fragment Profiler 1.2 software (GE Healthcare). Typing data was imported into BioNumerics 4.6 software (Applied Maths, Sint-Martens-Latem, Belgium) and analyzed using the multi-state categorical similarity coefficient with UPGMA clustering. Isolates possessing alleles with the same number of repeat units in all 9 loci were defined as a "clonal" cluster. Microvariants were defined as epidemiologically related isolates that differed by less than 2 repeat units in a single locus.

Results

Results of the STR*Af* assay for cluster OB1 showed that 3 clinical isolates 607, 683 and 686 belonged to a clonal cluster while all other isolates representing mostly environmental isolates consisted of unrelated genotypes (Fig. 1A). In cluster OB2, 11 isolates formed a clonal cluster and 1 clinical isolate had an unrelated genotype (Fig. 1B). Here, isolates B5850, B5864, B5852 and 38 recovered from a single patient (whose abdominal wound was implicated as the source of the outbreak), isolate B5856 from a second patient housed in the vicinity, and several other environmental isolates recovered from the air of this room grouped together as one clonal cluster. One environmental isolate collected from the same environment several days after the outbreak, isolate B5865, differed from this clonal cluster by a change in locus STR*Af*3C from allele 35 to allele 36, a change of one trinucleotide repeat unit (Fig. 1B, circled). Isolates from OB3 (Fig. 1C), OB4 (Fig. 1D) and the poultry outbreak (OB5, Fig. 1E) had unrelated genotypes when analyzed with the STR*Af* assay.

Seventeen isolates obtained from OB6 were grouped into 3 different clonal clusters. Twelve isolates formed one clonal cluster that included clinical isolates, isolates recovered from the air vent and from the environment of the infected patient. Within this clonal cluster, 2 isolates, one clinical (B6074) and an environmental isolate (B6081) differed at a single locus from the genotypes of the clonal group in that there was a change from allele 33 to allele 34 in one locus, STR*Af*3A (Fig. 1F, shown within a circle).

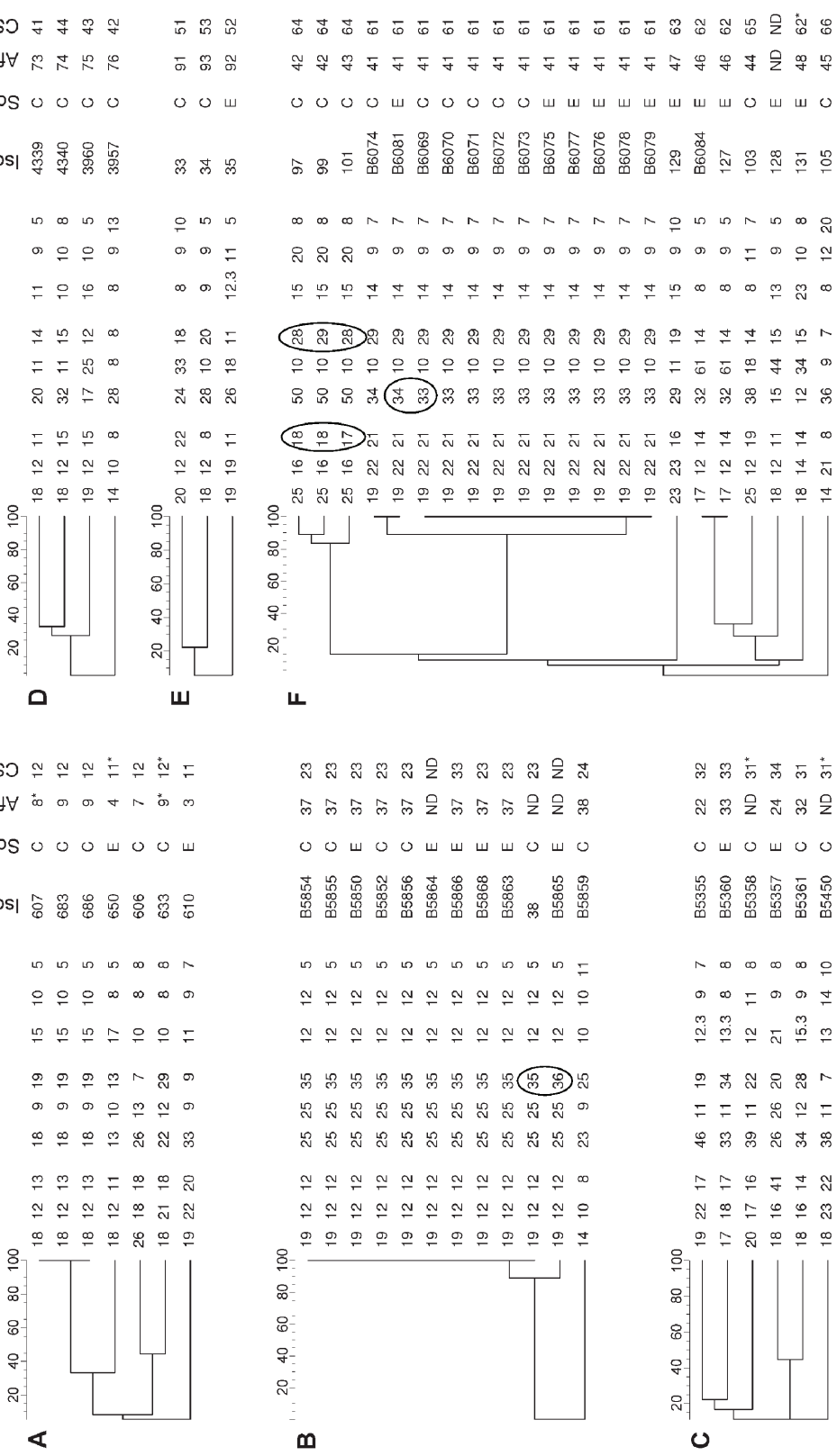


Figure 1 Dendrograms generated from genotyping 55 *A. fumigatus* isolates from outbreak 1 (A), OB2 (B), OB3 (C), OB4 (D), OB5 (E) and OB6 (F) using the STR*4f* assay. The respective Afut1 and CSP types are shown adjacent to the STR*4f* type. An asterisk indicates that the particular typing method was discordant with the STR*4f* typing result. Isolates recovered from clinical samples are denoted as C and strains isolated from the environment are denoted as E. Circled are the genotypes that show microvariation. The scale bars above the dendrograms indicate the percentage identity between the genotypes.

Three isolates (97, 99 and 101) formed the second clonal cluster and included clinical isolates recovered from 3 different patients who were housed in a different ward of the same hospital; here isolates 97 and 99 differed from each other in STR*Af* 3C, while isolate 101 had a change of allele 17 to 18 in STR*Af*2C (Fig. 1F, highlighted within a circle). The third clonal cluster included 2 environmental isolates (B6084 and 127) also recovered from the air of the patient's room. This outbreak collection also included 4 isolates with unrelated genotypes.

There was good agreement between the STR*Af* and Afut1 typing method. Of the 55 outbreak isolates typed by both methods, only 2 isolates (607 and 633) belonging to OB1 showed discordant genotypes (Fig. 1A). When the results of the CSP typing were compared with that of the STR*Af* assay, a total of 5 isolates had discordant genotypes (Figs. 1A, C and F, denoted with an asterisk).

Discussion

Recently, a method that employs a panel of short repeats from 9 loci in multiplex PCRs where the reaction amplified three di-, tri-, or tetranucleotide repeats respectively (the STR*Af* assay) has been described for fingerprinting *A. fumigatus* isolates [3].With this assay, the combination of all nine markers yielded a Simpson's diversity index of 0.9994, indicating that the method had a high discriminatory power. Theoretically this panel of markers can discriminate between up to 27×10^9 genotypes. Although the STR*Af* assay has been shown to be highly discriminatory and reproducible [3, 4], the method has never been rigorously examined for its ability to identify clonally related or epidemiologically linked *A. fumigatus* isolates.

The present study demonstrated that the STR*Af* assay results were in overall agreement with previous genotyping results obtained using the Afut1 and CSP genotyping methods [1], in that the clusters OB2 and OB6 were outbreaks that could be traced to a common source exposure while all other outbreaks were multi-source outbreaks with several unrelated genotypes identified.

The STR*Af* and CSP typing method were not concordant for all outbreak isolates emphasizing a previous observation that the CSP typing method has a lower discriminatory power and at best can be a first line typing strategy for *A. fumigatus* isolates [1]. Conversely, except for one outbreak cluster (OB1) results from both the Afut1 and the STR*Af* assays were in excellent agreement (Figs. 1A–F). Thus, the STR*Af* assay appears to be a useful molecular tool that is suitable for use in *A. fumigatus* outbreak related epidemiological investigations. The only disadvantage of the STR*Af* assay appears to be the requirement for expensive equipment and skilled personnel and thus limiting its use to specialized laboratories. A few serial isolates in clusters OB2 and OB6 showed variation at a single locus over time (Figs. 1B and F). Interestingly, both these isolates were recovered several days later than the other isolates in the respective outbreak cluster. In another instance, 3 isolates recovered from 3 different patients with documented infection (OB6, isolates 96, 99, 101) also showed a single repeat difference. These observations are suggestive of microvariation in the *A. fumigatus* population. Similar microvariation events have also been recently documented in *C. parapsilosis* and *Candida albicans* using microsatellite markers [7, 14]. Nevertheless, the present study demonstrates that the STR*Af* assay can be used effectively to detect subtle genetic changes in *A. fumigatus* isolates that were not previously observed with other genotyping methods. An alternate and plausible explanation for the slight genetic variation in the genotype of these isolates could be attributed to instability: it is well known that microsatellite instability can occur due to polymerase and strand slippage during DNA replication. In practice, changes in tandem repeats are expected to occur at faster rates than do spontaneous point mutations, in the end contributing to high discriminatory power of the microsatellite based typing assay.

References

1. Balajee, S. A., S. T. Tay, B. A. Lasker, S. F. Hurst, and A. P. Rooney. 2007. Characterization of a novel gene for strain typing reveals substructuring of *Aspergillus fumigatus* across North America. Eukaryot Cell 6:1392-9.
2. Bretagne, S., E. Bart-Delabesse, J. Wechsler, M. Kuentz, N. Dhedin, and C. Cordonnier. 1997. Fatal primary cutaneous aspergillosis in a bone marrow transplant recipient: nosocomial acquisition in a laminar-air flow room. J Hosp Infect 36:235-9.
3. de Valk, H. A., J. F. G. M. Meis, I. M. Curfs, K. Muehlethaler, J. W. Mouton, and C. H. W. Klaassen. 2005. Use of a novel panel of nine short tandem repeats for exact and high-resolution fingerprinting of *Aspergillus fumigatus* isolates. J Clin Microbiol 43:4112-20.
4. de Valk, H. A., J. F. G. M. Meis, B. E. de Pauw, P. J. Donnelly, and C. H. W. Klaassen. 2007. Comparison of two highly discriminatory molecular fingerprinting assays for analysis of multiple *Aspergillus fumigatus* isolates from patients with invasive aspergillosis. J Clin Microbiol 45:1415-9.
5. de Valk, H. A., J. F. G. M. Meis, and C. H. W. Klaassen. 2007. Microsatellite based typing of *Aspergillus fumigatus*: Strengths, pitfalls and solutions. J Microbiol Methods 69:268-72.
6. Lasker, B. A. 2002. Evaluation of performance of four genotypic methods for studying the genetic epidemiology of *Aspergillus fumigatus* isolates. J Clin Microbiol 40:2886-2892.
7. Lasker, B. A., G. Butler, and T. J. Lott. 2006. Molecular genotyping of *Candida parapsilosis* group I clinical isolates by analysis of polymorphic microsatellite markers. J Clin Microbiol 44:750-9.
8. Leenders, A., A. van Belkum, S. Janssen, S. de Marie, J. Kluytmans, J. Wielenga, B. Lowenberg, and H. Verbrugh. 1996. Molecular epidemiology of apparent outbreak of invasive aspergillosis in a hematology ward. J Clin Microbiol 34:345-51.
9. Munoz, P., J. Guinea, T. Pelaez, C. Duran, J. L. Blanco, and E. Bouza. 2004. Nosocomial invasive aspergillosis in a heart transplant patient acquired during a break in the HEPA air filtration system. Transpl Infect Dis 6:50-4.
10. Panackal, A. A., A. Dahlman, K. T. Keil, C. L. Peterson, L. Mascola, S. Mirza, M. Phelan, B. A. Lasker, M. E. Brandt, J. Carpenter, M. Bell, D. W. Warnock, R. A. Hajjeh, and J. Morgan. 2003. Outbreak of invasive aspergillosis among renal transplant recipients. Transplantation 75:1050-3.
11. Pegues, D. A., B. A. Lasker, M. M. McNeil, P. M. Hamm, J. L. Lundal, and B. M. Kubak. 2002. Cluster of cases of invasive aspergillosis in a transplant intensive care unit: evidence of person-to-person airborne transmission. Clin Infect Dis 34:412-6.
12. Singh, A., R. V. Goering, S. Simjee, S. L. Foley, and M. J. Zervos. 2006. Application of molecular techniques to the study of hospital infection. Clin Microbiol Rev 19:512-30.
13. van Belkum, A., P. T. Tassios, L. Dijkshoorn, S. Haeggman, B. Cookson, N. K. Fry, V. Fussing, J. Green, E. Feil, P. Gerner-Smidt, S. Brisse, and M. Struelens. 2007. Guidelines for the validation and application of typing methods for use in bacterial epidemiology. Clin Microbiol Infect 13 Suppl 3:1-46.

14. Verduyn-Lunel, F., L. Licciardello, S. Stefani, H. A. Verbrugh, W. J. G. Melchers, J. F. G. M. Meis, S. Scherer, and A. van Belkum. 1998. Lack of consistent short sequence repeat polymorphisms in genetically homologous colonizing and invasive *Candida albicans* strains. J Bacteriol 180:3771-3778.

Chapter 9

Molecular typing and colonization patterns of *Aspergillus fumigatus* in patients with cystic fibrosis

Hanneke A. de Valk, Corné H.W. Klaassen, Jan-Bart Yntema, Alexandra Hebestreit, Marc Seidler, Gerhard Haase, Frank-Michael Müller and Jacques F.G.M. Meis

Submitted.

Abstract

Aspergillus fumigatus is a chronic colonizer of the respiratory tract of patients with cystic fibrosis (CF). A total of 204 *A. fumigatus* isolates from 36 CF patients from three different medical centers, collected over a period of four months till 9½ years, were genotyped using the short tandem repeat panel for *A. fumigatus* (STR*Af* assay). Four different colonization patterns were observed. Colonization patterns with only unique genotypes were found in 36% of the patients. In contrast 17% of the patients were chronically colonized by a single genotype. The remaining patients showed a predominant genotype or genotypes that succeed each other. In this collection no relation was found between colonization pattern and allergic bronchopulmonary aspergillosis.

