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SPECIAL ARTICLE

Typing tools for the investigation of epidemic fungal infection

The epidemiology of bacterial infection is investigated by the use of identification procedures at a sub-species level by such techniques as serotyping, phage typing, various antigen recognition tests, plasmid profiling and DNA probes. Fungal epidemiology has tended to lag behind in the use of this technology, particularly with the filamentous fungi, though several techniqués have been developed for yeasts and especially for *Candida albicans*. This review will briefly describe the application of these methods to outbreaks of *C. albicans* infection, describe the limited methods available for the investigation of filamentous fungal infection and indicate the necessity for increased research in this area.

The need for better epidemiological tools for pathogenic fungi is growing with the increasing numbers of immunosuppressed and immunocompromised patients, longer life expectancy, improved survival of premature babies and the ever increasing use and numbers of antibiotics, since these factors influence the numbers of fungal infections experienced. The 1984 USA National Nosocomial Infection Surveillance figures showed *Candida* species to account for 5·5% and other fungi 1·7% of all pathogenic isolates identified [1]. In leukaemia patients, fungi caused 37% of all fatal infections in the late 1980s compared with 5% in the early 1950s [2]. The increased reporting of some fungi, such as *Cryptococcus neoformans*, can be attributed directly to specific diseases such as AIDS. With the number of AIDS patients in Britain projected to increase to about 5000 by 1993 the incidence of fungal infections in this group will increase dramatically.

Detailed epidemiological investigation is unnecessary when the infecting organism is unusual and the source readily identifiable, as for example when an interstate outbreak of eye infection in the USA with *Candida parapsilosis* was traced to contaminated ocular irrigation fluid [3], though in fact isoenzyme profiles were used to establish the identity of the isolates. Equally the association of Rhizopus infections with contaminated elastoplast was clear without the use of typing methods [4], whilst an outbreak of ringworm in a hospital ward was readily traceable to a heavily infected patient who suffered a cerebrovascular accident and thus required extensive contact care [5]. Most outbreaks are not so easily defined. One outbreak of *Candida tropicalis* sternal infections, following coronary bypass surgery, was attributed to the presence of a scrub nurse heavily colonized in the nasopharynx and fingertips with this yeast [6]. The outbreak terminated upon the nurse's departure from the unit but the mode of transmission was uncertain since, apart from her substitution of hypoallergenic soap for the scrub solution, she was not observed to break surgical hygienic procedures. Transmission was likely, therefore, to have been by dispersal of the yeast through her face mask during surgery. Typing techniques may have confirmed or excluded her as the source of organisms.

Establishment of an exogenous or endogenous source of an apparent epidemic has important implications for infection control. Richet and colleagues [7] proposed exogenous transmission of *Malassezia furfur* in a neonatal intensive care unit when three preterm infants, all catheterized and receiving intravenous emulsions, developed bronchopneumonia in a 72-h period. In contrast Surmont and co-workers [8] suggested that *M. furfur* fungaemia was likely to have resulted from endogenous sources following the observation that embrocation with sunflower oil increased *M. furfur* skin colonization so that blood stream inoculation was more likely to occur on insertion of a venous catheter. Others have reported similar findings of preterm infant fungaemia with the source of *M. furfur* colonization of venous catheters most likely to have been the skin [9]. Typing techniques could have confirmed single or multiple source outbreaks since in the outbreak described by Surmont and colleagues [8] each infant was probably infected with an individual strain. Study of an outbreak of systemic *Candida krusei* infections affecting seven newborns over a 2-month period failed to reveal the source, no staff or patients appeared to disperse the yeast [10], again knowledge of the identity of the *C. krusei* isolates could have pointed to multiple or single sources of this epidemic. A cluster of nine cutaneous *Aspergillus flavus* infections in a 4-month period associated with Hickman catheters, was found to be related to a group of 4 of 18 operating rooms though no contamination of ducting was detected [11]. Again a demonstration that all were the same clone could have indicated a source other than airborne contamination, since this should be composed of a variety of strains.

Fungal epidemiological techniques currently available include biotyping, morphotyping, resistotyping, serotyping, killer toxin typing, karyotyping and the use of restriction endonuclease digestion and Southern blot hybridization patterns. These techniques have been developed principally to analyse *Candida albicans* infection, although some have been applied to other yeast species.

The yeasts

Biotyping was developed by Odds and Abbott [12, 13] to differentiate between *C. albicans* strains, but has been difficult to reproduce due to factors such as variable media composition [14]. In an interlaboratory study Odds and co-workers [15] assessed the reproducibility of the technique for 18 strains in a blind trial involving four laboratories and described the intralaboratory but not the interlaboratory reproducibility as good. Biotyping has nevertheless yielded important information on strain carriage and possible reinfection routes. Identical biotypes were obtained from 99% of paired vaginal and urethral, and 94% of paired genital and anal isolates from 198 women attending a genitourinary clinic. Furthermore, carriage of the same biotype lasted for more than 15 weeks in 66% of patients [16].

