1 Molecular Epidemiology of Pathogenic Fungi Involved in Outbreaks

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CONTENTS

I. Introduction

Various techniques may be used to identify organisms accurately. For example, diversity in the ubiquitous ribosomal RNA genes can be identified easily and adequately in medically relevant fungal species (de Hoog et al. 1997), even in instances where the precise etiological pathogens have not yet been described in great detail. Using rDNA-based molecular taxonomy, most species can be included in a general scheme on the basis of which important physiological parameters, such as biosafety levels, can be predicted (Bowman 1993; de Hoog 1996; de Hoog et al. 1998). However, in order to perform epidemiological studies into the dissemination of a fungal isolate, additional technologies and types of investigation are required. Especially in the current era of steadily increasing numbers of nosocomially infected patients, technologies allowing the precise identification of strains are essential. Large groups of susceptible patients, like those suffering from neoplastic diseases (Walsh and Lee 1993), AIDS (Diamond 1991; Rubin 1993), or other forms of immunocompromising diseases or treatments (Vartivarian et al. 1993; Collins et al. 1994), are at a clear risk for developing infections with a wide spectrum of different fungal species. Most of these infections are hospital-acquired. This worrying development necessitates the execution of detailed studies into the mode of fungal dissemination (Fridkin and Jarvis 1996), but the identification of factors which may be important in fungal pathogenesis are of extreme importance as well (Hogan et al. 1996). Fortunately, medically significant fungal species show extensive genetic variability that can be used to identify particularly pathogenic strains that may cause disease in humans. In this chapter, the current state of affairs concerning the molecular laboratory methods that are at the disposal of the modern fungal epidemiologist are described. The use of these methods to document outbreaks of infections caused by important categories of human-pathogenic fungi is illustrated. The organisms included in this chapter are the most common nosocomial pathogens.

II. The Technological Armamentarium

The number of molecular techniques aimed at the subspecies identification of microbial isolates has expanded enormously over the past 15 years and during this period a number of reviews on this topic have been published.

For a historical description and technical detail, the reader is referred to this large body of literature (e.g., Eisenstein 1990; Maslow et al. 1993;

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The Mycota XII
Human Fungal Pathogens
J.E. Domer and G.S. Kobayashi (Eds.)

Versalovic et al. 1993; Maslow and Mulligan 1996). In addition, a number of papers describing the various individual techniques in intricate detail have been published over the years. Techniques such as ribotyping (Kostman et al. 1995), random amplification of polymorphic DNA (RAPD) analysis (Power 1996) and amplification fragment length polymorphism (AFLP) analysis (Savelkoul et al. 1999) have been discussed at length, often in the context of a critical review of the shortcomings of the various methodologies (e.g., Tyler et al. 1997). Of particular interest is a short technical review by Richard Goering (2000) published in *Reviews in Medical Microbiology*.

In this paper, the author categorizes the current array of molecular techniques into four different, but major groupings, based solely on historical considerations. First-generation technology included plasmid analysis, followed by second-generation technology such as the use of restriction enzymes and DNA probes (Southern 1975). Pulsed-field gel electrophoresis (Schwartz et al. 1983) and PCR-mediated methods (van Belkum 1994) constituted the third technical wave, whereas nucleotide sequence-based typing is currently giving rise to the fourth generation typing procedures (Maiden et al. 1998). The essentials of these four fundamental technologies are outlined schematically in Fig. 1.1.

Technical improvements have been implemented frequently over the past two decades. This does not imply, however that molecular-epidemiological typing can be practiced with impunity. One important issue is the interpretation of data and another is reproducibility. With respect to the interpretation of data, there is only one test (IS6110 typing of *Mycobacterium tuberculosis*) where there is consensus on the mode of interpretation of the experimental data and large-scale comparisons have been performed successfully (van Embden et al. 1993; Tenover et al. 1995). For most other technologies, data interpretation is being performed in a manner that is quite diverse and depends upon the experience of the individual handling the technical procedures. Improvement in this field is still urgently needed (Pitt 1994). Secondly, the interlaboratory reproducibility of typing methods is generally quite poor (van Belkum et al. 1995, 1998b). The ability to exchange primary experimental data is limited, although various promising initiatives in the typing field as they relate to fungi are currently underway.