Introduction

Cystic fibrosis (CF) or mucoviscidosis is an autosomal recessive disorder with an incidence of approximately one in 2500 live births in the Caucasian population. Mutations in the gene encoding the chloride channel cystic fibrosis transmembrane regulator (CFTR) result in dysfunction of the exocrine glands. As a consequence of this deficient transport mechanism, copious amounts of viscous respiratory mucus are secreted, which is difficult to clear and provides a breeding ground for microorganisms [2]. Early in life, CF patients become infected with a limited spectrum of bacteria, mostly *Staphylococcus aureus* and *Haemophilus influenzae*. As the disease progresses, *Pseudomonas aeruginosa* becomes the most common pathogen [16]. *Aspergillus fumigatus* is by far the most isolated filamentous fungus from the respiratory secretions of CF patients and up to 57% are chronically colonized with this fungus. A commonly recognized complication of *Aspergillus* infection in CF patients is allergic bronchopulmonary aspergillosis (ABPA), with a prevalence of approximately 1 to 15% [18, 22, 23]. Although persistent inflammation and the resulting pulmonary injury can finally progress to fibrosis [24], knowledge about the epidemiology of *A. fumigatus* in CF patients is still scarce [4, 15].

A better understanding of the airway colonization by *A. fumigatus* in CF patients may be achieved by accurate molecular typing of sequential isolates from sputum samples. For *A. fumigatus*, several typing techniques have been described previously, including microsatellite based fingerprinting assays. The STR*Af* assay, based on nine microsatellite or short tandem repeat (STR) markers, is a robust [11], reproducible [8] typing technique with a very high discriminatory power [9]. These characteristics differentiate the STR based assays from many other previously employed typing techniques, such as random amplified polymorphic DNA (RAPD) [3] and restriction fragment length polymorphism (RFLP) [20], which suffer from poor interlaboratory reproducibility and subjective interpretation of the fingerprinting data [14].

Up to now, four studies comprising a total of 20 CF patients have described the colonization pattern of *A. fumigatus* [7, 19, 21, 26]. Verweij *et al.* [26] performed molecular typing of sequential isolates from two patients using RAPD.

With the *Afut*1-RFLP method, a predominant genotype in sequential sputum samples of one patient was detected. In another patient about nine different genotypes were seen in 12 sputum samples. Neuveglise *et al.* [19] collected 412 *A. fumigatus* isolates from 6 patients over a 2½-year-period. These isolates yielded 54 unique genotypes with the *Afut*1-RFLP method. In all samples of the six patients, several *A. fumigatus* genotypes were found, whereas two patients harboured a predominant genotype. The two other studies [7, 21] used solely the low discriminatory molecular typing assay RAPD, which suggested the majority of isolates to be of the same genotype [7].

In this study we report the colonization patterns of 196 *A. fumigatus* genotypes in 36 different CF patients from 3 European medical centres. The isolates were collected from sequential sputum samples and analyzed using the highly discriminatory STR*Af* assay.

Material and Methods

Isolates. A total of 204 isolates from 36 CF patients were included in this study. Sputum samples were collected from these patients during routine clinical visits or during an admission to hospital because of exacerbation of their pulmonary disease. The time period between the first and the last *A. fumigatus* isolate per patient varied from four months to nine and a half years. The numbers of isolates per patient varied from two till 27. The patients originated from three different centers; 100 isolates from 14 patients were obtained from the Cystic Fibrosis Center Dekkerswald (Nijmegen, The Netherlands); 58 isolates from 15 patients were collected from the University of Aachen (Aachen, Germany); another 46 isolates from 7 patients were from the University Medical Center Würzburg (Würzburg, Germany)

Collection of A. fumigatus isolates. Sputum samples obtained from patients were routinely cultured on Sabouraud agars for 7 days at 30 and 35ºC. *A. fumigatus* isolates were identified at the time of collection by their macroscopic and microscopic characteristics and their ability to grow at 48ºC.

The obtained isolates were stored as spore suspensions in regular microbial freezing broth containing 12.5% (vol/vol) glycerol at -80ºC until testing. The isolates were revived by scraping off part of the frozen broth, plated on Sabouraud's or potato agar, and cultivated at 30ºC.

STR*Af* assay. Genomic fungal DNA was extracted and purified from all isolates with the MagNA Lyzer and MagNA Pure LC Instruments (Roche Diagnostics, Almere, The Netherlands) and PCR primers and conditions were exactly as described before [9]. The obtained PCR fragments were diluted 30 fold with distilled water. One µl of diluted PCR products was combined with 0.25 µl of ET-ROX 400 marker (GE Healthcare, Diegem, Belgium) and denaturated for 1 min at 95ºC followed by rapid cooling to 4ºC in a total volume of 10 µl. The denaturated fragments were analyzed on a MegaBACE 500 automated DNA platform (GE Healthcare), according to the manufacturer's instructions.

Data analysis. Electropherograms were analyzed using Fragment Profiler 1.2 software (GE Healthcare). Identical isolates were those that possessed alleles with the same number of repeat units in all nine loci. Isolates with genotypes that differed in a single locus were considered to be genetically related. The term unique was used for genotypes that were only found once in a patient.

Results

A. fumigatus isolates from 36 CF patients were analyzed using the STR*Af* assay. For 196 isolates of the 204 isolates, a single genotype was obtained. Eight samples displayed multiple peaks for all nine loci, indicating to be a mixture of two or more different *A. fumigatus* isolates. An overview of the remaining 196 genotypes is shown in figure 1 and described below.

Six CF-patients (#1, #6, #8, #9, #17 and #29) harbored only identical or related *A. fumigatus* isolates. From five of these patients, two to four isolates were cultured with a maximum time interval of 1½ year. Moreover, patient #29 even showed 7 related isolates over a 3½ years period. From 7 patients (#3, #7, #16, #18, #26, #34 and #35) it turned out that most of the isolates were identical, since all patients had one or two unique isolates besides the predominant genotype. For patient #35 this predominant isolate was observed consecutively over a period of 9½ years.

In three patients (#25, #28 and #32), identical isolates were found successively in a period of about one year. The isolates cultured before or after this period demonstrated unique genotypes. Patient #27 and #30 also showed identical and unique isolates. However in these patients during a period in which identical genotypes were found, also unique genotypes were present. For example, genotype P from patient #27 was found in month 11, two years later three other genotypes were found in the sputum samples of this patient, but after three years genotype P appeared again.

In the following group of patients, several genotypes were found more than once. For instance, in patient #20 genotype M was found twice, followed by genotype N that was also found twice. In patient #36 a genotype '**ε**' was found four times in a period of 9½ years, whereas second genotype '**λ**' was observed after 8 years and onwards. In patient #31, three genotypes (T, V and W) were observed in successive time intervals varying from one to five months. They alternated with unique genotypes. Patient #11 and #33 demonstrated the most complex colonization patterns. Patient #11 with 9 isolates showed six different genotypes of which two types were isolated more than once. Patient #33 yielded 16 different genotypes for 27 isolates. Six genotypes were found more than once. The remaining 12 patients only showed unique genotypes.

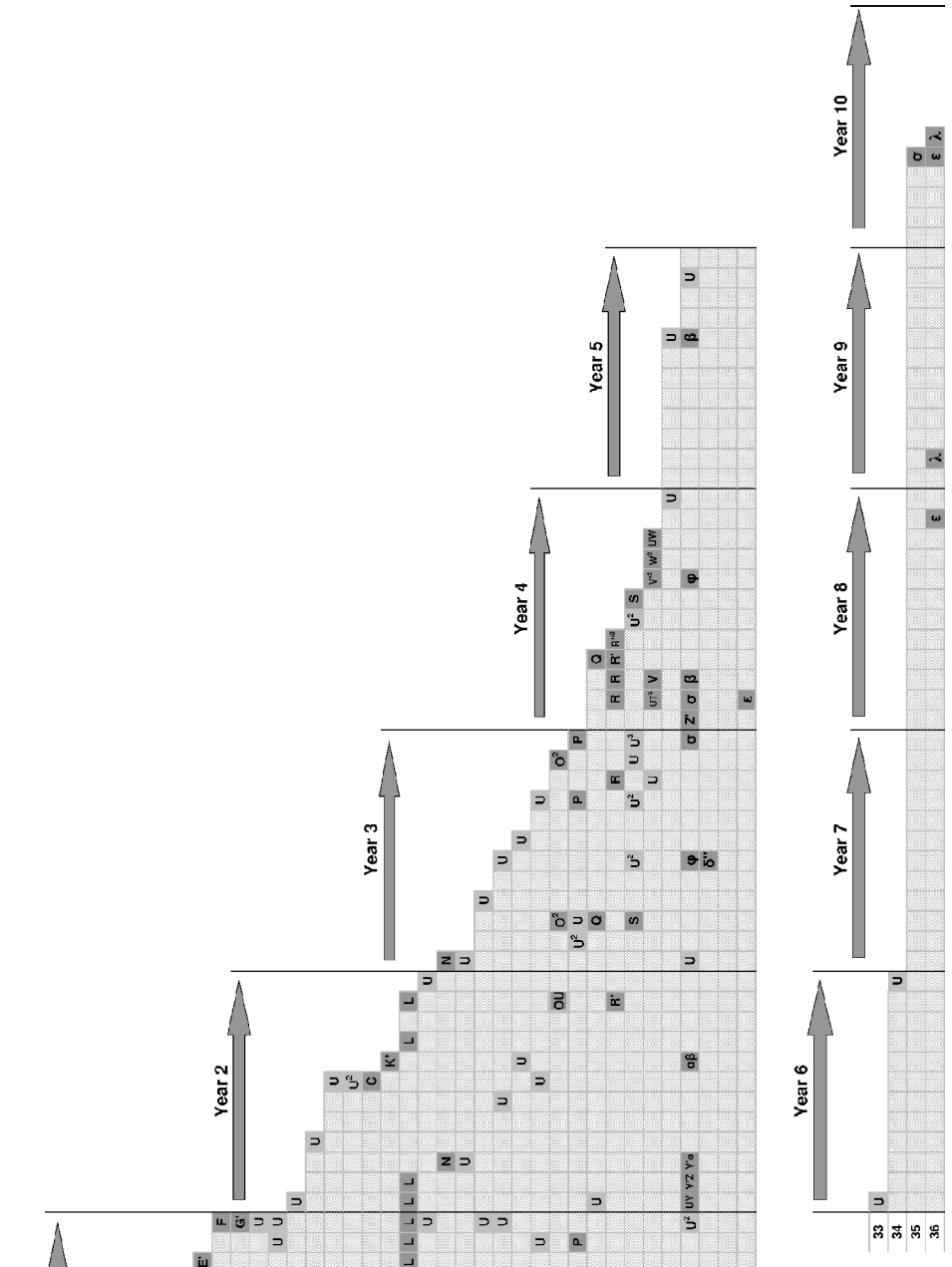


Figure 1. Graphical representation of the genotyping results from *A. fumigatus* isolates from 36 CF patients**.** Each patient is represented horizontally. The arrows indicate one year whereas each squares indicates one month. Unique isolates were depicted with the symbol U, these genotypes were only found once within a patient. Isolates from a patient possessing alleles with the same number of repeat units in all nine loci are identified by a letter. The ' symbol after a letter indicates that an isolates differed only in a single locus. The ^{2,3} and ^{5} after a letter showed the number of isolates found within a month, for example U^2 means that two unique isolates were found within one month.

Discussion

The *A. fumigatus* colonization patterns in 36 CF patients were investigated by typing 204 isolates with the STR*Af* assay, the largest study so far. From 196 *A. fumigatus* isolates a genotype was obtained, the remaining isolates were mixed genotypes. In general, four different colonization patterns can be discriminated as shown in figure 2. First, continuous colonization patterns were found. The patients yielded identical or related genotypes for all isolates over a prolonged period of time, indicating that they were not able to clear the *A. fumigatus* isolate. In this collection 17% of the patients showed colonization with only one genotype. The second colonization pattern consisted of a predominant genotype, meaning that most of the isolates are identical. These groups of patients were also not able to clear the *A. fumigatus* isolate and were occasionally co-colonized with a second genotype. This was the case in 19% of the patients. The third colonization pattern, found in 28% of the patients, contained genotypes which succeed each other. These patients were eventually able to clear the *A. fumigatus* isolate, but were easily recognized. The remaining 36% of the patients yielded colonization with only unique *A. fumigatus* isolates, indicating that these patients were continuously able to clear the fungus from the respiratory tract. In some other studies the relation between colonization patterns of CF patients and clinical symptoms was investigated [17, 24]. In our study population, we did not found an obvious correlation between colonization pattern and the development of ABPA, because only three CF patients in this study were diagnosed with ABPA. To get more insight in these relations, this study should be expanded with a larger number of CF patients with proven ABPA.

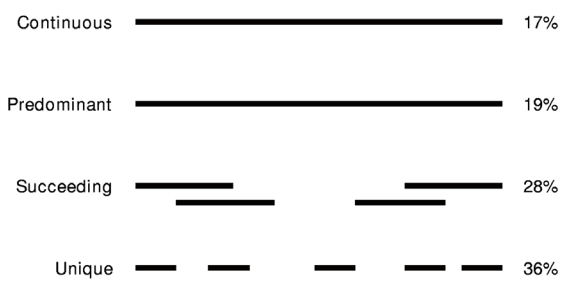


Figure 2. Schematic view of the colonization patterns of *A. fumigatus* in CF patients.

Molecular fingerprinting of *A. fumigatus* isolates from the environment and from clinical samples showed that a high extent of genetic variability can be found [6]. Therefore typing techniques with high discriminatory power are necessary to differentiate isolates from each other. Both the STR*Af* assay and the Afut1-RFLP method have shown to fulfill this criterion [6, 9]. Individuals may constantly be exposed to a large variety of different genotypes; this is in concordance with the 36% of the patients where only unique genotypes were found. It is therefore even more surprising that in the remaining group of patients identical genotypes were found more than once over longer periods. In patient #35 and #36 identical genotypes were found for more than 9 years. It is likely that inside the CF lung a different environment (local immunosuppression, different pH and growth nutrition) is present compared to healthy lungs. This may cause a different growth and virulence of *A. fumigatus*. Beauvais *et al.*[5] and Mowat *et al.* [17] recently demonstrated that *Aspergillus* has the ability to produce a matrix that surrounds hyphae which protects from the action of antifungals. Future studies may show if the mucus and the self produced matrix causes a barrier against the human defense and antifungals and therefore lead to a longer persistence. However, despite the presence of the viscous mucus, 36% of the patients in our collection with a unique colonization pattern were able to clear the fungus from the respiratory tract.