Subspecies identification may also yield information on pathogenicity and virulence. Krogh and colleagues [17], examined the carriage of particular *C. albicans* biotypes from the normal and pathological oral mucosa of 17 leukoplakia and 19 oral lichen planus patients. Yeast carriage in the leukoplakia patients was 82%, mostly *C. albicans*, compared with 37% in the lichen planus patients, which is approximately the same carriage rate as in normal subjects. In 5/11 leukoplakia and 1/3 lichen planus patients the biotypes from pathological mucosa were

different from those on normal mucosa, even from the same individual. Three biotypes were associated with nodular lesions, a form of nonhomogeneous leukoplakia which is more likely to undergo malignant transformation than the homogeneous types; Krogh and colleagues speculate on a possible role for *C. albicans* in the transformation of this condition to carcinoma. Korting and co-workers [18] reported poor reproducibility and discrimination when typing oral *C. albicans* strains from AIDS patients, using the API 20C carbohydrate assimilation system, but did find that 21·3% of the strains examined exhibited distinctive assimilation profiles not reported in other epidemiological studies using this test.

Morphotyping was originally described by Brown-Thomsen [19] and more recently modified by Phongpaichit and colleagues [20]. The method, which depends on variations in colony morphology, was reported to provide 84% reproducible results on the re-examination of 50 isolates after 2·5 years. Hunter and colleagues [21] tried to use morphotype as a marker for virulence but found no single morphotype to be associated with a particular body site, though 67% of strains from fatal infections exhibited discontinuous fringe morphology compared to 11% from other infections.

Resistotyping is based on the differing agar plate resistances to a panel of chemicals and dyes. The method has been used to demonstrate repeated recovery of particular *C. albicans* strains from individual vulvovaginitis patients over a time period and indicated that clinical relapse was due to inadequate elimination or to reinfection from a partner [22]. In another study the mouth has been shown to be the source of cutaneous colonization for sites of irradiation treatment for oral and laryngeal cancer [23].

Killer typing. Yeasts secrete toxins lethal to other members of the same or related species, a phenomenon similar to bacteriocin typing. This phenomenon was used by Polonelli and co-workers [24] to develop a killer typing system. *C. albicans* strains were collected from a variety of sources and although 25 killer types were distinguished amongst 100 isolates none was correlated with the source of isolation.

Pyrolysis-mass spectrometry (py-ms) involves heating an organic sample to more than 300 °C in a non-oxidizing environment and examining the resultant mixture of low molecular mass volatile compounds by mass spectrometry. The pyrolysis fingerprint is characteristic of the quantity and composition of the sample. Magee and co-workers [25] used py-ms to distinguish between two strains of *C. albicans*, which it had been claimed were identical. The method enabled the reference strains to be distinguished and authenticated the identity of 13/20 isolates of those strains received from various sources. Although py-ms is a fast method of analysis, it is not readily available to most routine microbiology laboratories.

Serotyping of *C. albicans* is limited as an epidemiological tool as only two serotypes, A and B, have been demonstrated by agglutination and precipitin tests. Approximately 80% of clinical isolates are type A, with 1–11% exhibiting flucytosine resistance compared to 49–89% of serotype B [14]. There is evidence to suggest that strains of serotype B, strains with increased flucytosine resistance and a reduced susceptibility to imidazoles and amphotericin B, are more likely to be isolated from immunocompromised than immune competent individuals [18, 26]; findings with obvious implications in the long-term control of infection.

Immunoblotting compares the antigenic variation between strains by blotting

electrophoretically separated yeast antigen with hyperimmune antiserum. Burnie and colleagues [27] demonstrated that four *C. albicans* outbreaks in different London hospitals were each due to a single strain. Three of the strains were indistinguishable from each other by immunoblotting but could be differentiated from other clinical strains.

Protein patterns analysed by sodium dodecylsulphate–polyacrylamide gel electrophoresis (SDS–PAGE), successfully applied to bacterial classification and epidemiology, have been used in a retrospective analysis of seven systemic *C. albicans* infections in a neonatal intensive care unit. Two strains were found to be involved; the result was confirmed by restriction endonuclease digestion of whole-cell DNA by *Eco*RI [28]. Cross-infection was originally suspected as the strains were isolated from patients linked spatially and temporally in the unit. Others have found PAGE patterns to be too variable for use in either taxonomic or epidemiological studies [29]. However, isoenzyme patterns of *Candida* species enabled 23 biotypes to be recognized amongst 37 strains of *C. albicans* and also revealed biotype information for *C. tropicalis* and *C. paratropicalis* [30].