Despite some of the problems mentioned above, molecular epidemiology has been very successful over the past few years and even in hospitals outside of metropolitan areas versatile molecular techniques are being introduced more frequently. The usefulness of nucleic acid-mediated methods is undisputed and acknowledged, as evidenced by a number of thorough reviews on the topic (e.g., Miller 1993; Bingen 1994; Jarvis 1994; Maslow and Arbeit 1996).

In conclusion, DNA technology has been adapted to the study of epidemiology and in combination with good hygienic practices in the hospital setting can be used for the evaluation of hospital infection control policies. Currently, research is focussing on the development of systems that will allow for more accurate data processing, storage and exchange between laboratories, but much remains to be done to achieve these goals (Struelens et al. 1998). Finally, one has to expand one's view beyond mere strain typing and (local) molecular epidemiology. Integration of genetic epidemiology of infectious diseases, population genetics and the analysis of mechanisms leading to mutation, will surely enhance our insights into the fundamental aspects of clonality or panmicticism among the sometimes pandemic spread of infectious agents (Musser 1996; Tibayrenc 1998; Spratt and Maiden 1999; van Belkum et al. 2001).

In principle, any of the molecular techniques mentioned can be applied to determine genetic variation among fungal isolates, although plasmid analysis is not considered a method of choice. Since fungal genomes are larger than those of bacteria, the complexity of experimental data will most certainly be greater. In the case of pulsed-field gel electrophoresis (PFGE), for example, prior restriction of the DNA is not required since entire chromosomes can be physically separated (Pfaller 1992). In some instances, this necessitated the development of simplified methods for the assessment of fungal genome variability employing combinations of various methods. Many authors have discussed modern molecular technology from a user's perspective, thereby highlighting various aspects of molecular epidemiology of fungi in a reader-friendly manner (Fridkin and Jarvis 1996; Reiss et al. 1998). Various molecular methods will be discussed below in combination with more classical methodologies and their value in investigations of outbreaks of fungal disease caused by distinct species of yeasts and molds.

