What can be learned from genotyping of fungi?

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Multiple genotyping studies have been carried out in order to clarify the epidemiology of fungal infections, more specifically to determine the sources, transmission routes, and colonization patterns of fungal isolates. In this review, the results obtained in genotyping investigations of *Aspergillus* isolates are summarized and discussed. Furthermore, we examine the epidemiologic studies of *Candida albicans*, *Exophiala dermatitidis* and *Scedosporium apiospermum* infections in patients with cystic fibrosis. Relative to *Aspergillus fumigatus*, colonization of the respiratory tract by multiple strains, and of deep organs by only a single strain were observed. On the other hand, the few studies which focused on other fungi isolated from patients with cystic fibrosis have suggested that colonization occurs primarily by a dominant genotype.

**Keywords** genotyping, *Aspergillus*, cystic fibrosis

Introduction

Typing is defined as the phenotypic and/or genetic analysis of isolates, below the species level, performed in order to generate strain/clone-specific fingerprints or datasets [1]. Typing methods can be based on phenotypic characteristics or can include direct or indirect analyses of differences in DNA sequences (genotyping). Because of their technical complexity, poor reproducibility and/or low discriminatory power, the use of phenotype-based methods is decreasing. More importantly, a phenotype does not always accurately reflect the genotype of a microorganism, and therefore may not provide a reliable and stable epidemiological marker [1–5].

Because of the growing incidence of fungal infections, typing methods of fungal isolates are increasingly being used. These methods can be employed to study the spread and population dynamics of fungi in clinical and environmental settings, at levels ranging from a single host to large-scale ecosystems. For example, the epidemiological relationship between clinical and environmental isolates can be analysed and hence, the significance of patient-to-patient transmission or the presence of an environmental source generating a cluster of infections can be studied. This understanding may lead to the design of better infection control procedures [1,6,7].

Typing is also useful in the study of colonization or infection of a single patient. The methods permit the differentiation between strains isolated only once and those able to persist in a patient. In this way, the factors involved in persistent colonization can be evaluated. Additionally, isolates retrieved from hosts with an increased susceptibility to colonization and/or infection can be typed in order to determine host risk factors. When typing, results of groups of strains that are either virulent or non-virulent can be compared, pathogenesis-related markers may be identified and ultimately translated into clinically relevant diagnostic targets [1,5].

Finally, some molecular typing methods are used to determine the population structure of a species. These studies provide valuable information concerning the evolution and diversification of the species. However, in this review, we will focus on the epidemiological conclusions that can be drawn from genotyping studies and not on the information concerning population structure [1,8–10].

In this presentation, the results obtained in genotyping studies of *Aspergillus fumigatus* and other *Aspergillus* species are discussed. In addition, the epidemiology of
Practical aspects of genotyping studies on fungal isolates

Isolates

An important prerequisite for conducting valuable typing studies is the availability of isolates for which there is clinical, epidemiological and demographical information [1]. For investigations focusing on the pathogenesis of a disease in a single patient, it is crucial that multiple isolates obtained from a single clinical sample be typed [11,12].

A marked difference between typing of fungal and bacterial isolates is that the former may be diploid, causing greater difficulty in the interpretation of genotyping data. For example, multilocus sequence typing (MLST) data may show two bases at the same variable site, indicative of the presence of two diploid alleles. Therefore, it is crucial to investigate the possibility of diploidism in the species being analysed [13].

Methods

Numerous typing methods such as amplified fragment length polymorphism (AFLP), MLST, pulsed-field gel electrophoresis (PFGE), random amplified polymorphic DNA (RAPD), restriction fragment length polymorphism (RFLP), retrotransposon insertion-site context (RISC), PCR-RFLP, repetitive-sequence-based PCR (rep-PCR), sequence-specific DNA primer (SSDP) and variable number of short tandem repeat (VNTR) typing, have been used for fungal genotyping. The principles of these methods are outlined in several reviews [1–6,12,14,15] and the different procedural steps are illustrated in Fig. 1. Additionally, detailed explanations of the principles of MLST and VNTR are found in the section in this review on the data obtained by genotyping A. fumigatus isolates.

Several criteria, including reproducibility, portability, ease of use, ease of interpretation, discriminatory power, cost and time to result, need to be considered when evaluating the performance of genotyping methods. While reproducibility pertains to the ability to obtain the same result after multiple analyses, portability refers to the possibility to exchange data between different laboratories. Finally, the discriminatory power of a method is its ability to assign a different type to two unrelated strains sampled randomly from the population of a given species [1,2,6,14,16]. In Table 1 an overview of the characteristics of different typing methods used for fungal genotyping is presented.

