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Epidemiology: surveillance of fungal infections

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Surveillance for fungal diseases is essential to improve our understanding of their epidemiology and to enable research and prevention efforts to be prioritized. In order to conduct better surveillance for fungal diseases, it is important to develop more accurate and timely diagnostic tests, to follow rigorous epidemiological methods and to have adequate support from public health agencies and the pharmaceutical industry. Investigations of nosocomial and community outbreaks of fungal infection have also resulted in a better understanding of the sources and routes of transmission of these diseases, and of the risk factors for infection. This has led to more effective prevention and control strategies. In addition, outbreak investigations have offered excellent opportunities to develop new molecular sub-typing methods, and to evaluate and validate older ones. For example, results obtained from a global epidemiological study of the genomic structure of *Cryptococcus neoformans* have led to a better understanding of the epidemiology of cryptococcosis. Similarly, a study of variations in the genotype of *Trichophyton rubrum* has found that patients may become infected with multiple strains, which has important implications for study design when looking at the epidemiology of dermatophyte infections.

Keywords *Cryptococcus*, dermatophytes, epidemiology, surveillance

Surveillance for fungal diseases

Since 1980 there has been a dramatic increase in the occurrence of serious fungal infections, largely as a result of an increase in the size of the population at risk. The acquired immune deficiency syndrome (AIDS) pandemic accounts for much of this increase, but other factors, such as the widespread use of immunosuppressive agents in transplantation and cancer treatment, have also contributed. Two recent studies from the Centres for Disease Control and Prevention (CDC) have highlighted the importance of fungal infections. In the first study, analysis of the US National Center for Health Statistics death records showed that fungal infections were the seventh most common cause of infectious disease-related mortality in the USA, and that fungal diseases-related fatalities increased more than 3-fold between 1980 and 1992 [1]. Candidiasis and aspergillosis were the two specific diseases that accounted for most of these deaths. In the second study, analysis of the US National Center for Health Statistics hospitalizations data showed that, in 1994, fungal infections resulted in about 30000 hospitalizations; these diseases accounted for the fourth highest annual percentage increase (10%) in number of hospitalizations since 1980 [2].

Surveillance for fungal diseases is important to define their burden and trends, to provide the infrastructure needed to perform various epidemiological and laboratory studies, to evaluate interventions and to detect new

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pathogens. Surveillance systems require the following basic elements: a clear case definition, a defined population, mechanisms for reporting, analysing and disseminating the data, and incentives to conduct surveillance. For fungal diseases, each one of these elements presents distinct challenges. In particular, diagnostic difficulties remain a major problem in developing adequate definitions for fungal infections. Culture is still the mainstay of diagnosis for many diseases, but it lacks sensitivity for many important fungal infections, such as invasive candidiasis, where blood culture sensitivity may be as low as 50% [3]. For some fungal infections, such as pulmonary aspergillosis, isolation of the organism is not proof of disease, and histopathological evidence is often required. Obtaining tissue samples from many of the patients at high risk for fungal infections is often difficult and may cause serious health risks. Fungal infections, especially the community-acquired endemic fungi, result in a wide spectrum of clinical manifestations, ranging from asymptomatic infections, to mild and life-threatening illnesses. Therefore, determining the overall burden of infection is often very difficult, and surveillance usually focuses on determining the burden of severe disease. Clinical consensus is often difficult to reach for fungal case definitions. There are few standardized definitions for invasive fungal infections. Indeed, a recent review of the medical literature revealed 25 different adjectives that had been used just to indicate the level of certainty of the diagnosis; of these, proven, probable, and possible were the most commonly used [4].