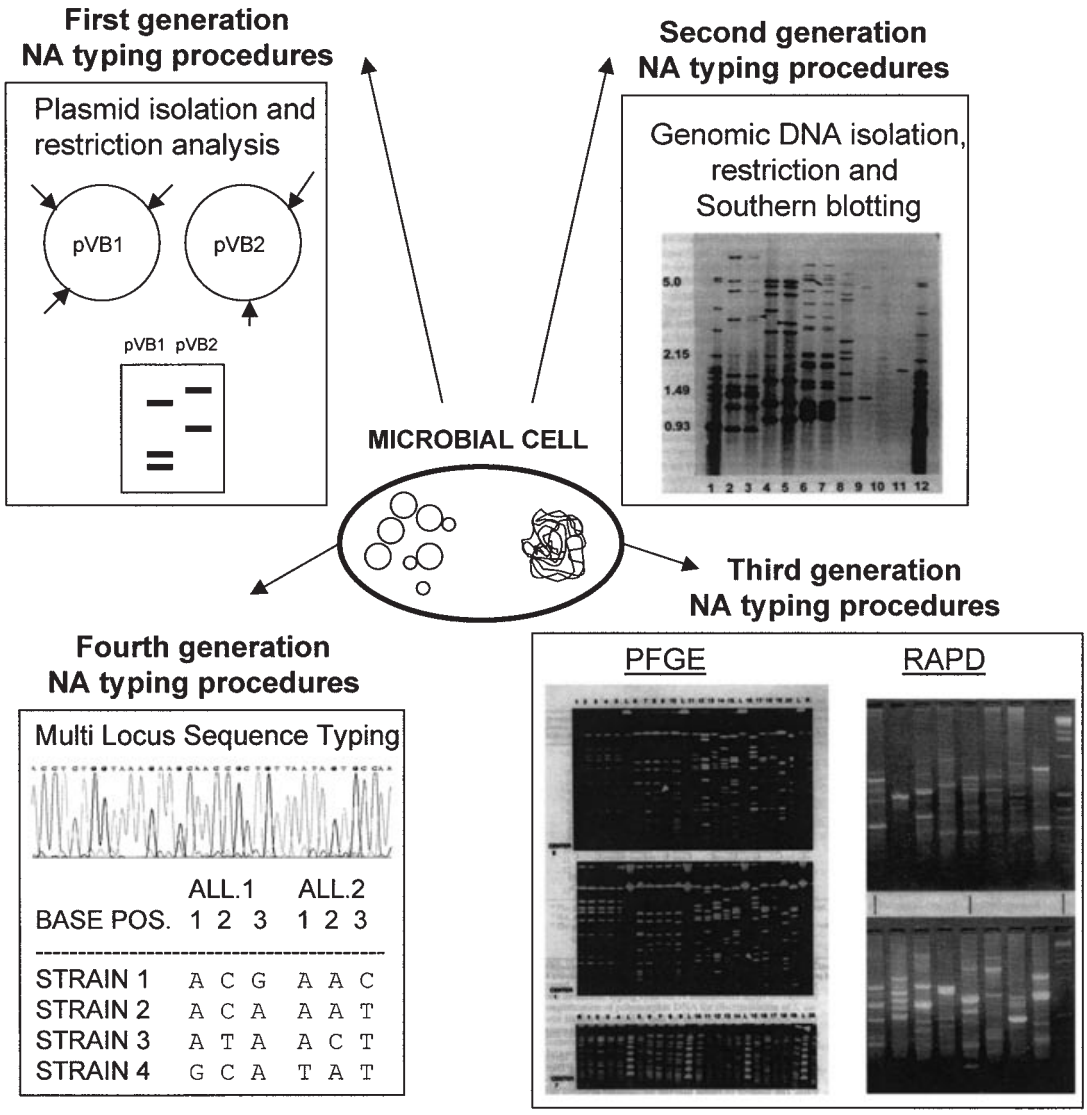

Fig. 1.1. Schematic outline of the four categories of epidemiological typing strategies as defined by Goering (2000). The *four panels* illustrate the end result of the typing methods. The first generation methods relied on plasmid isolation and characterization of polymorphism in plasmid number and size, with or without restriction enzyme analysis. Different plasmids can be identified on the basis of selective absence or presence in a microbial cell or on the basis of restriction site polymorphism (see pVB1 and pVB2 restriction maps). The second generation typing methods involved procedures for enzymatic digestion of DNA, Southern blot transfer and DNA probe hybridization for the detection of genomic restriction site polymorphism. As an example, an autoradiograph showing *IS6110*-based RFLP for various strains of *Mycobacterium tuberculosis* is included (courtesy of Drs. Dick van Soolingen and Kirstin Kremer, RIVM, Bilthoven, The Netherlands). Pulsed field gel electrophoresis (PFGE) and random amplification of polymorphic DNA (RAPD) methods are at the core of the third generation typing methods. Examples of banding patterns generated for *Staphylococcus aureus* by PFGE and *Naegleria fowleri* by RAPD are shown (van Belkum et al. 1992, 1998b). Variant allele identification (ALL. 1 and ALL. 2) for various housekeeping genes based on DNA sequencing of PCR products and computer-mediated annotation of multilocus sequence types (MLST analysis) is the current state-of-the-art technology. This fourth-wave technology is portable, data are interchangeable from laboratory to laboratory, and database management is relatively straightforward