Results of genotyping studies on fungal isolates

Aspergillus species

An astonishing variety of diseases caused by Aspergillus species has been described. The clinical manifestation is primarily driven by the host response. Based on host immunocompetence, three main categories of disease can be described, i.e., invasive, allergic and saprobic, which lead to invasive aspergillosis (IA), allergic bronchopulmonary aspergillosis (ABPA) and aspergilloma.

Aspergillus fumigatus

Because A. fumigatus is the etiologic agent of most Aspergillus infections, a large number of genotyping studies have focused on this pathogen [for reviews see 2,5,6,17–20]. An overview of these previous investigations is given in Table 2.

Genotyping methods

Numerous typing methods for A. fumigatus have already been described and in several of these studies the results obtained with these methods have been compared [16,21–27]. In these comparative investigations, no marked differences were found in the results obtained with multilocus enzyme electrophoresis (MLEE), microsatellite polymorphism (MSP), RAPD, RFLP, and SSDP [21–25]. However, a combination of several of these methods was suggested in order to obtain a sufficiently high discriminatory power.

Recently, two new typing methods for A. fumigatus have been developed, namely MLST and VNTR [28–30]. MLST is based on the principles of MLEE but the alleles are assigned directly by nucleotide sequencing instead of analysing the electrophoretic mobilities of the corresponding enzymes. The internal fragments (450 to 500 base pairs) of typically seven housekeeping genes are sequenced and an allele number is given to each locus. Combining these numbers results in a final overall allelic profile or sequence type that can be made available through public databases (for example: http://pubmlst.org) [31,32]. In VNTR typing, a number of repetitive DNA regions, called microsatellites or short tandem repeats, is amplified by PCR using a fluorescently labeled primer. Afterwards, the size of the fragments can accurately be determined with capillary electrophoresis using allelic ladders. This is in contrast with MSP typing for which fragment sizes are determined by traditional gel electrophoresis [29,30,33]. The inherent variability in the number of the short repetitive sequences is caused by slipped-strand mispairing, which
**MLST, VNTR typing led to the delineation of patient-specific clusters [16]. In general, an excellent agreement was found between the results obtained with VNTR typing and AFLP, but the former proved to be easier to use and interpret [26]. Additionally, a combination of MLEE, MSP, RAPD and SSDP typing methods resulted in the same clustering pattern and discriminatory power as VNTR typing, which is now widely accepted as the first choice for *A. fumigatus* genotyping [16].**

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Clinical versus environmental isolates of A. fumigatus

Numerous studies have used typing to identify the sources of A. fumigatus strains causing infections [7,21,24,25,28,36–53]. Several of these investigations described the genotyping of isolates obtained during outbreaks of aspergillosis [38,43–45,50,51]. Although it is generally accepted that transmission of A. fumigatus conidia from the air to the lungs of the immunocompromised patient can lead to infection, only a few studies have been able to demonstrate this link by identifying similar genotypes of isolates recovered from clinical and environmental samples [44,50,51]. Additionally, large-scale prospective studies were carried out to investigate the correlation between the number of Aspergillus conidia present in the hospital air and the incidence of aspergillosis. In most studies, an identical strain could be isolated from both environmental and clinical samples [37,40,47–49]. However, other studies failed to identify the source of A. fumigatus, often because the number of environmental isolates recovered was too low [33,39,41,42].

The impact of patient-to-patient transmission appears rather low as little or no genotypes are shared between patients [36,38,43,45,47,48,50]. Additionally, genotypes from patients residing at the same center are generally no more similar to each other than those from patients at different centers [25,37,41,43,51,53]. However, one study focusing on the epidemiology of aspergillosis in a transplant intensive care unit found evidence of person-to-person transmission, probably due to aerosolization of spores during debriding and dressing of wounds [46].

A different way of analyzing the typing results is to compare the genetic variability among clinical and environmental strains in order to determine if aspergillosis can be caused by all or only by selected strains of A. fumigatus. Several studies agree that all A. fumigatus strains are potentially infectious as the same genetic variability has been observed between isolates retrieved from air and patients [7,40,41,48,53]. Results of other studies suggest the presence of some form of selection since only a limited number of genotypes were identified among clinical isolates as opposed to those from environmental samples [37,42,47,49], however, none of these studies was able to determine what caused this selection. These results should be interpreted carefully as the genetic variability among clinical strains may vary a great deal depending on the origin of the isolates, as discussed below.

Genotyping of isolates from patients with IA

In multiple studies the A. fumigatus isolates retrieved from patients with IA were typed [16,22,23,26,29,30,36–38,40,42,43,45–47,49,50,53–59]. Most studies found that no or only a limited number of genotypes were shared among patients [23,26,37,38,42,45,58]. In contrast, some studies suggested patient-to-patient transmission or a common environmental source, as several patients were infected by the same strain [30,46,47,50,56].