Various surveillance systems and large databases have been used to estimate the burden of fungal diseases, including hospital-based surveillance, hospital discharge databases, and passive reporting systems. In the past, researchers have often relied on retrospective analysis of large databases, such as deaths records and hospitalization data, to estimate the burden of fungal diseases [1,2,5]. Although helpful for investigating trends in various diseases, these databases tend to significantly underestimate the incidence of these infections; for example, in death records, whenever malignancy or human immunodeficiency virus (HIV) infection are present, they are listed as the principal (underlying) cause of death, regardless of the immediate cause of death. In addition, validation of the fungal diagnosis is usually difficult since no medical records are available for review. USA national hospitalization data are derived from a representative sample of USA hospitals; in the case of the geographically restricted endemic mycoses, or the AIDS-associated mycoses, this sample may not be adequate to assess the burden of hospitalizations due to fungal diseases. Hospital-based systems, such as the National Nosocomial Infections Surveillance (NNIS) system, are important to quantify the mortality and morbidity associated with nosocomial fungal infections, especially in comparison to other nosocomial infections [6]. However, NNIS is a sentinel surveillance system of self-selecting hospitals and it may not be a representative sample of all hospitals. In addition, nosocomial infections constitute only a proportion of fungal infections. With the recent change in health care delivery, even infections historically considered as nosocomial are now seen in out-patients; population-based surveillance for candidemia has recently demonstrated that 19% of all candidemia infections are acquired outside hospitals [7]. Passive reporting is not ideal for fungal diseases; these rarely require immediate intervention and this leads to minimal incentive for the clinicians to report cases in a timely fashion, if at all. In the USA, there is only one nationally notifiable fungal disease, coccidioidomycosis, and its reporting is only required by states in the southwestern region [8]. Even when volunteer networks are formed, passive reporting underestimates incidence rates. Moreover, reporting varies for different diseases and risk groups, and this may lead to inaccurate descriptions of the epidemiological features of these diseases.

Active population-based surveillance for fungal infections is expensive and may be difficult to conduct, but it can provide accurate incidence rates and better descriptive epidemiology. Recently, active surveillance at selected USA sites has, for the first time, enabled population-based rates for invasive fungal diseases, such as candidemia and cryptococcosis, to be ascertained [7,9,10]. This form of surveillance has also enabled better risk factor studies to be conducted, because cases detected are more truly representative of the population. For example, following surveillance for cryptococcosis, we conducted a risk factor study for this disease among HIV-infected persons, and found that exposures to pigeons were not associated with an increased risk of disease; we also confirmed that fluconazole chemoprophylaxis is associated with decreased risk of disease [10]. These findings were helpful in designing the USA Public Health Service (PHS)/Infectious Diseases Society of America (IDSA) guidelines for prevention of opportunistic infections in HIV-infected persons [11]. Another example of how surveillance for fungal diseases is helpful is coccidioidomycosis. Following analysis of surveillance data for coccidioidomycosis in Arizona [12] and California [13], we conducted multiple studies to determine the risk factors for this disease in HIV-infected persons, the elderly, and risk factors for severe disease [13–15].

In addition to epidemiological studies, surveillance provides an excellent infrastructure for laboratory-based

studies. Specimens obtained during surveillance are ideal for the development of new diagnostic and subtyping procedures. For example, isolates from cryptococcosis surveillance have been used to develop subtyping methods for *Cryptococcus neoformans* and better understand the mechanisms of recurrent cryptococcosis [16,17]. Isolates from candidemia surveillance have been used to better understand the population genetics of *Candida albicans* [18]. Host genetic factors have been evaluated in conjunction with coccidioidomycosis surveillance in California, and have contributed to improved understanding of the pathogenesis of this disease [19].

Continuing surveillance for fungal diseases will be critical to improve our understanding of their epidemiology and to enable research and prevention efforts to be prioritized. In order to conduct better surveillance for fungal diseases, it is important to develop more accurate and timely diagnostic tests, to follow rigorous epidemiological methods and to have adequate support from public health agencies, and the pharmaceutical industry. Ultimately, improved surveillance will provide critical guidance for both clinicians and mycologists in management of patients and development of better diagnostic and subtyping tools. It will justify the need to develop more drugs and provide the isolates for *in vitro* testing, and will help institutions make better research decisions.

The role of outbreak investigations

Outbreak investigations are an important and challenging component of public health. Careful investigation of outbreaks has increased our understanding of fungal diseases, their sources and modes of transmission, and the risk factors for infection, and in so doing has assisted in the design of improved control measures for these infections. Investigations of nosocomial outbreaks of aspergillosis and candidiasis have led to the development of more effective strategies for prevention and control of these infections in hospitals. Investigations of outbreaks have also provided a great deal of useful information about the transmission and risk factors for the endemic fungal infections, such as histoplasmosis, blastomycosis and coccidioidomycosis, thus helping to reduce the public health burden of these diseases.