The colonization patterns of *A. fumigatus* in CF patients was comparable with other patients categories. In previous studies successive *A. fumigatus* isolates were typed from non CF-patients. Several patients with invasive aspergillosis [10, 13] and aspergilloma [12] were colonized with multiple genotypes, whereas other patients harbored unique isolates. However, there is a difference between our study and the latter, since almost all of the isolates were collected within one month. Symoens *et al.* [25] collected *A. fumigatus* isolates from eight lung transplant recipients. From three of these patients several isolates were collected over a period of 1 to 3 years also showing a continuous, predominant and unique type of colonization. To compare the colonization patterns in CF patient with those with other underlying diseases, similar studies should be performed with more non CF patients colonized with *A. fumigatus*. Such a group could be the patients with other chronic respiratory diseases, like bronchial asthma and chronic obstructive pulmonary diseases, since these patients also are prone to develop ABPA [1].

References

1. Agarwal, R., R. Srinivas, and S. K. Jindal. 2008. Allergic bronchopulmonary aspergillosis complicating chronic obstructive pulmonary disease. Mycoses 51:83-5.
2. Atkinson, R. M., J. J. Lipuma, D. B. Rosenbluth, and W. M. Dunne, Jr. 2006. Chronic colonization with *Pandoraea apista* in cystic fibrosis patients determined by repetitive-element-sequence PCR. J Clin Microbiol 44:833-6.
3. Aufauvre-Brown, A., J. Cohen, and D. W. Holden. 1992. Use of randomly amplified polymorphic DNA markers to distinguish isolates of *Aspergillus fumigatus*. J Clin Microbiol 30:2991-3.
4. Bakare, N., V. Rickerts, J. Bargon, and G. Just-Nubling. 2003. Prevalence of *Aspergillus fumigatus* and other fungal species in the sputum of adult patients with cystic fibrosis. Mycoses 46:19-23.
5. Beauvais, A., C. Schmidt, S. Guadagnini, P. Roux, E. Perret, C. Henry, S. Paris, A. Mallet, M. C. Prevost, and J. P. Latge. 2007. An extracellular matrix glues together the aerial-grown hyphae of *Aspergillus fumigatus*. Cell Microbiol 9:1588-600.
6. Chazalet, V., J. P. Debeaupuis, J. Sarfati, J. Lortholary, P. Ribaud, P. Shah, M. Cornet, H. Vu Thien, E. Gluckman, G. Brucker, and J. P. Latge. 1998. Molecular typing of environmental and patient isolates of *Aspergillus fumigatus* from various hospital settings. J Clin Microbiol 36:1494-500.
7. Cimon, B., F. Symoens, R. Zouhair, D. Chabasse, N. Nolard, A. Defontaine, and J. P. Bouchara. 2001. Molecular epidemiology of airway colonisation by *Aspergillus fumigatus* in cystic fibrosis patients. J Med Microbiol 50:367-74.
8. de Valk, H. A., J. F. G. M. Meis, S. Bretagne, J. Costa, B. A. Lasker, S. A. Balajee, A. C. Pasqualotto, M. J. Anderson, L. Alcazar-Fuoli, E. Mellado, and C. H. W. Klaassen. 2008. Interlaboratory compatibility of a microsatellite based typing assay for *Aspergillus fumigatus* through the use of allelic ladders: proof of concept. Clin Microbiol Infect, in press.
9. de Valk, H. A., J. F. G. M. Meis, I. M. Curfs, K. Muehlethaler, J. W. Mouton, and C. H. W. Klaassen. 2005. Use of a novel panel of nine short tandem repeats for exact and high-resolution fingerprinting of *Aspergillus fumigatus* isolates. J Clin Microbiol 43:4112-20.
10. de Valk, H. A., J. F. G. M. Meis, B. E. de Pauw, P. J. Donnelly, and C. H. W. Klaassen. 2007. Comparison of two highly discriminatory molecular fingerprinting assays for analysis of multiple *Aspergillus fumigatus* isolates from patients with invasive aspergillosis. J Clin Microbiol 45:1415-9.
11. de Valk, H. A., J. F. G. M. Meis, and C. H. W. Klaassen. 2007. Microsatellite based typing of *Aspergillus fumigatus*: Strengths, pitfalls and solutions. J Microbiol Methods 69:268-72.
12. Girardin, H., J. Sarfati, H. Kobayashi, J. P. Bouchara, and J. P. Latge. 1994. Use of DNA moderately repetitive sequence to type *Aspergillus fumigatus* isolates from aspergilloma patients. J Infect Dis 169:683-5.
13. Girardin, H., J. Sarfati, F. Traore, J. Dupouy Camet, F. Derouin, and J. P. Latge. 1994. Molecular epidemiology of nosocomial invasive aspergillosis. J Clin Microbiol 32:684-90.

14. Lasker, B. A. 2002. Evaluation of performance of four genotypic methods for studying the genetic epidemiology of *Aspergillus fumigatus* isolates. J Clin Microbiol 40:2886-92.
15. Mastella, G., M. Rainisio, H. K. Harms, M. E. Hodson, C. Koch, J. Navarro, B. Strandvik, and S. G. McKenzie. 2000. Allergic bronchopulmonary aspergillosis in cystic fibrosis. A European epidemiological study. Epidemiologic Registry of Cystic Fibrosis. Eur Respir J 16:464-71.
16. Mishra, A., R. Greaves, and J. Massie. 2005. The relevance of sweat testing for the diagnosis of cystic fibrosis in the genomic era. Clin Biochem Rev 26:135-53.
17. Mowat, E., J. Butcher, S. Lang, C. Williams, and G. Ramage. 2007. Development of a simple model for studying the effects of antifungal agents on multicellular communities of *Aspergillus fumigatus*. J Med Microbiol 56:1205-12.
18. Nelson, L. A., M. L. Callerame, and R. H. Schwartz. 1979. Aspergillosis and atopy in cystic fibrosis. Am Rev Respir Dis 120:863-73.
19. Neuveglise, C., J. Sarfati, J. P. Debeaupuis, H. Vu Thien, J. Just, G. Tournier, and J. P. Latge. 1997. Longitudinal study of *Aspergillus fumigatus* strains isolated from cystic fibrosis patients. Eur J Clin Microbiol Infect Dis 16:747-50.
20. Neuveglise, C., J. Sarfati, J. Latge, and S. Paris. 1996. Afut1, a retrotransposon-like element from *Aspergillus fumigatus*. Nucl Acids Res 24:1428-1434.
21. Rath, P. M., F. Ratjen, and R. Ansorg. 1997. Genetic diversity among isolates of *Aspergillus fumigatus* in patients with cystic fibrosis. Zentralbl Bakteriol 285:450-5.
22. Shoseyov, D., K. G. Brownlee, S. P. Conway, and E. Kerem. 2006. *Aspergillus* bronchitis in cystic fibrosis. Chest 130:222-6.
23. Skov, M., K. McKay, C. Koch, and P. J. Cooper. 2005. Prevalence of allergic bronchopulmonary aspergillosis in cystic fibrosis in an area with a high frequency of atopy. Respir Med 99:887-93.
24. Stevens, D. A., R. B. Moss, V. P. Kurup, A. P. Knutsen, P. Greenberger, M. A. Judson, D. W. Denning, R. Crameri, A. S. Brody, M. Light, M. Skov, W. Maish, and G. Mastella. 2003. Allergic bronchopulmonary aspergillosis in cystic fibrosis--state of the art: Cystic Fibrosis Foundation Consensus Conference. Clin Infect Dis 37 Suppl 3:S225-64.
25. Symoens, F., S. Bertout, M. A. Piens, J. Burnod, F. Renaud, N. Nolard, F. Chapuis, and R. Grillot. 2001. A longitudinal study of lung transplant recipients infected with *Aspergillus*: Genetic polymorphism of *A. fumigatus*. J Heart Lung Transplant 20:970-8.
26. Verweij, P. E., J. F. G. M. Meis, J. Sarfati, J. A. Hoogkamp-Korstanje, J. P. Latge, and W. J. Melchers. 1996. Genotypic characterization of sequential *Aspergillus fumigatus* isolates from patients with cystic fibrosis. J Clin Microbiol 34:2595-7.

Chapter 10

Detection and direct genotyping of *Aspergillus fumigatus* in tissue and serum samples in patients with invasive aspergillosis

Hanneke A. de Valk, Ineke M. de Kievit, Corné H.W. Klaassen and Jacques F.G.M. Meis

Submitted.

Abstract

Based on nine highly polymorphic short tandem repeat loci, genotyping of *Aspergillus fumigatus* was achieved from cultured isolates and tissue samples from five patients with invasive aspergillosis. In one patient it was also possible to obtain a genotype from several serum samples. Within three patients identical genotypes were found in all samples, one patient showed a dominant genotype, which was also found in the lung tissue. In two of the three lung biopsies of the fifth patient more than two genotypes were found. With this STR assay the presence of *A. fumigatus* in combination with a genotypic profile was obtained directly from tissue and serum samples, without culture. This allows (retrospective) examination of dissemination routes of *A. fumigatus* in patients with IA.

Introduction

Aspergillus fumigatus is the most important species responsible for invasive fungal disease. Invasive aspergillosis (IA) affects immunocompromised patients and despite treatment options, the mortality is high [13]. It is difficult to diagnose IA, as cultivation of *Aspergillus* has poor sensitivity and tissue biopsies are difficult to obtain [9]. Even if a biopsy is performed they are mostly sent to the laboratory in formalin fixative because IA is often not suspected clinically. It is therefore impossible to culture *A. fumigatus* from such tissues [6]. Unlike with many other serious pathogens, *A. fumigatus* is virtually never isolated from blood cultures [8]. Major advances have been achieved in the past decade by the determination of galactomannan in blood [7]. There are also several PCR-based assays developed for the direct detection of *A. fumigatus* DNA in serum, bronchoalveolar lavage (BAL) and lung tissue samples [8, 9, 11].

Closer insight in the pathogenesis of IA can be obtained, when the interstrain relatedness of *A. fumigatus* isolates obtained from respiratory and tissue samples is investigated. A few studies have already examined the genetic relatedness between isolates obtained from different body sites using highly discriminatory typing techniques [4, 5]. It was established that respiratory isolates are not necessarily identical to the isolate found in tissue samples. Isolation of *Aspergillus* from respiratory specimens may reflect colonization of the airway instead of invasive infection and even environmental contamination of the culture is possible [9]. To exclude contamination and to compare the isolates obtained from respiratory samples with the isolate which is responsible for invasive infections, it is important to obtain a genotype. IA is often diagnosed solely on histopathology of paraffin embedded samples. This is a definitive diagnosis of invasive fungal infection but this does not necessarily mean diagnosis of invasive aspergillosis [13]. PCR assays for direct detection of *A. fumigatus* in clinical samples have been described before [8, 9, 11, 12]. Recently direct genotyping of *Paracoccidioides brasiliensis* from paraffin embedded tissue has been reported with success [10]. In contrast, typing of *A. fumigatus* and most other fungal pathogens has always been performed on cultured isolates.

Our aim was to genotype *A. fumigatus* by high-resolution STR*Af* typing [3] directly from formalin-fixed paraffin-embedded (FFPE) tissue and serum samples, and to compare these genotypes with cultured isolates from respiratory samples.

Material and Methods

Patients. Isolates, FFPE tissue and serum samples of five patient (A-E) in the age of 58 to 67 years old, hospitalized on the ICU from our hospital, were collected. All patients were histologically diagnosed with invasive aspergillosis and all five patients were treated with antifungal antibiotics. An overview of the patients is given in table 1.

Table 1. Patients with histologically proven invasive aspergillosis

Patient	Age	Underlying disease	Diagnosis	Treatment	Outcome
1	67	COPD, Influenzae pneumoniae ICU treatment	Disseminated IA	Itraconazole Caspofungin	Died
2	62	COPD, Legionella pneumoniae ICU treatment	Disseminated IA	Itraconazole Caspofungin	Died
3	59	COPD, Lung carcinoom ICU treatment	Pulmonary aspergillosis	Caspofungin	Died
4	58	COPD, ?	Pulmonary aspergillosis	Itraconazole	Survived
5	65	COPD, ?	Pulmonary aspergillosis	Itraconazole	Survived

***A. fumigatus* isolates and DNA extraction.** Samples obtained from patients were routinely cultured on Sabouraud agars for 7 days at 30 and 35ºC. *A. fumigatus* isolates were identified at the time of collection by their macroscopic and microscopic characteristics and their ability to grow at 48ºC. The obtained isolates were stored as spore suspensions in regular microbial freezing broth containing 12.5% (vol/vol) glycerol at -80ºC until testing. The isolates were revived by scraping off part of the sample, plating on Sabouraud's agar and cultivate at 30ºC until sporulation. DNA from *A. fumigatus* spores was extracted and purified with the MagNA Lyzer and MagNA Pure LC Instruments (Roche Diagnostics, Almere, The Netherlands) as described before [3].

DNA extraction from serum. DNA was extracted from 200µl serum using the High Pure Viral Nucleic Acid Kit (Roche Diagnostics) according to the instructions from the manufacturer.

DNA extraction from FFPE tissue samples. For each FFPE tissue sample, 5 sections (thickness, 10µM) were cut using a sterile microtome blade and transferred to a 2 ml centrifuge tube. To remove paraffin wax, the samples were washed twice with 500 µl xylene, twice with 500 µl ethanol absolute and once with 500 µl acetone. The pellet was dried at 56ºC. Next, 300µl of Puregene cell lysis solution from Gentra Systems (Biozym B.V., Landgraaf, The Netherlands) and ProteïnaseK was added and incubated overnight at 56ºC during continue mixing. Following mechanical lysis in a MagNA Lyzer instrument (Roche Diagnostics), the DNA was purified using the method described by Boom *et al.* with minor modifications [2].

Genotyping using the STR*Af* assay. Reaction conditions and PCR primers were as described by de Valk *et al.* [3], Amplification mixtures contained 1 ng of genomic DNA for isolates or 5 µl of the total DNA extracted from tissue or serum samples. The total volume of all PCR reactions was kept constant at 25µl. The number of amplification cycles was increased to 40. Electropherograms were analyzed using Fragment Profiler 1.2 software (GE Healthcare, Roosendaal, The Netherlands).