Analysis of respiratory samples from patients with IA has led to the identification of a single genotype [23,37,42,47,54,56] or multiple genotypes per patient [23,26,36,40,45,47,49,50,54,55,59]. The discrepancies in these results might be explained by differences in host factors as all lung transplant recipients were infected by multiple strains. However, other transplant patients often harboured only a single strain [54,55]. Another explanation for the differences lies in the variety of the methods used and differences in their discriminatory power [26]. Finally, one study suggested that genetic changes occurring during colonization may explain the subtle differences observed between two strains isolated from the same patient [23].

When deep organ isolates were genotyped, a single strain per patient was found in all samples [26,58]. However, more research is required to understand why only a limited number of strains is able to spread to the deep organs in contrast to the colonization of the lungs by multiple strains.

Genotyping of isolates from patients with aspergilloma

Isolates retrieved from patients with aspergillomas have been genotyped in only a few studies [60–63]. Genotyping...
Table 2  Overview of genotyping studies for Aspergillus fumigatus.

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BMT, bone marrow transplant; CF, cystic fibrosis; IA, invasive aspergillosis; SOT, solid organ transplant; NS, not specified. *Calculated from the data provided in the original study. If either the number of isolates or the number of genotypes was not provided, no value is calculated. The closer to 1, the more discriminatory the method is. The average number of isolates/genotype was 3.63 (RAPD), 5.57 (SSDP), 2.26 (RFLP), 2.01 (VNTR), 3.23 (MLST), 5.55 (MLEE), and 1.97 (MSP) (averages were only calculated for methods for which data were reported in at least two studies).
the isolates recovered from the aspergillomas of 17 patients revealed the presence of a single strain in 10 and multiple strains in the remaining seven patients.

**Genotyping of isolates from people with CF**

Genotyping of isolates retrieved from CF patients led to the observation that the lungs of practically all individuals studied (55/61) were colonized by multiple genotypes of *Aspergillus* [64–69]. Moreover, in the limited number of studies in which multiple isolates retrieved from the same sample were analysed, co-colonization by several *A. fumigatus* strains at the same time was found with most patients [66–68].

Additionally, upon analysis of sequential isolates collected from CF patients who had histories of long-term fungal colonization, some strains were found recurrently [64,66–69]. Two studies found that these persistent strains differed between patients [68,69]. However, one study, using less discriminatory genotyping methods, found that upon long-term colonization, the same persistent genotype was retrieved from different persons with CF [67].

**Other Aspergillus species**

For *Aspergillus flavus* isolates, the most frequently used genotyping technique is RAPD [38,43,44,70–74], although AFLP typing schemes based on restriction analysis of mitochondrial DNA [75], restriction and hybridization with ß-tubulin probes [76], and restriction and hybridization with a repetitive DNA probe [77] have also been reported. In several studies, isolates obtained from hospital air and patients belonged to the same genotype [38,44,70–72]. Additionally, environmental surveillance of the hospital environment revealed that high numbers of *A. flavus* conidia, all belonging to the same genotype, were present in the air during an aspergillosis outbreak [43,44,71,72]. These studies suggest that a point source was responsible for *A. flavus* aspergillosis outbreaks. In some cases, the source appeared to be environmental (e.g., a contaminated air conditioning system) [72,77].

Recently, AFLP, RAPD and VNTR typing schemes have been developed for *A. niger* [78,79]. However, all isolates included in these studies were recovered from plants and therefore no information has been obtained concerning the epidemiology of *A. niger* in human infections. Molecular techniques have mainly been applied to species-level identification in this group of aspergilli.

Epidemiological studies focusing on *Aspergillus terreus* infections have mainly used RAPD typing [80–87]. Several centers have set up genotyping studies in order to get a greater insight into the epidemiology of such infections. These studies showed that all patients, including those with IA and CF, were infected or colonized by a single, unique strain [82–87]. On occasion, these same strains were also retrieved from the environment [82,87]. Additionally, the genetic diversity among environmental strains generally was very high [83–87], except in one study in which contamination of the environment with a single genotype was found [82].

**Candida albicans**

Multiple typing schemes, such as MLEE, MLST, RAPD, RFLP and VNTR, have been developed for *C. albicans* [13,88–95]. Numerous studies have investigated the sources of systemic *Candida* infections and revealed that the majority of infectious strains are acquired endogenously from the patient’s own commensal flora, with only a few reports indicating an environmental origin of the infections [88,94]. Patients are generally infected by a single, unique strain but during outbreaks strains can be shared between patients [88,92,94]. Additionally, genetic changes have frequently been encountered with *C. albicans* during prolonged infection.