Outbreak investigations of fungal infections present us with major challenges. These include limited sample size, difficulties with the case definition and exposures that are ubiquitous or too restricted. In many outbreaks, the number of cases is limited and, therefore, the statistical power of the investigation is limited, making it difficult to identify the source of the infection (by detecting significant differences in exposure between cases and control subjects). If, as is often the case with outbreaks of fungal infection, detection of the outbreak is delayed, important clinical and environmental samples may be difficult to obtain. However, investigating these outbreaks may still be useful and may lead to the prevention of further cases.

In some outbreaks, formulating the case definition and exclusion criteria are straightforward; in others, the case definition and exclusion criteria are complex, particularly if the disease is new or if the range of clinical manifestations is very broad. In many investigations of outbreaks of fungal infection (e.g. aspergillosis), multiple case definitions are needed (e.g. laboratory-confirmed case, clinical case, proven case, probable case, possible case) and the resulting data are analysed by using different case definitions. In the case of outbreaks of endemic mycoses, such as coccidioidomycosis and histoplasmosis, several different approaches are possible. For example, in the case of the coccidioidomycosis outbreak that followed the Northridge earthquake in California, population-based skin testing was used to determine the attack rate [20]. In other instances, the case definition may be limited to individuals with symptomatic disease.

Once it is clear that a suspected outbreak is not the result of laboratory error (e.g. due to specimen contamination) [21], the next step is to establish whether the observed number of cases is in excess of the usual number (i.e., that an outbreak has occurred), and to find all the cases in a given population over a certain period. Establishing the background rate of a disease is generally more straightforward if good laboratory tests are available than if such tests are unavailable or infrequently used. In the case of fungal diseases, establishing the background rate in a hospital or a community suspected of having an outbreak may be difficult because health-care providers may not have considered the diagnosis, or ordered the appropriate tests, or such tests may not have been available. This has certainly been the case when outbreaks of coccidioidomycosis or histoplasmosis have occurred among residents of regions not endemic for these diseases. Other factors that can affect the background rate of a disease include changes in the population at risk. For example, even small changes in the number of cases of aspergillosis in a particular hospital unit may appear to represent a cluster when in fact it is not. An increase in the number of patients undergoing bone marrow transplantation (BMT) or a change in the proportion undergoing unrelated donor BMT are among the factors that could account for the apparently higher incidence of aspergillosis. Outbreaks can also be difficult to detect when, during the period under investigation, changes occur in the level of suspicion and test-ordering practices of health-care providers, and the diagnostic tests used by laboratories.

By collecting detailed patient data, case-finding provides important information about the descriptive epidemiological features of an outbreak. By reviewing the times of onset of the cases, and by examining the characteristics (e.g. age, sex, residence, occupation, recent travel) of those affected, epidemiologists can often generate hypotheses about the cause and source of the outbreak. For example, careful investigation of a large outbreak of blastomycosis in Wisconsin during 1984 enabled the incubation period for the infection to be determined, and the risk factors for human disease to be identified, as well as leading to a much clearer understanding of the natural habitat of the fungus and the sources of human infection [22]. Likewise, bird roosts and bat guano have been clearly implicated in outbreaks of histoplasmosis [23,24], and archaeological digs and construction work in endemic regions are among the factors that have been implicated in outbreaks of coccidioidomycosis [25,26]. It is notable that, for those fungal infections that have never been associated with outbreaks (e.g. cryptococcosis), information about the precise source(s) of human infection and the incubation period remains very limited.