III. *Aspergillus fumigatus*

A. Typing Methods

Fungi belonging to the genus *Aspergillus* are ubiquitous in the environment and even tobacco and marijuana generally can be quite heavily contaminated with these organisms (Verweij et al. 2000). *A. fumigatus* and *A. flavus* are regularly encountered as contaminants in the clinical environment as well, and the effect of infections caused by these organisms may be devastating, especially among neutropenic patients. In addition to deep seated infections, *A. fumigatus* can also cause cutaneous infections. Cutaneous disease is usually associated with immunosuppression, burns or other forms of serious trauma, such as trauma caused by surgery (Pla et al. 1992) or vascular grafting (Motte et al. 1993). Complicating the situation is that effective therapeutic options are limited for the patients involved, and straightforward rapid diagnosis can be difficult (Andriole 1993). Nucleic acid isolation itself can present a bottleneck, since fungal cell walls are sturdy and quite resistant to lysis. This has resulted in the development of a wide variety of DNA isolation protocols appearing in peer-reviewed journals over the past 10 years (Bainbridge et al. 1990; Graham et al. 1994; Liu et al. 2000 and references therein). Pure cultures required for epidemiological studies, however, can be obtained in quantities large enough to overcome even the most daunting fungal isolate.

Nucleic acid preparations, even relatively impure ones, allow for the monitoring of genetic polymorphism among *Aspergillus* spp strains. Many techniques have been evaluated with respect to their potential applicability to various *Aspergillus* species, but the most emphasis has been placed on one of the most clinically significant, viz. *A. fumigatus*. In early, now classical studies, multilocus enzyme electrophoresis (MLEE) was the best evaluated method for phenotyping of *Aspergillus* strains (Nealson and Garber 1967), and the method is deemed, even today, a valuable tool for assessing inter-relatedness between fungal strains. Recently, this method was applied to a study of the population structure of *A. fumigatus* leading to the hypothesis that this fungus primarily reproduces in a clonal manner (Rodriguez et al. 1996). However, in the early 1990s, when molecular typing was introduced into mycology laboratories world-wide, restriction analysis of genomic DNA became an attractive alternative to phenotyping. The use of enzymes in combination with high-resolution gel-based systems became quite popular (Denning et al. 1990; Birch et al. 1995a,b).

These methods showed adequate resolution and were simple in execution. When combined with Southern blotting procedures and DNA probes suited to the detection of hybridization polymorphism the method gained in popularity (Girardin et al. 1993). At the present time, this type of restriction fragment length polymorphism (RFLP) mapping appears to be the preferred standard (Soll 2000). PCR-mediated procedures, such as RAPD, were developed in parallel and are, to date, still frequently applied as laboratory methods well suited to monitoring variability in small, local collections of clinical and environmental isolates. RAPD tests were developed by several researchers who each used different primers or combinations thereof (Loudon et al. 1993; van Belkum et al. 1993; Anderson et al. 1996). These tests are continuously being refined and are applied successfully in epidemiologic studies, providing easily obtained and highly discriminatory data (Mellado et al. 2000). More recently, assays aimed at size-variable repetitive DNA targets became available as highly discriminatory alternatives (Fig. 1.2; Bart-Delabesse et al. 1998; Bart-

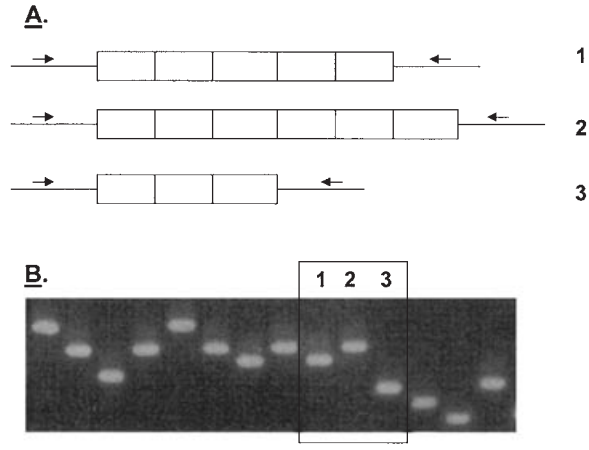

Fig. 1.2. Repetitive DNA moieties are often inherently unstable, in that the number of repeat units may differ between individual alleles, hence the terms variable number of tandem repeat (VNTR) loci or short sequence repeats. Variation in the number of repeat units can be visualized by anchoring two PCR primers to conserved sequence motifs upstream and downstream of the repeat locus (**A**). Simple agarose gel electrophoresis can be used to visualize polymorphism among the PCR products (**B**). (see also van Belkum et al. 1998a)