Results

An overview of the results is shown in table 2. From patient A five identical genotypes of *A. fumigatus* isolates were found, four of these were cultured from sequential sputum samples and one from bronchoalveolar lavage (BAL) in a one week period. Another, different genotype was obtained from a lung abscess. Galactomannan negative serum samples from this patient showed no characteristic amplification products using the STR*Af* assay. Lung biopsies were obtained on two different days.

In two of these lung biopsies (A8 and A10) multiple peaks were found for all nine markers, being a typical result of a mixture of two or more different genotypes in these samples. In another lung biopsy (A11) the same genotype was found as in the isolates from the four sputum samples and the BAL.

A total of 15 *A. fumigatus* isolates were obtained from clinical samples from patient B. Thirteen sequential isolates were cultured from sputum samples collected over a six-week period, one from ascitis fluid and one from a BAL. The genotypes from all these isolates are identical. From this patient three serum samples were analyzed using the STR*Af* assay. The value obtained with the galactomannan test was 0.4, 4.0 and 5.6 for the samples B10, B16 and B18, respectively. No genotype was obtained with the serum sample with the lowest galactomannan value, the other two serum samples yielded an identical genotypes as the 15 sputum isolates. The same genotype was found in the FFPE lung tissue sample.

From patient C, a total of 22 *A. fumigatus* isolates were cultured from sequential sputum samples during a nine-week period. Nineteen of these *A. fumigatus* isolates were of the same genotype. Isolates C2, C5 and C22 showed unique genotypes. The four, galactomannan negative, serum samples of this patient yielded no genotype in the STR*Af* assay. The first lung biopsy (C3) of this patient showed peaks in 6 of the nine loci, the remaining three loci were negative. The values in the six positive loci were identical to the genotype of the 19 sputum isolates. The second biopsy (C28) was also of the same genotype as the 19 sputum isolates.

Direct genotyping of the FFPE lung tissue of patient D yielded a genotype that was identical to five cultured *A. fumigatus* isolates from this patient. The isolates were collected from sputum, BAL, a lung abscess and lung tissue. Samples from patient E showed identical genotypes in an isolate obtained from BAL and in the FFPE bronchial biopsy.

Table 2: An overview of the typing results of all samples.

Patient + no.	Origin	Date of isolation (day-mo-yr)	Cultured isolate	STR*Af* type								
				2A	2B	2C	3A	3B	3C	4A	4B	4C
A1	Sputum	03-01-2005	Yes	25	22	19	28	9	27	10	9	5
A2	Sputum	05-01-2005	Yes	25	22	19	28	9	27	10	9	5
A3	Serum, GM 0.1	06-01-2005	No	neg	neg	neg	neg	neg	neg	neg	neg	neg
A4	Serum, GM 0.1	09-01-2005	No	neg	neg	neg	neg	neg	neg	neg	neg	neg
A5	Sputum	10-01-2005	Yes	25	22	19	28	9	27	10	9	5
A6	Lung abscess	10-01-2005	Yes	23	21	8	46	9	6	8	9	5
A7	Sputum	10-01-2005	Yes	25	22	19	28	9	27	10	9	5
A8	Biopsy lung	10-01-2005	No	> 2 genotypes								
A9	BAL	12-01-2005	Yes	25	22	19	28	9	27	10	9	5
A10	Biopsy lung	12-01-2005	No	> 2 genotypes								
A11	Biopsy lung	12-01-2005	No	neg	22	19	28	9	27	10	9	5
B1	Sputum	26-09-2005	Yes	18	12	8	28	10	18	9	8	5
B2	Sputum	27-09-2005	Yes	18	12	8	28	10	18	9	8	5
B3	Sputum	29-09-2005	Yes	18	12	8	28	10	18	9	8	5
B4	Sputum	03-10-2005	Yes	18	12	8	28	10	18	9	8	5
B5	Sputum	06-10-2005	Yes	18	12	8	28	10	18	9	8	5
B6	Sputum	10-10-2005	Yes	18	12	8	28	10	18	9	8	5
B7	Sputum	12-10-2005	Yes	18	12	8	28	10	18	9	8	5
B8	Sputum	16-10-2005	Yes	18	12	8	28	10	18	9	8	5
B9	Sputum	26-10-2005	Yes	18	12	8	28	10	18	9	8	5
B10	Serum, GM 0.4	26-10-2005	No	neg	neg	neg	neg	neg	neg	neg	neg	neg
B11	Sputum	27-10-2005	Yes	18	12	8	28	10	18	9	8	5
B12	Sputum	31-10-2005	Yes	18	12	8	28	10	18	9	8	5
B13	Sputum	03-11-2005	Yes	18	12	8	28	10	18	9	8	5
B14	BAL	07-11-2005	Yes	18	12	8	28	10	18	9	8	5
B15	Ascitis fluid	07-11-2005	Yes	18	12	8	28	10	18	9	8	5
B16	Serum, GM 4.0	08-11-2005	No	18	12	8	28	10	18	9	8	5
B17	Sputum	08-11-2005	No	18	12	8	28	10	18	9	8	5
B18	Serum, GM 5.6	09-11-2005	No	18	12	8	28	10	18	9	8	5
B19	Biopsy lung	09-11-2005	No	18	12	8	28	10	18	9	8	5
C1	Sputum	10-05-2007	Yes	21	25	18	27	12	7	21	10	8
C2	Sputum	10-05-2007	Yes	18	20	15	28	11	21	26	26	8
C3	Bronchial biopsy	11-05-2007	No	neg	neg	18	27	12	7	neg	10	8
C4	Serum	11-05-2007	No	neg	neg	neg	neg	neg	neg	neg	neg	neg
C5	Sputum	14-05-2007	Yes	18	28	15	16	10	19	8	8	5
C6	Sputum	15-05-2007	Yes	21	25	18	27	12	7	21	10	8
C7	Sputum	31-05-2007	Yes	21	25	18	27	12	7	21	10	8
C8	Sputum	04-06-2007	Yes	21	25	18	27	12	7	21	10	8
C9	Serum	04-06-2007	No	neg	neg	neg	neg	neg	neg	neg	neg	neg
C10	Sputum	11-06-2007	Yes	21	25	18	27	12	7	21	10	8
C11	Sputum	18-06-2007	Yes	21	25	18	27	12	7	21	10	8
C12	Sputum	21-06-2007	Yes	21	25	18	27	12	7	21	10	8
C13	Sputum	24-06-2007	Yes	21	25	18	27	12	7	21	10	8
C14	Sputum	25-06-2007	Yes	21	25	18	27	12	7	21	10	8
C15	Sputum	28-06-2007	Yes	21	25	18	27	12	7	21	10	8
C16	Sputum	02-07-2007	Yes	21	25	18	27	12	7	21	10	8
C17	Sputum	09-07-2007	Yes	21	25	18	27	12	7	21	10	8
C18	Sputum	11-07-2007	Yes	21	25	18	27	12	7	21	10	8
C19	Sputum	16-07-2007	Yes	21	25	18	27	12	7	21	10	8
C20	Sputum	19-07-2007	Yes	21	25	18	27	12	7	21	10	8

Patient + no.	Origin	Date of isolation (day-mo-yr)	Cultured isolate	STR*Af* type								
				2A	2B	2C	3A	3B	3C	4A	4B	4C
C21	Sputum	23-07-2007	Yes	21	25	18	27	12	7	21	10	8
C22	Sputum	23-07-2007	Yes	18	12	18	27	22	21	18	9	5
C23	Serum	26-07-2007	No	neg	neg	neg	neg	neg	neg	neg	neg	neg
C24	Sputum	26-07-2007	Yes	21	25	18	27	12	7	21	10	8
C25	Serum	28-07-2007	No	neg	neg	neg	neg	neg	neg	neg	neg	neg
C26	Sputum	30-07-2007	Yes	21	25	18	27	12	7	21	10	8
C27	Sputum	30-07-2007	Yes	21	25	18	27	12	7	21	10	8
C28	Bronchial biopsy	30-07-2007	No	21	25	18	27	12	7	21	10	8
D1	Sputum	12-12-2004	Yes	11	12	17	26	21	21	14	8	5
D2	BAL	13-12-2004	Yes	11	12	17	26	21	21	14	8	5
D3	BAL	13-12-2004	Yes	11	12	17	26	21	21	14	8	5
D4	Lung tissue	15-12-2004	Yes	11	12	17	26	21	21	14	8	5
D5	Lung abscess	15-12-2004	Yes	11	12	17	26	21	21	14	8	5
D6	Serum	16-12-2004	No	neg	neg	neg	neg	neg	neg	neg	neg	neg
D7	Serum, GM 0.2	16-12-2004	No	neg	neg	neg	neg	neg	neg	neg	neg	neg
D8	Biopsy lung	16-12-2004	No	11	12	17	26	21	21	14	8	5
E1	BAL	22-02-2006	Yes	18	23	16	38	11	46	10	9	8
E2	Bronchial biopsy	22-02-2006	No	18	23	16	38	11	46	10	9	8

Discussion

We have shown here that it is technically feasible to genotype *A. fumigatus* directly from serum and FFPE tissue samples without the need for cultivation of the isolate. To be able to obtain genotypes directly from FFPE tissue and serum samples, it is important that only DNA of the fungus is amplified. With previously described high resolution typing assays for *A. fumigatus* such as AFLP [4] and RAPD [1], human DNA will also be amplified and will corrupt the banding patterns. Current PCR protocols to specifically detect *A. fumigatus*, like real-time assays targeting the ITS region [8, 11] or mitochondrial DNA [9] in clinical samples are not suitable to distinguish between isolates from different origin. With the STR*Af* assay, not only an identification of the histopathologically identified fungus is possible, but also a STR*Af* genotype can be assigned to the fungus seen by histology. This assay only amplified specific *A. fumigatus* targets and the method is highly discriminating [3]

The genotypes found within the patients B, D and E were identical for all samples. Patient C showed isolates obtained from sputum with different genotypes, the dominant genotype was also found in the lung tissue. These results are in concordance with previous studies, where *A. fumigatus* isolates obtained from respiratory and deeper tissue samples were investigated [4, 5]. Five of the six cultured isolates of patient A were identical to a lung biopsy sample of this patient. A remarkable result in this patient was that more than 2 genotypes were found in two other lung biopsies. An explanation would be that the sample was contaminated by several isolates from the respiratory tract of this patient during the biopsy.

In one patient (B) it was also possible to obtain a genotype from several serum samples. The samples with the highest galactomannan values contained sufficient fungal DNA to determine the genotype of the isolate. To obtain signals from the low amount of fungus DNA in FFPE tissue and serum samples optimal PCR conditions are required. All samples used in this study were amplified using multiplex PCR reactions. These multiplex reactions should be perfectly balanced to obtain optimal amplification of all the targets. Therefore, another option to increase the sensitivity of PCR amplification is to use monoplex reactions for each target. In this study no monoplex reaction were tested, because in only one biopsy (C3) two dinucleotide repeats (STR*Af*2A and STR*Af* 2B) and one tetranucleotide repeat (STR*Af* 4A) remained negative, while the six remaining loci yielded good interpretable peaks. These six loci showed identical repeat numbers as the cultured isolate of this patients and were considered to be identical to each other. Therefore, there is no guarantee that monoplex reaction will yield good interpretable results, since each individual PCR reactions may have a different sensitivity leading to failure of amplification with some markers in samples with very low loads.

In conclusion, the STR*Af* assay allows simultaneous detection as well as high resolution genotyping of *A. fumigatus* directly in clinical samples without the need for culture. This approach allows (retrospective) examination of dissemination routes of *A. fumigatus* in patients with IA and might be used towards a better understanding of the pathogenesis of this disease.

References

1. Aufauvre-Brown, A., J. Cohen, and D. W. Holden. 1992. Use of randomly amplified polymorphic DNA markers to distinguish isolates of *Aspergillus fumigatus*. J Clin Microbiol 30:2991-3.
2. Boom, R., C. J. Sol, M. M. Salimans, C. L. Jansen, P. M. Wertheim-van Dillen, and J. van der Noordaa. 1990. Rapid and simple method for purification of nucleic acids. J Clin Microbiol 28:495-503.
3. de Valk, H. A., J. F. Meis, I. M. Curfs, K. Muehlethaler, J. W. Mouton, and C. H. Klaassen. 2005. Use of a novel panel of nine short tandem repeats for exact and high-resolution fingerprinting of *Aspergillus fumigatus* isolates. J Clin Microbiol 43:4112-20.
4. de Valk, H. A., J. F. Meis, B. E. de Pauw, P. J. Donnelly, and C. H. Klaassen. 2007. Comparison of two highly discriminatory molecular fingerprinting assays for analysis of multiple *Aspergillus fumigatus* isolates from patients with invasive aspergillosis. J Clin Microbiol 45:1415-9.
5. Girardin, H., J. Sarfati, F. Traore, J. Dupouy Camet, F. Derouin, and J. P. Latge. 1994. Molecular epidemiology of nosocomial invasive aspergillosis. J Clin Microbiol 32:684-90.
6. Lau, A., S. Chen, T. Sorrell, D. Carter, R. Malik, P. Martin, and C. Halliday. 2007. Development and clinical application of a panfungal PCR assay to detect and identify fungal DNA in tissue specimens. J Clin Microbiol 45:380-5.
7. Mennink-Kersten, M. A., J. P. Donnelly, and P. E. Verweij. 2004. Detection of circulating galactomannan for the diagnosis and management of invasive aspergillosis. Lancet Infect Dis 4:349-57.
8. O'Sullivan, C. E., M. Kasai, A. Francesconi, V. Petraitis, R. Petraitiene, A. M. Kelaher, A. A. Sarafandi, and T. J. Walsh. 2003. Development and validation of a quantitative real-time PCR assay using fluorescence resonance energy transfer technology for detection of *Aspergillus fumigatus* in experimental invasive pulmonary aspergillosis. J Clin Microbiol 41:5676-82.
9. Rantakokko-Jalava, K., S. Laaksonen, J. Issakainen, J. Vauras, J. Nikoskelainen, M. K. Viljanen, and J. Salonen. 2003. Semiquantitative detection by real-time PCR of *Aspergillus fumigatus* in bronchoalveolar lavage fluids and tissue biopsy specimens from patients with invasive aspergillosis. J Clin Microbiol 41:4304-11.
10. Ricci, G., U. Zelck, F. Mota, C. Lass-Florl, M. F. Franco, and R. Bialek. 2008. Genotyping of *Paracoccidioides brasiliensis* directly from paraffin embedded tissue. Med Mycol 46:31-4.
11. Schabereiter-Gurtner, C., B. Selitsch, M. L. Rotter, A. M. Hirschl, and B. Willinger. 2007. Development of novel real-time PCR assays for detection and differentiation of eleven medically important *Aspergillus* and *Candida* species in clinical specimens. J Clin Microbiol 45:906-14.
12. Vollmer, T., M. Stormer, K. Kleesiek, and J. Dreier. 2008. Evaluation of a novel broad-range real-time PCR assay for the rapid detection of human pathogenic fungi in various clinical specimens. J Clin Microbiol. 46:1919-26

13. Walsh, T. J., E. J. Anaissie, D. W. Denning, R. Herbrecht, D. P. Kontoyiannis, K. A. Marr, V. A. Morrison, B. H. Segal, W. J. Steinbach, D. A. Stevens, J. A. van Burik, J. R. Wingard, and T. F. Patterson. 2008. Treatment of aspergillosis: clinical practice guidelines of the Infectious Diseases Society of America. Clin Infect Dis 46:327-60.