It is essential to determine the source and route of exposure in order to understand why an outbreak occurred, how to prevent similar outbreaks in the future, and, if the outbreak is ongoing, how to prevent others from being infected. In some outbreaks, the source and route are obvious to those involved. In others, this is not the case. In these instances, hypotheses can be generated in a number of ways. Existing epidemiological and microbiological data describing previous outbreaks or sporadic cases of the same disease are often very useful. For example, sporotrichosis has long been associated with handling of sphagnum moss [27]. Therefore, when in 1994 an outbreak of lymphocutaneous disease occurred among a group of workers using sphagnum moss and metal frames covered with wire to create ornamental topiaries for an entertainment park, it was possible to identify specific behaviours that increased the risk of disease, and to formulate prevention measures that led to the disappearance of the disease [28].

When neither review of the descriptive epidemiological features of the cases, nor review of existing published literature yields the correct hypothesis, other methods must be used. Interviews of those affected are one such method. Whether a hypothesis explaining the occurrence of an outbreak is easy or difficult to generate, an analytical epidemiological study to test the hypothesis is usually the next step. In many instances, a case-control study is used, but in others a retrospective cohort or cross-sectional study may be more appropriate. One problem, which often arises during investigations of fungal disease outbreaks, is that only a small number of cases are involved, and consequently the statistical power of the analysis to find a true difference in exposure between cases and controls is very limited. Moreover, if the persons involved in the outbreak do not provide accurate information about their exposure to suspected sources of infection because of poor recall, language problems, or other reasons, the resulting misclassification of exposure status can prevent the source of infection from being identified.

Environmental specimens can support epidemiological findings. However, these need to be collected as soon as possible, either before they are no longer available, as in the case of contaminated parenteral nutrition fluids etc, or before environmental interventions are implemented, as in the case of repairing a malfunctioning air-filtration unit. Finding or not finding the causative organism in environmental samples is often perceived as powerful evidence implicating or exonerating an environmental source; however, both positive and negative findings can be misleading. For example, finding a ubiquitous organism such as *Aspergillus fumigatus* in an item of hospital food does not prove that the food (rather than some other source) is responsible for an outbreak of aspergillosis. Likewise, not finding the causative organism in an environmental sample does not conclusively rule out a source as the cause of the problem. This is especially true for difficult-to-culture organisms, such as *Blastomyces dermatitidis*.

Central to any outbreak investigation is the implementation of appropriate control measures to minimize further illness and death. For example, although most cases of candidiasis are endogenous in origin, investigation of outbreaks in neonatal and surgical intensive care units has demonstrated that carriage of organisms on the hands of health-care providers is a common cause of nosocomial transmission [29,30]. In turn, this has facilitated the development of rational preventive measures, such as rigorous hand washing before and between all patient contacts in units dealing with high-risk patients. Investigation of nosocomial outbreaks of *Aspergillus* infection has contributed to the development of measures for the control and prevention of this devastating disease. For example, outbreaks of cutaneous aspergillosis have sometimes been traced to the use of contaminated biomedical devices, or associated with contaminated adhesive tape used to secure intravenous infusion sets [31–33]. These outbreaks have served to highlight the risk that the use of non-sterile items, which often become contaminated with environmental moulds, can pose to low-birth weight infants and other immunocompromised individuals.

There are also numerous reports of apparent outbreaks of aspergillosis associated with sources or activities, which might have released *Aspergillus* conidia into hospital air. These include construction or demolition work, and malfunctioning or contaminated ventilation or air filtration systems [34–37]. However, most studies that have documented potential sources of increased conidia concentrations in the hospital air, such as construction or demolition work, have done so retrospectively, and in so doing may have resulted in biased conclusions. More importantly, there are often no data available on the baseline air concentrations of conidia to determine whether these clusters were associated with increased exposure to airborne *Aspergillus* conidia. It now seems probable that many patients are colonized or infected before their admission to hospital. Indeed, Patterson *et al*. [38] estimated that 70% of cases of aspergillosis diagnosed during a 3-year period of surveillance during hospital construction were community acquired. Clearly, it is important to ascertain whether this disease is predominantly a hospital or community-acquired infection, because hospital infection control measures, such as housing patients in rooms supplied with high efficacy particulate (HEPA)-filtered air, will not prevent community-acquired cases.