The advantage of most of the techniques described so far is that they can readily be applied to large numbers of strains and could be automated to some extent.

Karyotype separation of chromosomes using pulsed field gel electrophoresis (PFGE) is based on the DNA molecules' ability to relax and realign through the agarose pores in a changing electric field. This technique enabled Merz and co-workers [31] to detect 7–9 chromosomes in clinical strains of *C. albicans* revealing 14 discrete karyotypes among 17 isolates. Isolates from the same individual exhibited the same karyotype in 10/12 patients from whom multiple isolates were obtained.

A variation of PFGE, called contour-clamp homogeneous field gel electrophoresis (CHEF), was used to distinguish 22 karyotypes amongst 33 clinical isolates of *Torulopsis glabrata* [32]. Nosocomial transmission of this organism was assessed by karyotyping strains from multiple individuals hospitalized in the same unit during a 3-week period. No cross-infection was detected, for none of the isolates from patients within any single unit shared the same karyotype.

Polacheck and colleagues [33] examined the electrophoretic karyotypes of the two varieties of *Cryptococcus neoformans*, isolated from a range of sources, using orthogonal field alternation gel electrophoresis (OFAGE). Three distinctive patterns were observed: serotypes B and C of the variety *gattii* exhibited nine chromosome bands, serotype A variety *neoformans* had eight bands, while strains of serotype D variety *neoformans* were different from each other and the other serotypes. Sexual crossings of serotype D with other serotypes always produced progeny of serotype D; the authors proposed this could account for the variation. However, when isolates of all four serotypes were karyotyped by CHEF gel electrophoresis 10–12 chromosome bands were visualized and there were differences between strains and within serotypes [34].

Karyotyping would appear to be a useful epidemiological tool for some species of yeast, but enables only species or varietal recognition in others. Comparison of the karyotypes of *Malassezia* (*Pityrosporum*) species in the authors' laboratory (unpublished results) have enabled three morphological variants of *M. furfur* var. *ovale* to be distinguished from each other and from *M. furfur* var. *orbiculare* and *M. pachydermatis*, but has not revealed any significant strain variation.

Restriction endonuclease analysis of genetic material currently provides the definitive comparison for strains and species. Patterns produced by restriction endonuclease digestion of whole cell DNA (restriction fragment length polymorphism, RFLP) should provide a greater potential for differentiation than karyotyping. Scherer and Stevens [35] showed restriction digestion patterns to be stable through several hundred doublings from single colonies. These analyses also separated *Candida* species and subgrouped 17 *C. albicans* strains from random clinical samples. One subgroup of six strains contained four isolates from urine specimens and one further isolate from a stool specimen from one of the patients providing a urine strain. This suggested an association between genotype and site of infection.

This approach was used by Matthews and Burnie [36] to re-examine the outbreak strains of *C. albicans* from London hospitals that had been previously characterized by immunoblotting and morphotyping [27]. In one outbreak 11 patients were infected systemically and a further 8 patients and 2 nurses were suspected of harbouring the same strain. RFLP analysis using *Eco*RI revealed 4 of these latter patients and the hands of the 2 nurses to be colonized with the outbreak strain. In an unpublished ongoing study of *C. albicans* in HIV-positive patients, we have shown that small changes in morphotype and resistotype were not reflected in the RFLP patterns of strains from some patients. Equally, however, a diversity of RFLP patterns may be obtained within a group of isolates which exhibit very similar morphotype and resistotype obtained from a single patient. The relationship between types designated by various methods remains to be explored fully.

Discrimination using RFLP analysis relies on polymorphisms and repeat sequences that are visualized as bright bands superimposed on the general background. Greater sensitivity and specificity can be achieved using Southern blot transfer and specific DNA probes. A probe containing *Saccharomyces cerevisiae* DNA was used by Magee and colleagues [37] to distinguish *Candida* species and found 6 patterns among 12 strains of *C. albicans*. Others have divided strains of *Histoplasma capsulatum* from environmental and clinical sources by Southern blot hybridization with a cloned mtDNA probe. Further subdivision was achieved using rDNA polymorphisms [38]. *Sporothrix schenckii* and related species can be divided into several RFLP types [39] though this appears not to have been used yet in epidemiology.

Filamentous fungi

Few methods, analogous to those for the yeasts, exist for the subspecies recognition of clinically important filamentous fungal species. Identification of filamentous fungi relies heavily on morphological characteristics and a few biochemical/nutritional tests. Morphological variants within species are known to occur, but strain recognition is a problem. Leslie and co-workers [40] examined morphological variation in a series of *Aspergillus fumigatus* isolates from aspergilloma patients and found isolation of strains exhibiting abnormal characteristics to increase during the course of the infection, perhaps as a result of therapy. The spread of a dysgonic morphological form of *Microsporum canis* across N.W. London has been reported. Tucker [41] described the possible zoophilic transmission of the fungus between cats and their owners in an area of

N.W. London. However, as this form was unstable during *in vitro* culture, reverting to more commonly isolated forms, the extent of the outbreak was impossible to assess. Midgley [42] found this morphological form was predominately isolated from dermatology patients from the West and South of Greater London, with few isolates from other London regions; but no epidemiological connection was proposed.