Although *C. albicans* is frequently found in respiratory samples from people with CF (78–93% of persons with CF) [96], genotyping data have only been reported in a single study [96]. In this particular investigation, 442 *Candida* isolates (332 of which were *C. albicans*) were recovered from sputa or oropharyngeal swabs of 56 CF patients. RFLP using hybridization of the CARE-2 probe was employed in genotyping *C. albicans* isolates while PCR-RAPD was applied to isolates of other *Candida* species. Long-term persistence of *C. albicans* strains in the respiratory tract of people with CF was found (average persistence is 16.4 months) and only related strains were isolated over the entire observation period, suggesting micro-evolution of a single strain. Additionally, similar genotypes were only found in the case of siblings, indicating that transmission of yeasts among persons with CF can occur but is probably limited to persons having close contact [96]. Despite the frequent isolation of *Candida* species from the respiratory tract, the clinical relevance is minimal as a recent study revealed no cases of *Candida* pneumonia in patients with positive BAL cultures for *Candida* species [97].

**Exophiala dermatitidis**

Through the use of selective media, *E. dermatitidis* is more frequently isolated from respiratory samples of CF patients and isolates of this organism have been included in several genotyping studies [98–102]. However, only limited epidemiologic results are available as most studies focused on the development, comparison and evaluation of methods.
such as RAPD [98–100,102] and sequencing of the internal transcribed spacer region (ITS) [100–102]. In a study comparing phenotypic and genotypic typing methods, no agreement between the methods was found and based on RAPD, all clinical isolates belonged to the same genotype although they were retrieved from multiple patients at multiple time points [99]. Several other studies confirmed the limited discriminatory power of RAPD for genotyping E. dermatitidis [98,100,102]. Sequencing of the ITS region seems promising but to date no studies with a sufficiently high number of isolates have been carried out to allow conclusions concerning the epidemiology of E. dermatitidis in people with CF [100–102].

Scedosporium apiospermum

Clinicians have described a large variety of diseases (ranging from skin to brain infections) caused by species of the Pseudallescheria boydii complex. In the present review we want to discuss the results of genotyping studies of Scedosporium isolates recovered from CF patients. Although Scedosporium species usually are saprobes, they may cause respiratory disease in susceptible hosts such as persons with CF. S. apiospermum is the only species with which genotyping studies have been performed in order to elucidate its epidemiology in persons with CF. Many molecular techniques have been employed including AFLP, ITS sequencing, MLEE, RAPD and RFLP [3,103–105]. Additionally, a MLST scheme for genotyping of S. apiospermum isolates is currently under development [3]. One large-scale study focused on the epidemiology of S. apiospermum in people with CF. To that end, 129 sequential and multiple isolates from nine persons were typed by RAPD. Although only 16 genotypes were identified, not a single genotype was shared among these individuals. While five persons were colonized by a single genotype, four had a predominant genotype which was found in conjunction with one or two others that were genetically close to the predominant type [105]. A similar pattern was found with 29 patients with chronic lung disease [104], in that some of the individuals were persistently colonized by one strain, while different strains were isolated from other patients of which one was the dominant type. No agreement in genotypes between clinical isolates and those recovered from dust, air or soil from potted plants was found. However, while a single common type was found in 11 respiratory medicine unit inpatients, for outpatients different strains were isolated [104].

Conclusions

Genotyping studies have led to a better understanding of the sources of infections, transmission routes and colonization patterns, resulting in more efficient infection control procedures. In this review, we summarized the results obtained in genotyping studies, focusing on Aspergillus fumigatus and C. albicans, E. dermatitidis and S. apiospermum isolates retrieved from patients with CF.

In general, the respiratory tract of patients with IA or CF is colonized by multiple Aspergillus strains while only a single strain is found in deep organs of patients with IA. For isolates retrieved from patients with aspergilloma, the epidemiology differs according to the patient with some infected by a single genotype, while for others multiple genotypes were isolated. Aspergillus strains are usually acquired from the environment although in some occasions patient-to-patient transmission has been documented.

Little is known about the importance of other fungi including C. albicans, E. dermatitidis and S. apiospermum that are frequently isolated from samples of CF patients. Despite the availability of typing methods and isolates, C. albicans isolates from patients with CF were only analysed in a single study. However, the significance of isolation of Candida species from the respiratory tract remains a subject of debate. The key question is whether these isolates are true colonizers of the respiratory tract or whether they were contaminants originating from the oral mucosa. For E. dermatitidis and S. apiospermum on the other hand, the limited number of isolates and the lack of typing methods with sufficient resolution have made genotyping studies very difficult. The few data available all seem to suggest that patient-to-patient transmission is limited and that patients with CF are colonized persistently with a single strain each. In some studies, genetic changes of fungal strains in the respiratory tract of patients with CF was suggested as certain strains were closely related.

Acknowledgements

The research of Lies Vanhee was financially supported by the Bijzonder Onderzoeksfonds of Ghent University (project B/07601/02).

Declaration of interest: None.

References


© 2010 ISHAM, Medical Mycology, 48(Suppl. 1), S60–S69
Learning from the genotyping of fungi