Outbreak investigations have also provided much useful information that has enabled prevention guidelines to be formulated for community-acquired infections. For example, the CDC has published detailed guidelines for protecting workers against histoplasmosis who are involved in activities where material contaminated with *Histoplasma capsulatum* might be disturbed [39]. Such activities include disturbance of soil at active or inactive bird roosts, excavations in regions where *H. capsulatum* is endemic, or removal of accumulations of bat or bird manure from buildings. Recommended control measures include dust suppression, and the wearing of respirators and other items of personal protective equipment.

In conclusion, investigations of nosocomial and community outbreaks of fungal infection have resulted in a better understanding of the sources and routes of transmission of these diseases, and of the risk factors for infection. This has led to more effective prevention and control strategies. In addition, outbreak investigations have offered excellent opportunities to develop new molecular sub-typing methods, and to evaluate and validate older ones. Findings of fungal outbreak investigations have increased our knowledge of the ecology, epidemiology, and prevention of these diseases and thereby have assisted in reducing illness and death from these infections.

A global molecular epidemiological survey of *Cryptococcus neoformans*

C. neoformans is an opportunistic, encapsulated basidiomycetous yeast which causes disease in immunocompromised and immunocompetent hosts. Cryptococcosis is among the most prevalent life-threatening mycoses with a worldwide distribution [40,41]. Human infection is acquired by inhalation of the infectious propagules from the environment, primarily causing disease in the lung, which can disseminate to the central nervous system. Immunocompromised hosts, such as patients with HIV/AIDS, haematological malignancies, and solid organ transplants are particularly at risk for infection; cryptococcosis in these hosts often progresses to meningoencephalitis. Approximately 5–15% of patients infected with HIV develop cryptococcal meningitis [40,42,43]. Three genetically distinct varieties of *C. neoformans* have been recognized: *C. neoformans* var. *grubii* (serotype A) [44] and *C. n.* var. *neoformans* (serotype D), both with the sexual stage *Filobasidiella neoformans* var. *neoformans* and *C. n.* var. *gattii* (serotypes B and C), with the sexual stage *F. neoformans* var. *bacillisporus*. The serotype AD appears to represent a recombinant between the serotypes A and D [45, T. Boekhout, unpublished results]. The majority of infections world-wide are caused by isolates of *C. n.* var. *grubii*, serotype A [40,42], which is ubiquitous in nature, in association with soil, avian habitats and decaying wood [40,42,46, E. Castaneda, personal communication]. Isolates of *C. n.* var. *gattii* are largely restricted to tropical and subtropical regions, where they are found in association with decaying wood (e.g. certain eucalyptus species: *E. camaldulensis*, *E. tereticornis*, *E. rudis* and *E. gomphocephala* [47,48, D. Ellis, personal communication], pink shower trees in Brazil [49, M. Lazera & B. Wanke, personal communication], and almond trees in Colombia [E. Castaneda, personal communication]). *C. n.* var *grubii* and *C. n.* var. *neoformans* predominantly affect immunocompromised hosts, while the variety *gattii* nearly always infects immunocompetent hosts [40,50].

To date, several molecular typing techniques have been used to analyse limited numbers of geographically restricted clinical and/or environmental isolates of *C. neoformans*. These techniques include Southern blot hybridization with DNA probes based on repetitive DNA sequences isolated from *C. neoformans* [51], restriction fragment length polymorphisms (RFLP) [52,53], karyotyping [54], PCR-fingerprinting [55–57], randomly amplified polymorphic DNA (RAPD) [48,58] sequence analysis [59] and more recently, amplified fragment length polymorphisms (AFLP) [T. Boekhout, unpublished results]. A study conducted on *C. neoformans* iso-

lates from several countries, maintained in a commercial culture collection, revealed little variation amongst isolates from the same geographical region, leading to the suggestion that clonal reproduction was occurring [60]. Our own previously obtained results showing a high variation within *C. neoformans* isolates from the USA and a nearly clonal population structure within Australian isolates, led to the establishment of an international epidemiological survey for *C. neoformans*. In this ongoing study we are analysing the global genomic structure of the three varieties of *C. neoformans* using polymerase chain reaction (PCR)-fingerprinting and RAPD analysis as standard typing techniques.