Chapter 11

Summary and general discussion

- Nederlandse samenvatting
- Dankwoord
- List of publications
- Curriculum vitae

Summarizing discussion

Aspergillus species are widely distributed fungi that release large amounts of airborne conidia that are dispersed in the environment. Several *Aspergillus* species have been described as human pathogens. *Aspergillus fumigatus* is the species most frequently isolated from human infections. To investigate the epidemiologic relation between environmental and clinical isolates molecular typing techniques have been developed. Fingerprinting techniques for *A. fumigatus* require a high discriminatory power and need to be highly reproducible, since a high extent of genetic variability has been demonstrated for this species [8, 10]. The RFLP (Restriction Fragment Length Polymorphism) method in combination with the Afut1 hybridization probe is highly discriminatory [11, 20]. The time-consuming and labor intensive performance together with the difficult interpretation of the complex RFLP banding patterns, makes the RFLP method far from ideal for typing large amount of isolates. In contrast, typing techniques which yield sequence data, like MLST [2] and CSP [3], are 100% reproducible and the data can be readily exchanged between laboratories. Unfortunately these methods lack sufficient discriminatory power for large scale studies. Microsatellites or short tandem repeats (STRs), however, have shown to provide a high level of discrimination between different isolates in a wide variety of micro-organisms. They are also extensively being used for high-resolution fingerprinting of the human genome. Bart-Delabesse et al. [4] firstly used STRs to type *A. fumigatus*, the so-called MLP method (Microsatellite Length Polymorphism). Because no genomic sequences were available at that time, among 17.000 clones were screened with a CA_{10} oligonucleotide probe. Four polymorphic CA-microsatellite loci were retained for further analysis. The discriminatory power of these four markers is almost equal to that of RFLP [5, 15]. STR analysis yielded highly reproducible and exact typing results, which allow the easy exchange of data. In two of the MLP loci a second repeat sequence was located next to the CA-repeat. Different combinations of two repeat sequences in a single PCR amplicon may lead to the formation of fragments of the same length. This makes it impossible to determine the exact repeat number for each of the two repeats and compromises the exact nature of the STR assay.

Therefore in **chapter 2** we describe the development of a novel assay based on nine short tandem repeats for exact and high-resolution fingerprinting of *A. fumigatus*, the so-called STR*Af* assay. The repeats were searched in silico in the available genomic sequences of *A. fumigatus*. The availability of genomic sequences simplified the analysis for the presence of STRs. Three multiplex PCRs were made for three perfect di-, tri- and tetranucleotide repeats. The STR*Af* assay was able to differentiate all unrelated *A. fumigatus* isolates from nonoutbreak situations. The Simpson's diversity index [14] for the nine loci is 0.9994, which confirms the high discriminatory power of this assay. In theory this panel is able to discriminate between 27 x 10^9 different genotypes. This is an improvement of close to 10^6-fold over that obtained with the previously reported MLP panel [4].

In the remaining part of this thesis, the technical aspects of the STR*Af* assay are described as well as the application of the assay for clinical purposes.

Part 1: Technical aspects

In **chapter 3** the performance of STR*Af* panel was compared to AFLP (Amplified Fragment Length Polymorphism), which is another high discriminatory molecular fingerprinting assay. A total of 55 *A. fumigatus* isolates, obtained from 15 different patients suffering from invasive aspergillosis, were analyzed using the STR*Af* panel and the AFLP method. This set of isolates was also analyzed with a new method, RISC (Retrotransposon Insertion-Site Context)-typing, which is described in detail in **chapter 4**. In general, there was an excellent agreement between the STR*Af*, RISC and AFLP methods. The slight differences that were observed were not entirely unexpected since each of the three methods target different parts of the fungal genome. A clear advantage of the STR*Af* assay compared to AFLP and RISC is its ability to identify mixture of isolates. It has been demonstrated that specimens from respiratory origin and environmental samples may contain multiple different genotypes [6, 9, 13, 26]. A major difference between the typing methods is the interpretation of the obtained fingerprint results. Amplification products of the STR*Af* assay are sized automatically and converted into repeat numbers, a genotype consist of nine repeat numbers, which is easy to interpret.

The AFLP and RISC results consist of complex banding patterns with multiple strong as well as weak bands. To determine the genetic relationship between multiple isolates, one has to rely on specialized computer software which rely on patterns based algorithms. Visual identical banding patterns were never calculated to be 100% identical with computerized analysis using a pattern based algorithm. The long-term reproducibility and intralaboratory comparisons may also be quite challenging for both the AFLP and RISC methods. Because small differences in the AFLP and RISC fingerprints exist, due to small variations occurring during restriction/ligation, PCR amplification and fragment analysis, these may affect the final peak intensity. However, the intralaboratory reproducibility of the STR*Af* assay is 100%. In order for any typing technique to be successfully used in other settings, the most important factor is reproducibility. The STR*Af* assay offers the most benefits compared to AFLP and RISC-typing. The robustness of the STR*Af* assay under different amplification conditions was investigated in **chapter 5**. Various components of the PCR reaction and thermocycling parameters were deliberately and systemically altered to investigate their effect on the outcome of this assay. Some extreme conditions let to a loss of signal, but, under all conditions where a signal was obtained, identical typing results were produced. Thus, the STR*Af* assay proved to be extremely robust and should be used in other settings without problems. A major pitfall concerning the exchange of STR*Af* data between different laboratories is the assignment of the repeat numbers to the principal peak, which is routinely established by sizing of the DNA fragment and comparing it to the size of the reference fragments with established repeat numbers. It is often not realized that sizing DNA fragments only yield relative data, because the electrophoretic mobility of a DNA fragment is influenced by multiple factors. Thus, sizing values alone are not suitable for exchange unless a careful calibration of the different platforms has been established. Such a calibration could consist of the generation of calibration curves as demonstrated by Pasqualotto et al. [22]. But the most straightforward method to achieve such a calibration is the use of allelic ladders. An allelic ladder consist of a mixture of well-defined alleles with predetermined repeat numbers.

Such allelic ladders have been introduced as reference tools in human forensic applications in the early 1990's [23, 24], but have not yet been described for calibration of microbial micosatellite based typing schemes.

In **chapter 6** allelic ladders were developed for the STR*Af* 3 marker. The reproducibility and compatibility of the STR*Af* assay was investigated in different settings, with the use of these ladders for calibration purposes. Five laboratories independently analyzed 18 samples that were provided either as DNA or as *A. fumigatus* conidia. All laboratories were allowed to use their own equipment. The results demonstrate that identical alleles, analyzed on different platforms with different size standards may differ up to 6.7 nucleotides. This results in interpretation errors up to 2 repeats units for the trinucleotide repeats. In contrast, with the use of the allelic ladders all participants were able to produce identical typing data for all the STR*Af* 3 markers in nearly all samples with a wide variety of experimental conditions.

In **chapter 7** the stability of the individual markers of the STR*Af* assay was investigated. Knowledge of the rate of repeat change is important when using STR*Af* as part of an outbreak investigation, in order to be able to interpret the relationship between isolates with different typing results. To investigate the dynamics of the STR*Af* markers, five unrelated *A. fumigatus* isolates were clonally expanded for 100 generations. All subcultures were generated from a single spore and analyzed with the nine STR*Af* loci. In total, five times a change in the number of repeat units was observed. All changes occurred in the trinucleotide repeats and four of the five alterations were found in markers with 50 or more repeat numbers. For the interpretation of the typing results this means that expansion of one repeat unit to a repeat consisting of five units may, for example, have a relative larger effect than adding a unit to a repeat that already contain 40 units.

Part 2: Clinical applications

A. fumigatus plays an important role in several clinical manifestations. Invasive aspergillosis, for example, is a life-threatening disease of immunocompromised patients. The incidence of IA has grown dramatically in recent years due to the increase in the number of patients undergoing bone marrow or solid organ transplantation [12, 16, 28]. The STR*Af* assay can be used to provide a better understanding of the uniqueness of each clinical isolate and of its dissemination route. Pulmonary infections are thought to arise by local tissue invasion, and eventually disseminate to other deep seated organs. In **chapter 3** the genetic relatedness between 55 isolates obtained from respiratory and tissue samples obtained from 15 patients with proven IA was examined. Within the patients all nonrespiratory isolates belonged to the same genotype. However, multiple genotypes were detected in isolates originated from respiratory samples from the same patient. These results are consistent with a scenario in which the respiratory tract may be colonized by several different genotypes, as inhalation of fungal conidia is assumed to be the first step towards infection, but where a single genotype gains supremacy and invades the surrounding tissue eventually disseminating to other organs. Besides comparing clinical isolates with each other, it is also important to compare patient isolates with isolates obtained from the environment, since evidence suggest that IA infections may be acquired from hospital environment [7, 18]. Therefore the STR*Af* assay was tested for its utility in outbreak settings in **chapter 8**, where it is critical to distinguish clonal clusters from genetically unrelated isolates. A panel of epidemiologically linked isolates obtained from 6 different outbreaks of IA were analyzed with the STR*Af* assay. These isolates have also been subjected to the RFLP method including the Afut1 DNA hybridization probe and to CSP-typing. Isolates from three of the six outbreaks showed no relation to each other, while in the three other outbreaks identical genotypes were found. In two of these outbreaks clinical and environmental isolates were of the same genotype. The results found with the STR*Af* assay were in good agreement with the Afut1-RFLP method.

Besides IA, *A. fumigatus* can also colonize the respiratory tract of patients with compromised airways [25]. The most common complication of *Aspergillus* colonization is allergic bronchopulmonary aspergillosis (ABPA), which can occur in children, adolescents and adults with cystic fibrosis (CF). In 57% of the CF patients the respiratory tract is colonized by *Aspergillus* species, 1 to 15% of these patients developed ABPA [1, 19, 27]. In **chapter 9** 204 *A. fumigatus* isolates obtained from 36 CF patients were typed, to gain more insight in the airway colonization by *A. fumigatus* in CF patients. The isolates were collected from sequential sputum samples over a period of 4 months till 9 ½ years. In general, four different colonization patterns can be discriminated in this collection of CF patients: i Continuous colonization patterns: all isolates consist of identical or related genotypes. ii Colonization patterns with a predominant genotype: most of the isolates are identical. iii Succeeding colonization patterns: isolates with identical genotypes that succeed each other. iv Unique patterns: all isolates within a patient have unique genotypes. Most patients have a colonization pattern with unique isolates (36%), the smallest group of patients (17%) have a continuous colonization pattern. A notable result in this study is, that at two patients identical isolates were found over a period longer than nine years. This also indicates that the STR*Af* markers are sufficiently stable for epidemiological questions.

The aim of the study described in **chapter 10** was to genotype *A. fumigatus* directly in formalin-fixed paraffin-embedded (FFPE) tissue and serum samples. This is desirable since *A. fumigatus* cultures are not available in all cases, because the tissue biopsy specimens are directly embedded into formalin fixative [17] and *A. fumigatus* is virtually never isolated from blood cultures [21]. The STR*Af* assay was performed on cultured isolates, tissue and serum samples of five patients. Genotypes were obtained from the lung biopsies and in one patient even in serum samples. This allows detection as well as genotyping of *A. fumigatus* directly on clinical samples without the need of cultivation.

In conclusion

In this thesis a novel assay for fingerprinting *A. fumigatus* is described and evaluated. This assay demonstrate several characteristics important for a good typing assay. Of major importance was the ability to discriminate between unrelated isolates. Typing results from epidemiological related collections showed identical genotypes and the results are in concordance with other high discriminatory typing techniques. Other advantages include the requirement of low amounts of DNA, and the automated sizing of amplification products result in the relatively easy interpretation of the data. The STR*Af* assay is also rapid and has a high throughput by multiplexing. The assay is robust, which makes it possible to successfully use the assay in other settings. Together with the use of allelic ladders it is even possible to exchange fingerprint typing data between laboratories, provided that they have access to high resolution electrophoresis platforms. In CF patients different colonization patterns were found with the STR*Af* assay. In IA patients the uniqueness of clinical isolate and the dissemination route are evaluated. With the option to genotype *A. fumigatus* directly in FFPE-tissue samples and serum samples, it is possible to retrospectively examine dissemination routes in IA patients without *A. fumigatus* cultures. In outbreak settings, where besides clinical isolates also environmental isolates were analyzed, variations in a single loci between epidemiological related isolates were found. The knowledge of the rate of change of the individual markers of the STR*Af* assay, may provide good guidelines for the interpretation of different STR*Af* typing patterns.

The high throughput of samples, the low costs, the robustness and the interlaboratory reproducibility together with the high discrimination power makes this assay suitable for fingerprinting large amount of isolates and exchange of results between labs worldwide.