Epidemiological surveillance is of most importance in hospital-acquired infections. Nuovo and colleagues [43] described the possible nosocomial spread of *Fusarium solani* from an infected car accident patient to the wound of a second patient with similar injuries 5 days after the arrival of the index patient. Both isolates were resistant to ketoconazole, miconazole and itraconazole, but the isolate from patient 1 was amphotericin B sensitive whereas that from patient 2 was resistant, clearly identity of strains had not been established despite the circumstantial evidence. Pulmonary aspergillosis has been regularly associated with the inhalation of fungal spores released by construction work. Increased recovery of *Aspergillus flavus* from respiratory specimens was found to be associated with hospital construction [44]. Similarly, increased numbers of aspergillosis cases were observed in a bone marrow transplant unit during the construction of a laminar air flow (LAF) isolation unit on an adjacent site. The outbreak ceased when the LAF was operational [45]. In these cases however it is improbable that typing would help. Typing schemes could have answered the epidemiological questions arising from an apparent outbreak of disseminated aspergillosis over a 6-month period involving four patients, all of whom had had a laparotomy and been placed on haemofiltration in the intensive care unit of one hospital [46]. Only two of the patients were considered to be at 'high risk' of aspergillosis. The hospital was of Victorian construction and constantly undergoing maintenance, although there was no major building work in the vicinity of the ICU at the time of the outbreak. The exact source of infection in these cases therefore remains unknown.

Until recently, typing schemes for filamentous fungi had not been explored. One approach examined fungal byproducts to assess the role of pit-head showers in coal mines as a source of foot infection in miners [47]. Antibiotics produced by dermatophytes isolated from miners' feet were compared using chromotographic mobility patterns. Approximately 70% of the strains that produced antibiotics gave distinctive chromatographic profiles, however, at least 40% of the isolates failed to produce antibiotics. The technique was therefore of limited applicability, but did indicate the pit-head showers were unlikely to be simply a reservoir for tinea pedis. Killer toxins, another sort of fungal byproduct, were reported as being an effective epidemiological marker in the differentiation of biotypes of *Pseudoallescheria boydii* and *A. fumigatus* [48].

Some immune recognition techniques have been developed, such as the differentiation of *M. canis* strains by Western blotting of electrophoretically separated exoantigen with monoclonal antibodies [49], and similarly, has permitted the differentiation of 14 isolates of *Penicillium camemberti* implying a role for these techniques in the control of industrial starter cultures [48]. Burnie and colleagues [50] examined strains of *A. fumigatus* by immunoblot fingerprinting and found 11 immunoblot types amongst 21 isolates obtained from 8 aspergilloma

patients. Multiple isolates were obtained from six patients, with the isolates from three of the patients showing identical patterns. Strains from the remaining three patients exhibited several immunoblot types, apparently reinforcing the morphological results of Leslie and co-workers [40] demonstrating a multiplicity of *A. fumigatus* strains in one infection.

The epidemiology of several outbreaks of *M. canis* has been examined using protein PAGE patterns [51]. The clinical and outbreak isolates were unrelated geographically and were of (−) mating type. All produced identical PAGE patterns probably because of a true lack of genetic diversity. Mating studies between (−) and (+) mating types (the latter recorded only in Japan) showed that genetically diverse strains had different PAGE patterns. The implication is that *M. canis* clinical strains are all derived from a clone probably inhabiting domestic cats. The technology to use genetic material for the analysis of filamentous fungi exists but has rarely, if ever, been used in a clinical context; the chief difficulty lies in devising methods for the lysis of the fungal cell wall and the extraction of DNA. Strains of *A. nidulans* have been karyotyped using CHEF gel electrophoresis, and genetic linkage groups assigned to the chromosomal bands by Southern blotting [52]. Restriction enzyme analysis of the mitochondrial DNA from several *Aspergillus* species was used as an aid to the taxonomy of this genus [53]. A similar approach was applied to the morphological variants of *Trichophyton rubrum* [54].

All of the techniques described have generally been developed and used in research establishments and not part of a routine clinical microbiology service. The use of broad spectrum antibiotics and immune suppression has provided niches for opportunistic fungal infection. The recognition of infection with these agents, instead of environmental contamination, and the instigation of effective infection control procedures requires more sensitive, specific and available epidemiological methods.

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