PCR-fingerprinting combines the specificity of conventional DNA hybridization-based fingerprinting with the efficiency of the PCR. Oligonucleotides originally designed as hybridization probes, which are specific to minisatellite (M13 core sequence 5′ GAGGGTGGCGGTTCT 3′) and microsatellite (simple repeats, e.g. $[GACA]_4$, $[GTG]_5$) DNA sequences, are used as single primers in the PCR to amplify the stretches of DNA lying between these hypervariable repetitive DNA sequences (inter-repeats) of genomic DNA from *C. neoformans*. This technique yields distinct species- and strain-specific multi-locus profiles, termed 'PCR-fingerprints'. DNA extraction, PCR amplification and analysis of the obtained PCR-fingerprinting patterns were carried out as described previously [57]. Major molecular types were assigned according to the major bands that were typical for each pattern. All visible bands were included in the analysis, regardless of their intensity. For the RAPD analysis, two combinations of three random primers, CN1 (5′-TACCCCCGCCCATATTCCAT-3′), MYC1 (5′-GAGGAAGGTGGGGATGACGT-3′) and 5SOR (5′-ATGGGGAATACGA CGTGCTGTAG-3′) were used as primer pairs CN1/MYC1 and CN1/5SOR for the PCR amplification. DNA extraction, PCR amplification and analysis of the obtained RAPD profiles were carried out as described previously [57]. Bands of 100–600 bp were included if they were visible, regardless of their intensity. RAPD profiles were classified into the major molecular types if both RAPD primer pairs produced the same differences. The obtained PCR-fingerprinting and RAPD patterns were analysed using the software package GelComparII, version 1.01 (Applied Maths, Kortrijk, Belgium).

Currently, there are approximately 300 clinical and environmental isolates included in our global epidemiological survey. The isolates were supplied from numerous laboratories around the world representing Argentina, Australia, Belgium, Brazil, Germany, Hong Kong, India, Italy, Malaysia, New Zealand, Papua New Guinea, South Africa, Thailand, Uganda, USA and Zaire. Both PCR-fingerprinting and RAPD analysis generated the same molecular types, separating all clinical and environmental isolates into eight major types. Within each major molecular type a number of subtypes have been identified. A correlation between the serotypes and the obtained major molecular types was found for *C. n.* var. *grubii* and *C. n.* var. *neoformans*. The molecular types VNI and VNII are characteristic for *C. n.* var. *grubii*, serotype A. The molecular type VNIII is characteristic for the recombinant strains between *C. n.* var. *grubii* (serotype A) and *C. n.* var. *neoformans* (serotype D), and serotype AD. The molecular type VNIV contains all isolates of *C. n.* var. *neoformans* (serotype D). There was no correlation between serotypes and the obtained major molecular types within *C. n.* var. *gattii*. All *C. n.* var. *gattii* strains (serotypes B and C) could be assigned to four major molecular types VGI, VGII, VGIII and VGIV. The distribution of the molecular types is shown in Table 1. The most common molecular types were VNI and VGI, which were present in most countries studied. In addition, the major molecular type VNI showed the largest number of subtypes. All isolates from AIDS patients had molecular types VNI and VNII, which agrees with other findings that *C. n.* var. *grubii* (serotype A) is causing the vast majority of infections in HIV-positive patients [40,50]. The molecular type VGIII was found to be geographically restricted to India and the USA and the molecular type VGIV was only found in India and South Africa.

Table 1 Global molecular type distribution of *C. neoformans*

Country	*C. n.* var. *neoformans*/ *C. n.* var. *grubii*	*C. n.* var. *gattii*
Australia	VNI, VNII, VNIII, VNIV	VGI, VGII
Argentina	VNI	–
Belgium	VNI	–
Brazil	VNI, VNII, VNIII	VGI, VGII
Germany	VNI, VNII, VNIII	–
Hong Kong	VNI	–
India	VNI, VNII	VGI, VGIII, VGIV
Italy	VNI, VNII, VNIII, VNIV	VGI
Malaysia	–	VGI, VGII
New Zealand	VNI, VNII, VNIV	VGI, VGII
Papua New Guinea	VNI	VGI
South Africa	VNI, VNII	VGI, VGIV
Thailand	VNI, VNIV	–
Uganda	VNI	–
USA	VNI, VNII, VNIV	VGI, VGII, VGIII
Zaire	VNI, VNII	–