References

1. Aufauvre-Brown, A., J. Cohen, and D. W. Holden. 1992. Use of randomly amplified polymorphic DNA markers to distinguish isolates of *Aspergillus fumigatus*. J Clin Microbiol 30:2991-3.
2. Bain, J. M., A. Tavanti, A. D. Davidson, M. D. Jacobsen, D. Shaw, N. A. Gow, and F. C. Odds. 2007. Multilocus sequence typing of the pathogenic fungus *Aspergillus fumigatus*. J Clin Microbiol 45:1469-77.
3. Balajee, S. A., S. T. Tay, B. A. Lasker, S. F. Hurst, and A. P. Rooney. 2007. Characterization of a novel gene for strain typing reveals substructuring of *Aspergillus fumigatus* across North America. Eukaryot Cell 6:1392-9.
4. Bart-Delabesse, E., J.-F. Humbert, E. Delabesse, and S. Bretagne. 1998. Microsatellite markers for typing *Aspergillus fumigatus* isolates. J Clin Microbiol 36:2413-2418.
5. Bart-Delabesse, E., J. Sarfati, J. P. Debeaupuis, W. van Leeuwen, A. van Belkum, S. Bretagne, and J. P. Latge. 2001. Comparison of restriction fragment length polymorphism, microsatellite length polymorphism, and random amplification of polymorphic DNA analyses for fingerprinting *Aspergillus fumigatus* isolates. J Clin Microbiol 39:2683-6.
6. Bertout, S., F. Renaud, R. Barton, F. Symoens, J. Burnod, M. A. Piens, B. Lebeau, M. A. Viviani, F. Chapuis, J. M. Bastide, R. Grillot, and M. Mallie. 2001. Genetic polymorphism of *Aspergillus fumigatus* in clinical samples from patients with invasive aspergillosis: investigation using multiple typing methods. J Clin Microbiol 39:1731-7.
7. Bretagne, S., E. Bart-Delabesse, J. Wechsler, M. Kuentz, N. Dhedin, and C. Cordonnier. 1997. Fatal primary cutaneous aspergillosis in a bone marrow transplant recipient: nosocomial acquisition in a laminar-air flow room. J Hosp Infect 36:235-9.
8. Chazalet, V., J. P. Debeaupuis, J. Sarfati, J. Lortholary, P. Ribaud, P. Shah, M. Cornet, H. Vu Thien, E. Gluckman, G. Brucker, and J. P. Latge. 1998. Molecular typing of environmental and patient isolates of *Aspergillus fumigatus* from various hospital settings. J Clin Microbiol 36:1494-500.
9. Cimon, B., F. Symoens, R. Zouhair, D. Chabasse, N. Nolard, A. Defontaine, and J. P. Bouchara. 2001. Molecular epidemiology of airway colonisation by *Aspergillus fumigatus* in cystic fibrosis patients. J Med Microbiol 50:367-74.
10. Debeaupuis, J. P., J. Sarfati, V. Chazalet, and J. P. Latge. 1997. Genetic diversity among clinical and environmental isolates of *Aspergillus fumigatus*. Infect Immun 65:3080-5.
11. Girardin, H., J. P. Latge, T. Srikantha, B. Morrow, and D. R. Soll. 1993. Development of DNA probes for fingerprinting *Aspergillus fumigatus*. J Clin Microbiol 31:1547-54.
12. Girardin, H., J. Sarfati, F. Traore, J. Dupouy Camet, F. Derouin, and J. P. Latge. 1994. Molecular epidemiology of nosocomial invasive aspergillosis. J Clin Microbiol 32:684-90.

13. Guarro, J., M. Sole, R. Castany, J. Cano, A. Teixido, I. Pujol, J. Gene, A. Castro, and P. Sarda. 2005. Use of random amplified microsatellites to type isolates from an outbreak of nosocomial aspergillosis in a general medical ward. Med Mycol 43:365-71.
14. Hunter, P. R., and M. A. Gaston. 1988. Numerical index of the discriminatory ability of typing systems: an application of Simpson's index of diversity. J Clin Microbiol 26:2465-6.
15. Lasker, B. A. 2002. Evaluation of performance of four genotypic methods for studying the genetic epidemiology of *Aspergillus fumigatus* isolates. J Clin Microbiol 40:2886-2892.
16. Latge, J.-P. 1999. *Aspergillus fumigatus* and aspergillosis. Clin Microbiol Rev 12:310-350.
17. Lau, A., S. Chen, T. Sorrell, D. Carter, R. Malik, P. Martin, and C. Halliday. 2007. Development and clinical application of a panfungal PCR assay to detect and identify fungal DNA in tissue specimens. J Clin Microbiol 45:380-5.
18. Leenders, A. C., A. van Belkum, M. Behrendt, A. Luijendijk, and H. A. Verbrugh. 1999. Density and molecular epidemiology of *Aspergillus* in air and relationship to outbreaks of *Aspergillus* infection. J Clin Microbiol 37:1752-7.
19. Mellado, E., T. M. Diaz-Guerra, M. Cuenca-Estrella, V. Buendia, J. Aspa, E. Prieto, J. R. Villagrasa, and J. L. Rodriguez-Tudela. 2000. Characterization of a possible nosocomial aspergillosis outbreak. Clin Microbiol Infect 6:543-8.
20. Neuveglise, C., J. Sarfati, J. Latge, and S. Paris. 1996. Afut1, a retrotransposon-like element from *Aspergillus fumigatus*. Nucl Acids Res 24:1428-1434.
21. O'Sullivan, C. E., M. Kasai, A. Francesconi, V. Petraitis, R. Petraitiene, A. M. Kelaher, A. A. Sarafandi, and T. J. Walsh. 2003. Development and validation of a quantitative real-time PCR assay using fluorescence resonance energy transfer technology for detection of *Aspergillus fumigatus* in experimental invasive pulmonary aspergillosis. J Clin Microbiol 41:5676-82.
22. Pasqualotto, A. C., D. W. Denning, and M. J. Anderson. 2007. A cautionary tale: Lack of consistency in allele sizes between two laboratories for a published multilocus microsatellite typing system. J Clin Microbiol 45:522-8.
23. Puers, C., H. A. Hammond, L. Jin, C. T. Caskey, and J. W. Schumm. 1993. Identification of repeat sequence heterogeneity at the polymorphic short tandem repeat locus HUMTH01[AATG]n and reassignment of alleles in population analysis by using a locus-specific allelic ladder. Am J Hum Genet 53:953-8.
24. Skowasch, K., P. Wiegand, and B. Brinkmann. 1992. pMCT 118 (D1S80): A new allelic ladder and an improved electrophoretic separation lead to the demonstration of 28 alleles. Int J Legal Med 105:165-8.
25. Stevens, D. A., R. B. Moss, V. P. Kurup, A. P. Knutsen, P. Greenberger, M. A. Judson, D. W. Denning, R. Crameri, A. S. Brody, M. Light, M. Skov, W. Maish, and G. Mastella. 2003. Allergic bronchopulmonary aspergillosis in cystic fibrosis--state of the art: Cystic Fibrosis Foundation Consensus Conference. Clin Infect Dis 37 Suppl 3:S225-64.

26. Symoens, F., J. Burnod, B. Lebeau, M. A. Viviani, M. A. Piens, A. M. Tortorano, N. Nolard, F. Chapuis, and R. Grillot. 2002. Hospital-acquired *Aspergillus fumigatus* infection: Can molecular typing methods identify an environmental source? J Hosp Infect 52:60-7.

27. Verweij, P. E., J. F. G. M. Meis, J. Sarfati, J. A. Hoogkamp-Korstanje, J. P. Latge, and W. J. Melchers. 1996. Genotypic characterization of sequential *Aspergillus fumigatus* isolates from patients with cystic fibrosis. J Clin Microbiol 34:2595-7.

28. Walsh, T. J., E. J. Anaissie, D. W. Denning, R. Herbrecht, D. P. Kontoyiannis, K. A. Marr, V. A. Morrison, B. H. Segal, W. J. Steinbach, D. A. Stevens, J. A. van Burik, J. R. Wingard, and T. F. Patterson. 2008. Treatment of aspergillosis: clinical practice guidelines of the Infectious Diseases Society of America. Clin Infect Dis 46:327-60.

Samenvatting

Aspergillus soorten zijn schimmels die wereldwijd veel voorkomen. Deze schimmels kunnen grote hoeveelheden sporen produceren die in de omgeving worden verspreid. Er zijn verschillende *Aspergillus* soorten beschreven die pathogeen blijken te zijn voor de mens. Van alle soorten is *Aspergillus fumigatus* het meest betrokken bij humane infecties. Om besmettingsroutes tussen omgeving en klinische isolaten beter in kaart te brengen zijn er veel verschillende moleculaire typeringsmethoden ontwikkeld. De genetische variabiliteit binnen *A. fumigatus* isolaten blijkt erg hoog te zijn, zodat typeringsmethoden een hoog discriminerend vermogen en een goede reproduceerbaarheid moeten hebben om ongerelateerde isolaten van elkaar te kunnen onderscheiden [8, 10]. De RFLP (Restriction Fragment Length Polymorphism) methode in combinatie met de Afut1 hybridisatie probe heeft een goed onderscheidend vermogen [11, 20]. De RFLP methode heeft een aantal nadelen als het gaat om het analyseren van grote hoeveelheden isolaten, omdat de techniek arbeidsintensief is en de complexe bandenpatronen die worden gegenereerd moeilijk te interpreteren zijn. In tegenstelling zijn typeringsmethoden waarbij sequentie data wordt verkregen, zoals MLST (Multilocus Sequence Typing)[2] en CSP (Cell Surface Protein) typing [3], 100% reproduceerbaar, waardoor data gemakkelijk kunnen worden uitgewisseld tussen verschillende laboratoria. Helaas hebben ook deze methoden te weinig discriminerend vermogen voor studies met grote hoeveelheden isolaten. Echter microsatellieten of short tandem repeats (STRs) laten een hoog discriminerend vermogen zien tussen verschillende isolaten uit een grote variëteit aan micro-organismen. Ze worden ook veel gebruikt als hoge resolutie *fingerprinting*-techniek voor het humane genoom. Bart-Delabesse et al. [4] gebruikte als eerste STRs voor het typeren van *A. fumigatus*, de zogenoemde MLP methode (Microsatellite Length Polymorphism). Aangezien er toentertijd geen genomische sequenties van *A. fumigatus* beschikbaar waren, werden zo´n 17.000 klonen gescreend met een CA_{10} oligonucleotide probe. Vier CA-microsatellieten merkers werden geselecteerd voor verdere analyse. Het onderscheidende vermogen van deze vier merkers was vergelijkbaar met de RFLP [5, 15]. STR analyses resulteren in goed reproduceerbare en exacte typeringsresultaten, die makkelijk uitwisselbaar zijn.

In twee MLP merkers zit een tweede repeat sequentie naast de CA-repeat. Hierdoor kunnen fragmenten van dezelfde lengte verschillende combinaties van deze twee repeat sequenties bevatten. Het wordt dan onmogelijk om op basis van de fragment grootte het precieze aantal repeats te bepalen, waardoor het exacte karakter van de techniek verloren gaat. Daarom wordt in **hoofdstuk 2** voor *A. fumigatus* een nieuwe typeringsmethode beschreven, de zogenoemde STR*Af* methode, gebaseerd op negen short tandem repeats voor exacte en hoge resolutie typering. Deze negen merkers zijn geselecteerd uit grote delen van genomische *A. fumigatus* sequenties die op dat moment bekend waren. Het zoeken naar STRs is sterk vereenvoudigd doordat genomische sequenties ter beschikking zijn gekomen. Er zijn drie multiplex PCRs ontwikkeld voor respectievelijk drie di-, tri- en tetranucleotide merkers. Met de STR*Af* methode was het mogelijk om ongerelateerde isolaten van elkaar te onderscheiden. De *Simpson's diversity index* [14] voor de negen merkers is 0.9994, hetgeen aangeeft dat het discriminerende vermogen erg hoog is. In theorie is het zelfs mogelijk om met deze negen merkers 3×10^{13} verschillende genotypen van elkaar te onderscheiden. Dit is een 10^6-voudige verbetering ten opzichte van de eerder genoemde MLP methode [4].

In de volgende onderdelen van dit proefschrift worden naast de technische aspecten van de STR*Af* methode ook de klinische toepassingen van deze methode beschreven.

Deel 1: Technische aspecten

In **hoofdstuk 3** wordt de STR*Af* methode vergeleken met de AFLP (Amplified Fragment Length Polymorphism) methode, een andere goed discriminerende moleculaire *fingerprint*-techniek. In totaal worden 55 *A. fumigatus* isolaten, verkregen van 15 verschillende patiënten met invasieve aspergillosis, geanalyseerd met zowel de STR*Af* als de AFLP methode. Deze isolaten zijn ook getest met een nieuwe methode, namelijk de RISC (Retrotransposon Insertion-Site Context)-typering. Deze techniek staat in detail beschreven in **hoofdstuk 4**. Over het algemeen was er een zeer goede overeenkomst van de resultaten van de STR*Af*, RISC en AFLP methode.

De verschillen die zijn gevonden waren niet geheel onverwacht omdat elke methode een ander deel van het genoom analyseert. Een duidelijk voordeel van de STR*Af* methode vergeleken met de AFLP en de RISC methoden is de mogelijkheid om mengsels van meerdere isolaten te identificeren. Het is gebleken dat klinische monsters uit de luchtwegen en omgevingsmonsters meerdere verschillende genotypen kunnen bevatten [6, 9, 13, 26]. Een ander verschil tussen de drie typeringsmethoden is de interpretatie van de resultaten. De bepaling van de grootte van een PCR product voor de STR*Af* methode is geautomatiseerd. Aan de hand van de grootte van een fragment worden het aantal kopieën van de STR merker bepaald. Een genotype bestaat uit negen getallen, die gemakkelijk te interpreteren zijn. De AFLP en de RISC resultaten bestaan uit complexe bandenpatronen met meerdere sterke en zwakke banden. Om de genetische relatie tussen meerdere isolaten te kunnen bepalen, moet er gebruik worden gemaakt van speciale computer software met patroon gebaseerde algoritmen. Met deze algoritmen worden op het oog identieke bandenpatronen nooit als 100% gelijk berekend. Een goede reproduceerbaarheid op de lange termijn en het vergelijken van resultaten tussen verschillende experimenten binnen een laboratorium is voor de AFLP en RISC methode moeilijk haalbaar, zoniet onmogelijk. Dit wordt veroorzaakt doordat kleine verschillen in de AFLP en RISC methode ontstaan door geringe variaties tijdens de restrictie/ligatie stap, gedurende de PCR amplificatie en de analyse van de PCR producten. De reproduceerbaarheid van de STR*Af* methode binnen een laboratorium daarentegen is 100%. De reproduceerbaarheid moet goed zijn om een methode succesvol te kunnen gebruiken in een ander laboratorium. In vergelijking met de AFLP en de RISC methode lijkt de STR*Af* methode hiervoor het meest geschikt. De robuustheid van de STR*Af* methode is onderzocht in **hoofdstuk 5** door de amplificatie condities van de methode te variëren. Hier is gekeken wat het effect is van het stapsgewijs variëren van verschillende PCR reactie componenten en thermocycler parameters. Extreme condities zorgde ervoor dat het signaal verdween, echter als er een signaal verkregen werd, dan waren de resultaten identiek. Dit betekent dat de STR*Af* methode zeer robuust is en dat deze zonder problemen in andere instellingen gebruikt zou moeten kunnen worden.