Delabesse and Bretagne 1999). Several methods have been combined for high-resolution genotyping of strains of *A. fumigatus.* Methods are often combined, which not only allows strains to be genotyped, but also allows the establishment of the most optimal typing method for this fungal species (Lin et al. 1995; Loudon and Burnie 1995; Rinyu et al. 1995; Rodriguez et al. 1999). Despite all these efforts, one single "gold standard" has not yet been adopted by the fungal typing community (Birch et al. 1995a; Rodriguez et al. 1999), and the application of more than one typing method is still strongly advocated (Bart-Delabesse et al. 2001; Bertout et al. 2001)

B. Epidemiological Studies

Outbreaks of aspergillosis have a longstanding history, not only among hospitalized individuals, but also among animal species such as penguins or other birds, particularly when confined to zoos (Khan et al. 1977; Dykstra et al. 1997). Although these outbreaks could not be explained easily on the basis of airborne dissemination of high numbers of fungal spores, some construction activities were noted in, or in the vicinity of, the facilities in which the animals were housed. Precise definition of the immune status of affected animals was lacking as well, so a precise epidemiological scenario could not be identified. Among humans, however, it is generally assumed that an immunocompromized status and construction activities in the vicinity are clear predisposing factors for the initiation of fungal infection. Increased numbers of airborne spores are considered to be one important driving factor. The increased spore densities can be monitored using gravity air-settling plates or other air-sampling devices, but elevated numbers of spores cannot always be substantiated at the time of the outbreak (Iwen et al. 1994). In 1998, however, a link between construction activities and elevated spore densities was demonstrated at one institution where there was an outbreak of ocular infections caused by *A. fumigatus* following cataract surgery (Tabbara and Al Jabarti 1998).

In the case of *A. fumigatus* it has been documented regularly that cases of aspergillosis occurring in a specific location were due to several nonclonally related strains; thus, increases in the incidence of infection did not appear to result from a point-source dissemination involving a single fungal genotype. This correlates well with the observation that airborne populations of spores are genetically heterogeneous. A large French study corroborated this conclusion. When over 700 strains of *A. fumigatus* from various French regions were typed genetically, it was concluded that clonal outbreaks had not occurred (Chazalet et al. 1998). However, two patients housed in the same environment may acquire infections caused by a single strain because both patients would breathe the same population of airborne spores. Similar conclusions were drawn from data presented by Dutch researchers (Leenders et al. 1996, 1999). In their studies, apparent outbreaks of invasive fungal infections were shown to be caused by *A. fumigatus* isolates that were genetically heterogenous, and in some cases there was the involvement of two separate species, viz., *A. fumigatus* and *A. flavus*. The same studies showed that despite the fact that some strains persisted for more than a year in a given clinical environment, they are not found to be responsible for infections arising during that time On the other hand, the existence of point sources of contamination of media over time in the diagnostic laboratory was documented.

IV. *Candida albicans*

A. Typing Methods

Isolation of DNA from dimorphic fungi, where the yeast form such as *C. albicans* dominates, is less demanding than nucleic acid extraction from species of fungi which exist only in filamentous states. Several protocols involving sequential treatment with cell-wall degrading enzymes and proteinases and an extraction step, have been published for yeast forms (e.g., Polaina and Adam 1991; Pospisek and Palkova 1991). The DNA extracted can be used for SPECIES identification, and occasionally the same technology has been developed for STRAIN identification (e.g., Meyer et al. 1991; Williams et al. 1995 and many others). However, since the topic of this chapter is limited to fungal epidemiology, the matter of molecular speciation will not be discussed further.

The methodology for subspecies identification of *C. albicans* strains has been available for several decades now. Initially, characteristics such as colony morphology, resistance to various chemicals, serologic reactivity, biochemical tests and