In order to study the genetic variation between the investigated cryptococcal isolates, the obtained banding patterns were analysed with the software package GelComparII, version 1.01. The analysis showed that both genotyping techniques clearly distinguished *C. n.* var. *grubii* and *C. n.* var. *neoformans* (serotypes A, D and AD) from *C. n.* var. *gattii* (serotypes B and C), supporting the genetic distinction between two potentially separate species: *F. neoformans* and *F. bacillispora*. Similar results have been found using AFLP analysis [T. Boekhout *et al.*, unpublished results]. The cryptococcal isolates clustered broadly according to their country of origin in the GelComparII analysis with the exception of the USA isolates, which were clustered between isolates obtained from all other countries. Some strains were common to different countries, implying some mechanism of strain transfer between countries, e.g., increasing travel between continents or export of biological materials, which may carry the fungus. The overall global similarity between strains was 60%. All USA isolates from different patients were characterized by a unique, strain-specific profile, despite their origin from within a localized area (most isolates had been obtained from patients treated at Duke University Medical Center, Durham, NC, USA), indicating substantial genetic diversity compared to isolates obtained from other areas of the world. Non-USA isolates were genetically highly homogeneous or even clonal in some cases. A high degree of genetic variation was also found amongst clinical isolates from another USA location, New York city. This variation was not observed amongst isolates from two Brazilian cities (Rio de Janeiro and Belo Horizonte) [59]. The authors speculate that this may be due to natural selection of certain clones by specific environmental or climatic conditions. Our Australian data support the hypothesis that local or regional factors could be involved in the evolution of pathogenic clones of *C. neoformans*, whereby strains obtained from all states of Australia showed much less genetic variability than that found in the USA [48,58]. Similar results were found for cryptococcal isolates from outside the USA, although sampling bias may explain some of these results. In most cases the clinical isolates from outside the USA came from a hospital within a major city. The observed degree of genetic variability within different countries may increase if more strains from geographically different locations in each country are included. Notably, substantial genetic heterogeneity was reported within *C. n.* var. *neoformans*, serotype D, isolates obtained from several regions in France [46].

Regardless of the country of origin, all isolates obtained from the same patient at different time points and/or different body sites (e.g. urine, cerebrospinal fluid or blood) showed in most cases identical banding patterns using both typing methods. These results are in agreement with previous studies [41] and confirm that a patient is in most cases infected with only a single fungal isolate. Isolates obtained over a period of several years after the initial infection presented identical profiles to those of the original strain, suggesting that a recurrent cryptococcosis is due to a persistent strain rather than an infection with a new strain.

Regional PCR-fingerprinting and RAPD profiles obtained from eucalyptus-derived and clinical isolates in Australia revealed concordant banding patterns [58]. Similar results were obtained from environmental isolates obtained from pink shower trees and clinical isolates in the north of Brazil [M. Lazera & B. Wanke, personal communication]. These findings support an epidemiological association between these trees and human infection.

This report is a preliminary summary of our current results obtained from our ongoing global epidemiological study aimed to investigate the genomic structure of *C. neoformans* on an international scale. Two genotyping methods have been used: PCR-fingerprinting and RAPD, which grouped all isolates into eight major molecular types. Within each molecular type there was only limited genetic variation found, with the exception of the USA isolates. Our ongoing, more extensive, global study of cryptococcal isolates should provide a better understanding of the genetic distribution, the epidemiology, evolution and the pathogenic potential of *C. neoformans*.