Een valkuil voor het uitwisselen van STR*Af* data tussen verschillende laboratoria wordt veroorzaakt door de toekenning van het aantal kopieën aan het PCR product. Normaal gesproken wordt het DNA fragment vergeleken met de lengte van een referentie product waarvan het aantal kopieën bekend is. Vaak realiseert men zich niet dat de lengte van een fragment alleen relatieve data oplevert, omdat de electroforetische mobiliteit van een DNA fragment beinvloedt wordt door vele factoren. Dus het bepalen van de lengte van PCR fragmenten alleen is niet voldoende voor uitwisseling, tenzij de verschillende electroforese platforms gekalibreerd worden. Een dergelijke kalibratie kan tot stand komen door het maken van kalibratie curven [22]. De beste manier om platforms te kalibreren is echter door gebruik te maken van allelische ladders. Een allelische ladder bestaat uit een mix van goed gedefinieerde allelen waarvan het aantal STR kopieën vooraf is bepaald. Deze allelische ladders zijn in de vroege jaren '90 als referentie middel geïntroduceerd in humane forensische bepalingen [23, 24], maar zijn nog niet eerder beschreven op microsatellieten gebaseerde microbiële typeringsmethoden.

Hoofdstuk 6 beschrijft de ontwikkeling van de allelische ladders voor de STR*Af* 3 merkers. De reproduceerbaarheid en de toepasbaarheid van de STR*Af* methode is getest in verschillende instellingen waarbij gebruik gemaakt is van allelische ladders. Vijf laboratoria hebben onafhankelijk van elkaar 18 monsters geanalyseerd, die toegestuurd zijn als DNA of als *A. fumigatus* sporen. Alle laboratoria hebben gebruik gemaakt van hun eigen apparatuur. De resultaten laten zien dat identieke allelen, geanalyseerd op verschillende platforms met verschillende interne standaarden een verschil kunnen opleveren tot 6,7 nucleotiden. Dit zou een interpretatiefout opleveren van meer dan 2 kopieën voor de trinucleotide repeats. Echter met het gebruik van de allelische ladders was het voor alle deelnemers mogelijk om identieke resultaten te genereren voor alle STR*Af* 3 merkers in bijna alle monsters met een grote verscheidenheid aan experimentele condities.

In **hoofdstuk 7** wordt de stabiliteit van de individuele markers onderzocht. Het is belangrijk om te weten hoe snel een repeat verandert wanneer de STR*Af* methode ingezet wordt om een uitbraak te onderzoeken, zodat het mogelijk is de relatie tussen isolaten met verschillende genotypen goed te interpreteren.

Om de veranderingen van de STR*Af* merkers te onderzoeken, zijn vijf ongerelateerde *A. fumigatus* isolaten 100 keer overgeënt. Alle subculturen zijn verkregen vanuit een enkele spore en geanalyseerd met de negen STR*Af* merkers. In totaal, zijn er vijf veranderingen opgetreden in het aantal kopieën. Alle veranderingen kwamen voor in de trinucleotide repeats en vier van de vijf veranderingen zijn gevonden in merkers met 50 of meer kopieën. Voor de interpretatie van typeringsresultaten betekent dit dat een verandering van één kopie in een merker met vijf kopieën een relatief groter effect heeft dan een toevoeging van een repeat aan een merker met 40 kopieën.

Deel 2: Klinische toepassingen

A. fumigatus speelt een belangrijke rol in meerdere klinische ziektebeelden. Invasieve aspergillosis (IA), bijvoorbeeld, is een levensbedreigende ziekte bij immuun-gecompromitteerde patiënten. Met de toename van het aantal patiënten met een beenmerg- of orgaantransplantatie, is ook het aantal patiënten met IA dramatisch gestegen [12, 16, 28]. De STR*Af* methode kan ingezet worden om een beter inzicht te krijgen in hoe uniek elk klinisch isolaat is en op welke wijze verspreidingsroute gaat. Van luchtweginfecties wordt gedacht dat deze dissemineren door lokale ingroei in het weefsel, waarna er een verdere verspreiding ontstaat naar dieper gelegen organen. In **hoofdstuk 3** wordt de genetische relatie van 55 isolaten bekeken. Dezw isolaten zijn afkomstig uit luchtweg en weefsel materialen van 15 patiënten met bewezen IA. Alle isolaten binnen een patiënt, die niet afkomstig zijn uit de luchtwegen, behoren tot hetzelfde genotype. Echter, als bij één patiënt meerdere genotypen gevonden zijn, zijn deze isolaten allemaal gekweekt uit respiratoire monsters. Deze resultaten tonen aan dat de luchtwegen gekoloniseerd zijn met meerdere verschillende genotypen, als gevolg van inhalatie van schimmelsporen, waarvan één enkel genotype dominant wordt en de omliggende weefsels binnendringt en zich uiteindelijk verspreidt naar andere organen.

Naast de vergelijking van klinische isolaten met elkaar, is het ook belangrijk om deze isolaten te vergelijken met isolaten verkregen uit de omgeving, ook omdat er gedacht werd dat IA infecties kunnen ontstaan uit de ziekenhuis omgeving [7, 18]. Daarom wordt in **hoofdstuk 8** gekeken wat het nut is van de STR*Af* methode in uitbraak situaties, waar het van belang is om klonale clusters te onderscheiden van genetisch ongerelateerde isolaten. Een set van epidemiologisch gerelateerde isolaten, verkregen uit 6 verschillende IA uitbraken, werden geanalyseerd met de STR*Af* methode. Deze isolaten zijn ook getest met de RFLP methode inclusief de Afut1 hybridisatie probe en met CSP typering. Isolaten afkomstig van drie van de zes uitbraken hebben geen relatie met elkaar, terwijl in de andere drie uitbraken identieke genotypen zijn gevonden. In twee van deze uitbraken zijn identieke genotypen gevonden in klinische- en omgevingsisolaten. De resultaten verkregen met de STR*Af* methode komen goed overeen met de Afut1-RFLP methode.

Naast het veroorzaken van IA, kan *A. fumigatus* ook de luchtwegen van patiënten met gecompromitteerde luchtwegen koloniseren [25]. De meest voorkomende complicatie van *Aspergillus* kolonisatie is allergische bronchopulmonale aspergillose (ABPA), hetgeen voorkomt bij kinderen, ouderen en volwassenen met cystic fibrosis (CF). Bij 57% van de CF patiënten zijn de luchtwegen gekoloniseerd met *Aspergillus* soorten en 1 tot 15% van deze patiënten ontwikkelen ABPA [1, 19, 27]. In **hoofdstuk 9** worden 204 isolaten verkregen van 36 CF patiënten getypeerd om meer inzicht te krijgen in de luchtweg kolonisatie van *A. fumigatus* bij CF patiënten. De isolaten zijn verzameld uit opeenvolgende sputum monsters over een periode van 4 maanden tot 9 ½ jaar. Over het algemeen kunnen er vier verschillende kolonisatie patronen onderscheiden worden in deze verzameling van CF patiënten namelijk: i Continue kolonisatie patroon: alle isolaten bestaan uit identieke of gerelateerde genotypen. ii Kolonisatie patroon met een overheersend genotype: bijna alle isolaten zijn identiek. iii Opeenvolgend kolonisatie patroon: isolaten met identieke genotypen wisselen elkaar af. iv Unieke patronen: alle isolaten binnen een patiënt hebben unieke genotypen. De meeste patiënten hebben een uniek kolonisatie patroon (36%), de kleinste patiënten groep heeft een continue kolonisatie patroon (17%).

Een opmerkelijk resultaat uit deze studie is dat bij twee patiënten identieke isolaten zijn gevonden over een periode langer dan 9 jaar. Dit laat tevens zien dat de STR*Af* merkers voldoende stabiel zijn voor epidemiologische vraagstukken.

Het doel van de studie beschreven in **hoofdstuk 10** was om *A. fumigatus* direct in formaline gefixeerde paraffine (FFPE) weefsels en sera monsters te typeren. Dit is van belang omdat *A. fumigatus* kweken niet altijd beschikbaar zijn, omdat weefsel biopten direct gefixeerd worden in formaline [17] en omdat *A. fumigatus* vrijwel nooit wordt geïsoleerd uit bloed [21]. De STR*Af* methode is ingezet om gekweekte isolaten, weefsel- en serummonsters van vijf patiënten te analyseren. In alle long weefsels was het mogelijk een genotype te genereren en in één patiënt was het zelfs mogelijk een genotype in serum aan te tonen. Dit maakt het mogelijk om *A. fumigatus* te detecteren en te typeren in klinische materialen, zonder deze schimmel eerst te kweken.

Conclusie

In dit proefschrift wordt een nieuwe methode, namelijk de STR*Af*, beschreven voor de typering van *A. fumigatus* en geëvalueerd. Deze methode laat verschillende karaktereigenschappen zien die evident zijn voor een goede typeringsmethode. Het belangrijkste is dat deze methode ongerelateerde isolaten van elkaar kan onderscheiden. Typeringsresultaten van epidemiologisch gerelateerde verzamelingen laten identieke genotypen zien en de resultaten komen overeen met andere goed discriminerende typeringstechnieken. Andere voordelen van deze methode zijn de lage hoeveelheid DNA die nodig is, het automatisch toekennen van lengtes aan PCR producten en het relatief gemakkelijk interpreteren van de data. De STR*Af* methode is snel en er kunnen veel monsters tegelijk geanalyseerd worden doordat de PCRs bestaan uit multiplex reacties. Samen met het gebruik van allelische ladders is het zelfs mogelijk om data uit te wisselen met andere labs, mits deze de beschikking hebben over een hoog resolutie electroforese platform. Met de STR*Af* methode worden verschillende kolonisatiepatronen gevonden bij CF patiënten. Bij IA patiënten is gekeken hoe uniek isolaten zijn en naar de verspreiding van isolaten binnen een patiënt.

Met de mogelijkheid om *A. fumigatus* direct in FFPE-weefsels en sera monsters te typeren, kan met terugwerkende kracht de verspreiding van isolaten in IA patiënten onderzocht worden, zonder dat een stam gekweekt is. In uitbraak situaties, waar naast klinische isolaten ook omgevingsisolaten worden geanalyseerd, worden tussen epidemiologische verwante isolaten variaties gevonden in een enkele merker. Door inzicht te krijgen in de mate van verandering van individuele merkers van de STR*Af* methode, kunnen goede richtlijnen opgesteld worden voor de interpretatie van verschillende STR*Af* typeringsresultaten.

Door de lage kosten, de robuustheid en de reproduceerbaarheid tussen verschillende instellingen, in combinatie met het hoge onderscheidend vermogen van deze methode is het mogelijk om grote hoeveelheden isolaten te analyseren en data uit te wisselen tussen verschillende laboratoria over de hele wereld.

Referenties

1. Aufauvre-Brown, A., J. Cohen, and D. W. Holden. 1992. Use of randomly amplified polymorphic DNA markers to distinguish isolates of *Aspergillus fumigatus*. J Clin Microbiol 30:2991-3.
2. Bain, J. M., A. Tavanti, A. D. Davidson, M. D. Jacobsen, D. Shaw, N. A. Gow, and F. C. Odds. 2007. Multilocus sequence typing of the pathogenic fungus *Aspergillus fumigatus*. J Clin Microbiol 45:1469-77.
3. Balajee, S. A., S. T. Tay, B. A. Lasker, S. F. Hurst, and A. P. Rooney. 2007. Characterization of a novel gene for strain typing reveals substructuring of *Aspergillus fumigatus* across North America. Eukaryot Cell 6:1392-9.
4. Bart-Delabesse, E., J.-F. Humbert, E. Delabesse, and S. Bretagne. 1998. Microsatellite markers for typing *Aspergillus fumigatus* isolates. J Clin Microbiol 36:2413-2418.
5. Bart-Delabesse, E., J. Sarfati, J. P. Debeaupuis, W. van Leeuwen, A. van Belkum, S. Bretagne, and J. P. Latge. 2001. Comparison of restriction fragment length polymorphism, microsatellite length polymorphism, and random amplification of polymorphic DNA analyses for fingerprinting *Aspergillus fumigatus* isolates. J Clin Microbiol 39:2683-6.
6. Bertout, S., F. Renaud, R. Barton, F. Symoens, J. Burnod, M. A. Piens, B. Lebeau, M. A. Viviani, F. Chapuis, J. M. Bastide, R. Grillot, and M. Mallie. 2001. Genetic polymorphism of *Aspergillus fumigatus* in clinical samples from patients with invasive aspergillosis: investigation using multiple typing methods. J Clin Microbiol 39:1731-7.
7. Bretagne, S., E. Bart-Delabesse, J. Wechsler, M. Kuentz, N. Dhedin, and C. Cordonnier. 1997. Fatal primary cutaneous aspergillosis in a bone marrow transplant recipient: nosocomial acquisition in a laminar-air flow room. J Hosp Infect 36:235-9.
8. Chazalet, V., J. P. Debeaupuis, J. Sarfati, J. Lortholary, P. Ribaud, P. Shah, M. Cornet, H. Vu Thien, E. Gluckman, G. Brucker, and J. P. Latge. 1998. Molecular typing of environmental and patient isolates of *Aspergillus fumigatus* from various hospital settings. J Clin Microbiol 36:1494-500.
9. Cimon, B., F. Symoens, R. Zouhair, D. Chabasse, N. Nolard, A. Defontaine, and J. P. Bouchara. 2001. Molecular epidemiology of airway colonisation by *Aspergillus fumigatus* in cystic fibrosis patients. J Med Microbiol 50:367-74.
10. Debeaupuis, J. P., J. Sarfati, V. Chazalet, and J. P. Latge. 1997. Genetic diversity among clinical and environmental isolates of *Aspergillus fumigatus*. Infect Immun 65:3080-5.
11. Girardin, H., J. P. Latge, T. Srikantha, B. Morrow, and D. R. Soll. 1993. Development of DNA probes for fingerprinting *Aspergillus fumigatus*. J Clin Microbiol 31:1547-54.
12. Girardin, H., J. Sarfati, F. Traore, J. Dupouy Camet, F. Derouin, and J. P. Latge. 1994. Molecular epidemiology of nosocomial invasive aspergillosis. J Clin Microbiol 32:684-90.

13. Guarro, J., M. Sole, R. Castany, J. Cano, A. Teixido, I. Pujol, J. Gene, A. Castro, and P. Sarda. 2005. Use of random amplified microsatellites to type isolates from an outbreak of nosocomial aspergillosis in a general medical ward. Med Mycol 43:365-71.
14. Hunter, P. R., and M. A. Gaston. 1988. Numerical index of the discriminatory ability of typing systems: an application of Simpson's index of diversity. J Clin Microbiol 26:2465-6.
15. Lasker, B. A. 2002. Evaluation of performance of four genotypic methods for studying the genetic epidemiology of *Aspergillus fumigatus* isolates. J Clin Microbiol 40:2886-2892.
16. Latge, J.-P. 1999. *Aspergillus fumigatus* and aspergillosis. Clin Microbiol Rev 12:310-350.
17. Lau, A., S. Chen, T. Sorrell, D. Carter, R. Malik, P. Martin, and C. Halliday. 2007. Development and clinical application of a panfungal PCR assay to detect and identify fungal DNA in tissue specimens. J Clin Microbiol 45:380-5.
18. Leenders, A. C., A. van Belkum, M. Behrendt, A. Luijendijk, and H. A. Verbrugh. 1999. Density and molecular epidemiology of *Aspergillus* in air and relationship to outbreaks of *Aspergillus* infection. J Clin Microbiol 37:1752-7.
19. Mellado, E., T. M. Diaz-Guerra, M. Cuenca-Estrella, V. Buendia, J. Aspa, E. Prieto, J. R. Villagrasa, and J. L. Rodriguez-Tudela. 2000. Characterization of a possible nosocomial aspergillosis outbreak. Clin Microbiol Infect 6:543-8.
20. Neuveglise, C., J. Sarfati, J. Latge, and S. Paris. 1996. Afut1, a retrotransposon-like element from *Aspergillus fumigatus*. Nucl Acids Res 24:1428-1434.
21. O'Sullivan, C. E., M. Kasai, A. Francesconi, V. Petraitis, R. Petraitiene, A. M. Kelaher, A. A. Sarafandi, and T. J. Walsh. 2003. Development and validation of a quantitative real-time PCR assay using fluorescence resonance energy transfer technology for detection of *Aspergillus fumigatus* in experimental invasive pulmonary aspergillosis. J Clin Microbiol 41:5676-82.
22. Pasqualotto, A. C., D. W. Denning, and M. J. Anderson. 2007. A cautionary tale: Lack of consistency in allele sizes between two laboratories for a published multilocus microsatellite typing system. J Clin Microbiol 45:522-8.
23. Puers, C., H. A. Hammond, L. Jin, C. T. Caskey, and J. W. Schumm. 1993. Identification of repeat sequence heterogeneity at the polymorphic short tandem repeat locus HUMTH01[AATG]n and reassignment of alleles in population analysis by using a locus-specific allelic ladder. Am J Hum Genet 53:953-8.
24. Skowasch, K., P. Wiegand, and B. Brinkmann. 1992. pMCT 118 (D1S80): A new allelic ladder and an improved electrophoretic separation lead to the demonstration of 28 alleles. Int J Legal Med 105:165-8.
25. Stevens, D. A., R. B. Moss, V. P. Kurup, A. P. Knutsen, P. Greenberger, M. A. Judson, D. W. Denning, R. Crameri, A. S. Brody, M. Light, M. Skov, W. Maish, and G. Mastella. 2003. Allergic bronchopulmonary aspergillosis in cystic fibrosis--state of the art: Cystic Fibrosis Foundation Consensus Conference. Clin Infect Dis 37 Suppl 3:S225-64.

26. Symoens, F., J. Burnod, B. Lebeau, M. A. Viviani, M. A. Piens, A. M. Tortorano, N. Nolard, F. Chapuis, and R. Grillot. 2002. Hospital-acquired *Aspergillus fumigatus* infection: Can molecular typing methods identify an environmental source? J Hosp Infect 52:60-7.

27. Verweij, P. E., J. F. G. M. Meis, J. Sarfati, J. A. Hoogkamp-Korstanje, J. P. Latge, and W. J. Melchers. 1996. Genotypic characterization of sequential *Aspergillus fumigatus* isolates from patients with cystic fibrosis. J Clin Microbiol 34:2595-7.

28. Walsh, T. J., E. J. Anaissie, D. W. Denning, R. Herbrecht, D. P. Kontoyiannis, K. A. Marr, V. A. Morrison, B. H. Segal, W. J. Steinbach, D. A. Stevens, J. A. van Burik, J. R. Wingard, and T. F. Patterson. 2008. Treatment of aspergillosis: clinical practice guidelines of the Infectious Diseases Society of America. Clin Infect Dis 46:327-60.

Dankwoord

Het tot stand komen van dit proefschrift is mede de verdienste, van een heel team van collega´s, van vrienden en familieleden.

Allereerst mijn co-promotores dr. C.H.W. Klaassen en dr. J.F.G.M. Meis. Corné, jouw enthousiasme en betrokkenheid bij dit onderzoek waardeer ik enorm. Van jouw kennis van de moleculaire biologie heb ik ontzettend veel geleerd en kan ik nog steeds veel leren. De discussies die we gevoerd hebben de afgelopen jaren werkte zeer stimulerend. Jacques, jouw kritische blik en jouw wetenschappelijk inzicht zijn bijzonder waardevol gebleken. Tijdens de laatste fase van mijn promotie heb je me enorm gestimuleerd om alles afgerond te krijgen, daar ben ik je dankbaar voor.

Mijn promotor prof dr. A. Voss; ik ben je dankbaar voor de belangstelling tijdens mijn onderzoek. Tevens wil ik jou en verder dr. A.M. Horrevorts, dr. J.W. Mouton en dr. A. Dofferhoff bedanken voor de geboden mogelijkheid om te promoveren. Jullie hebben een belangrijke bijdrage geleverd, met name op de donderdagochtenden tijdens het innovatie-overleg.

Mijn paranimfen Maaike de Ruiter en Ilse Curfs. Maaike, we kennen elkaar al meer dan 10 jaar. Jij hebt me geholpen bij het nemen van de beslissing om wel of niet te starten met dit onderzoek. Ik ben je dankbaar dat je al die tijd voor me hebt klaargestaan, ook buiten de werktijden. Ik vind het fijn dat je mijn paranimf bent. Ilse, je hebt me enorm geholpen bij het aanenten en registreren van isolaten, het overzetten van de generaties en nog veel meer, nooit was het teveel voor jou. Daarnaast was het ontzettend gezellig en leerzaam om samen met jou naar de *Aspergillus* meeting in Miami te gaan.

Corina en Kristel, vanaf het 2^{e} jaar van het HLO kennen we elkaar. Na het HLO zijn we alledrie in het CWZ beland. Het is fijn om met jullie te werken en wekelijks bij te kletsen. Corina, ik wil je ontzettend bedanken voor het ontwerp van de omslag.

Antoinette, jij bent de allereerste geweest die me kennis heeft laten maken met de wondere wereld van de moleculaire biologie door mij in 1998 te begeleiden bij mijn afstudeeropdracht. Daarna heb je me over weten te halen de bacteriologie achter me te laten en me te richten op de moleculaire biologie. Wat hebben we veel meegemaakt,

samen met Maaike achter het schotje. Ik vind het dan ook erg leuk dat je weer terug bent in het CWZ.

Jeroen, Kim, Sandra en Debby, als collega's van de moleculaire biologie hebben jullie gedurende de afgelopen jaren het onderzoeksproces van dichtbij meegemaakt. Het was voor jullie meer dan vanzelfsprekend dat ik van bepaalde werkzaamheden vrijgeroosterd werd, bedankt daarvoor. Jitske, als stagiaire heb je veel werk verzet voor dit onderzoek, ik ben blij dat je je plek hebt gevonden op het laboratorium kindergeneeskunde en neurologie van het UMCN St. Radboud in Nijmegen.

Aan de overige medewerkers van de afdeling medische microbiologie en infectieziekten van het Canisius Wilhelmina Ziekenhuis dank voor jullie belangstelling, die jullie getoond hebben tijdens mijn onderzoek. Dit geldt ook voor mijn collega's van de klinische chemie en pathologie.

Tot slot wil ik mijn familie en vrienden bedanken voor hun interesse en vriendschap tijdens mijn onderzoek. Pap, mam, Grad en Riek, wij zijn ontzettend dankbaar dat jullie altijd voor ons klaarstaan en voor jullie onvoorwaardelijke steun. Gerwin, zonder jou was het nooit gelukt! Inge en Bas, mama heeft de laatste bladzijde van het proefschrift echt af!

List of Publications

1. **de Valk HA**, Curfs IM, de Ruiter MT, Meis JFGM and Klaassen CHW. 2008. Stability of microsatellite markers in *Aspergillus fumigatus. Submitted*

2. **de Valk HA**, Klaassen CHW, Yntema J, Hebestreit A, Seidler M, Haase G, Müller F and Meis JFGM. 2008. Molecular typing and colonization patterns of *Aspergillus fumigatus* in patients with cystic fibrosis. *Submitted*

3. **de Valk HA**, de Kievit IM, Klaassen CHW and Meis JFGM. 2008. Detection and direct genotyping of *Aspergillus fumigatus* in tissue and serum samples in patients with invasive aspergillosis. *Submitted*

4. **de Valk HA**, Meis JFGM, Bretagne S, Costa J, Lasker BA, Balajee SA, Pasqualotto AC, Anderson MJ, Alcázar-Fuoli L, Mellado E and Klaassen CHW. 2008. Interlaboratory reproducibility of a microsatellite based typing assay for *Aspergillus fumigatus* through the use of allelic ladders: Proof of concept. Clin Microbiol Infect, *in press*

5. **de Valk HA**, Meis JFGM and Klaassen CHW. 2008. Molecular typing of *Aspergillus* species. Mycoses Digital published doi: 10.1111/j.1439-0507.2008.01538.x

6. Balajee SA, **de Valk HA**, Lasker BA, Meis JFGM, Klaassen CHW. 2008. Utility of a microsatellite assay for identifying clonally related outbreak isolates of *Aspergillus fumigatus*. J Microbiol Methods 73:252-6.

7. de Ruiter MT, **de Valk HA**, Meis JFGM, Klaassen CHW. 2007. Retrotransposon insertion-site context (RISC) typing: A novel typing method for *Aspergillus fumigatus* and a convenient PCR alternative to restriction fragment length polymorphism analysis. J Microbiol Methods 70:528-34.

8. Prinsen CF, Fles R, Wijnen-Dubbers CW, **de Valk HA**, Klaassen CHW, Mravunac M, Horrevorts AM, Thunnissen FB. 2007. Baseline human papillomavirus status of women with abnormal smears in cervical screening: a 5-year follow-up study in The Netherlands. Br J Obstet Gynaecol 114:951-7.

9. **de Valk HA**, Meis JFGM, de Pauw BE, Donnelly PJ, Klaassen CHW. 2007. Comparison of two highly discriminatory molecular fingerprinting assays for analysis of multiple *Aspergillus fumigatus* isolates from patients with invasive aspergillosis. J Clin Microbiol 45:1415-9.

10. **de Valk HA**, Meis JFGM, Klaassen CHW. 2007. Microsatellite based typing of *Aspergillus fumigatus*: strengths, pitfalls and solutions. J Microbiol Methods 69:268-72.

11. **de Valk HA**, Meis JFGM, Curfs IM, Muehlethaler K, Mouton JW, Klaassen CHW. 2005. Use of a novel panel of nine short tandem repeats for exact and high-resolution fingerprinting of *Aspergillus fumigatus* isolates. J Clin Microbiol 43:4112-20.

12. **de Valk HA,** Meis JFGM en CHW Klaassen. 2005. Moleculaire genotypering van *Aspergillus fumigatus*-isolaten. Ned Tijdschr Med Microbiol 2:40-43.

13. Neeleman C, **de Valk JA**, Klaassen CH, Meijers S, Mouton JW. 2005. In-vitro susceptibility and molecular characterisation of macrolide resistance mechanisms among *Streptococcus pneumonia* isolates in The Netherlands: the DUEL 2 study. Clin Microbiol Infect 2005 11:312-8.

14. Klomberg DM, **de Valk HA**, Mouton JW, Klaassen CHW. 2005. Rapid and reliable real-time PCR assay for detection of the macrolide efflux gene and subsequent discrimination between its distinct subclasses mef(A) and mef(E). J Microbiol Methods 60:269-73.

15. Neeleman C, Klaassen CHW, Klomberg DM, **de Valk HA**, Mouton JW. 2004. Pneumolysin is a key factor in misidentification of macrolide-resistant *Streptococcus pneumoniae* and is a putative virulence factor of *S. mitis* and other streptococci. J Clin Microbiol 42:4355-7.

16. Klaassen CHW, Prinsen CF, **de Valk HA**, Horrevorts AM, Jeunink MA, Thunnissen FB. 2004. DNA microarray format for detection and subtyping of human papillomavirus. J Clin Microbiol 42:2152-60.

17. Neeleman C, Klaassen CHW, **de Valk HA**, de Ruiter MT, Mouton JW. 2004. Amplified fragment length polymorphism fingerprinting is an effective technique to distinguish *Streptococcus pneumoniae* from other Streptococci and an efficient alternative to pulsed-field gel electrophoresis for molecular typing of pneumococci. J Clin Microbiol 42:369-71.

18. Klaassen CHW, **de Valk HA**, Horrevorts AM. 2003. Clinical *Staphylococcus aureus* isolate negative for the Sa442 fragment. J Clin Microbiol 41:4493.

19. Warris A, Klaassen CHW, Meis JFGM, de Ruiter MT, **de Valk HA**, Abrahamsen TG, Gaustad P, Verweij PE. 2003. Molecular epidemiology of *Aspergillus fumigatus* isolates recovered from water, air, and patients shows two clusters of genetically distinct strains. J Clin Microbiol 41:4101-6.

20. Klaassen CHW, **van Haren HA**, Horrevorts AM. 2002. Molecular fingerprinting of *Clostridium difficile* isolates: pulsed-field gel electrophoresis versus amplified fragment length polymorphism. J Clin Microbiol 40:101-4.

Curriculum vitae

Hanneke de Valk-van Haren werd geboren op 6 december 1976 te Nijmegen. In 1994 behaalde zij haar HAVO diploma op scholengemeenschap “de Griend” te Grave. Datzelfde jaar startte zij met de studie Medische Microbiologie aan de Hogere Laboratoriumopleidingen in Nijmegen, welke in 1998 met goed resultaat werd afgerond. Vanaf die tijd was zij werkzaam op de moleculair biologische unit van de afdeling Medische Microbiologie en Infectieziekten van het Canisius Wilhelmina Ziekenhuis te Nijmegen. In 2004 startte zij met het onderzoek dat tot het huidige proefschrift leidde, onder directe begeleiding van dr. C.H.W. Klaassen en dr. J.F.G.M. Meis. In 2007 ontving zij de Canisius wetenschapsprijs voor haar onderzoek.

Zij is getrouwd met Gerwin de Valk en moeder van Inge en Bas.