The molecular epidemiology of dermatophyte infections

Dermatophytes are a group of fungi that are specialized in the utilization of keratinous substrates and therefore in the infection of skin hair and nails. These infections include tinea capitis (infection of the scalp), tinea pedis (infection of the feet), tinea cruris (infection of the groin area), and tinea unguium or onychomycosis (infection of nails). Dermatophytes comprise a group of about 20 species from three genera: *Microsporum*, *Trichophyton* and *Epidermophyton*. Diagnosis of dermatophytoses is generally straightforward by microscopy and culture and treatment has improved greatly with the use of certain systemic antifungals in recent years. However, a number of epidemiological questions remain to be answered. Are all dermatophytes equally virulent? Are infections caused by a single strain of fungus or are multiple strains involved and are there changes strains over a period of time? What is the source of relapsing disease–re-infection with the same strain or a new source of infection? Many of the answers to these questions require an effective method of differentiating between strains of dermatophytes. In recent years several groups have worked to

achieve this using the tools of molecular biology. We have looked for variations in the genotype of the dermatophyte *Trichophyton rubrum* the cause of tinea pedis, tinea corporis, tinea cruris and onychomycosis. Variations in the genotype of *T. rubrum* have been sought through RFLPs in the mitochondrial DNA [61,62] and various PCR methods, primarily RAPD approaches [63,64]. RAPD is also notorious for problems of reproducibility and furthermore these methods have proven of only limited success in differentiating strains of *T. rubrum*, indeed Graser *et al.* [63] concluded that *T. rubrum* is very much a clonal species. For these reasons it was decided to seek more robust methods of demonstrating genetic polymorphisms between strains. Thus we have looked at variation in the non-transcribed spacer (NTS) regions of the ribosomal RNA (rRNA) gene repeats.

Using probes from the intervening transcribed sequences (ITS) 1 and 2 and 5·8S rRNA genes on digests of *T. rubrum* DNA, variations in the numbers and sizes of fragments that included the NTS region were observed between strains. Fifteen strain types were identified and it was proposed that this variation was due to the presence of internal mini-repeats in the NTS region [65] similar to that seen in *Candida krusei* [66]. When the NTS region of several strains of *T. rubrum* were sequenced this was confirmed. Within the NTS region, which was typically 2·5 kb in length, two repeat motifs of 200 bp (TRS-1) and 77 bp (TRS-2) were revealed. Variation in the copy number of these mini-repeats, from one to six for TRS-1 and zero to three for TRS-2, accounted for variations in hybridization patterns seen in the earlier study [A. Yazdanparest *et al.*, unpublished results]. By designing primers either side of TRS-1 and 2, the number of copies of these repeats could be counted for different strains. In a survey of *T. rubrum* strains from around the world the majority of strains displayed a simple pattern with up to six copies of TRS-1 (designated types 1 to 6) and two copies of TRS-2. In a few type 1 strains zero, one, three or two and three copies of TRS-2 were present. In 20% of strains of *T. rubrum*, amplification using the TRS-1 primers revealed a complex pattern suggesting combinations of numbers of copies of this repeat or other arrangements with in most cases two copies of TRS-2. These repeats were only amplified from *T. rubrum* and a few closely related species such as *T. violaceum*, *T. gourvilii* and *T. soudanense* and not from any other dermatophyte or other pathogenic fungi.

Among UK isolates of *T. rubrum* the most common was type 1 (one copy of TRS-1), the next type 2, and then in order of types 3, 4, 5 and 6. There were only one or two of the complex types. However, when eleven isolates from Japan were examined only one type 1 was seen. The significance of this divergence from the UK strain distribution in terms of the spread of *T. rubrum* to Europe from the Far East and Africa at the beginning of the 20th Century is an intriguing issue and requires further work with more strains.

When single colony isolates of *T. rubrum* from two to five nail specimens of eight patients with onychomycosis taken before, during and after unsuccessful antifungal therapy were examined a remarkable diversity of strains was seen with a mean of 2·5 strains per patient. Thus, five colonies of *T. rubrum* from plates seeded with nail specimens from another five patients were analysed. Surprisingly, in four of five onychomycosis patients there were two to three strains in a single specimen [67]. These findings suggest that patients become infected with multiple strains of *T. rubrum* and this has important implications for study design when looking at the epidemiology of dermatophyte infections.

Work with *T. mentagrophytes* and *T. violaceum* indicates that repeats are also present in the NTS region of other species, though in the case of *T. mentagrophytes* it is clear that the repeats are different from those in *T. rubrum*. Thus the variation in the numbers of these repeats between strains will be useful for strain typing for epidemiological purposes.

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Contributors

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