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Application of molecular typing methods to dermatophyte species that cause skin and nail infections

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Typing methods utilising DNA technology were applied to a collection of *Trichophyton mentagrophytes* and *T. rubrum* isolates from skin and nail infections. The methods included restriction enzyme analysis (REA), hybridisation with the DNA probe poly (dG-dT), randomly amplified polymorphic DNA (RAPD) by PCR and restriction analysis of a segment of PCR-amplified rDNA. All these tests successfully differentiated the species, but few intra-species differences were detected. REA demonstrated some isolate variation, but this was limited and difficult to interpret, making it unsuitable as a typing tool. RAPD demonstrated few variations amongst *T. mentagrophytes* and none in *T. rubrum*.

Introduction

Dermatophytes are capable of infecting nails and the skin from almost any body site. One of the frequently encountered dermatophyte infections is athlete's foot or tinea pedis. This can be a recurring infection and is most often caused by the dermatophyte species *Trichophyton rubrum* and *T. mentagrophytes*. When infection recurs it is not possible to establish if this is due to relapse from a previous infection or due to a newly acquired organism, as there are no typing techniques available to differentiate strains of these species. Furthermore, the ability to identify individual organisms would enable sources of infection to be identified, such as communal swimming and washing facilities, and appropriate control measures applied.

Dermatophyte species are well defined by morphological methods, but there have been relatively few attempts to devise typing techniques that would enable epidemiological studies to be performed. Some studies of cellular proteins by polyacrylamide gel electrophoresis have shown subspecies differences [1], but this has not been a universal finding [2]. There have been few reports of the application of molecular DNA

techniques for the study of dermatophytes. The G + C composition was in the narrow range of 48.7–50.3 mol% for species of the three dermatophyte genera [3], and the DNA homology of *T. rubrum* and *T. mentagrophytes* to *Arthroderma benhamiae*, one of the teleomorph species, was shown to be in the range 71–76% [4], indicating a closely related group of fungi. Other reports have compared the mitochondrial DNA composition by restriction enzyme analysis (REA) and found the *T. mentagrophytes* var. *interdigitale* isolates to be highly uniform in content and indistinguishable from *A. vanbreuseghemii*, its teleomorphic species [5, 6]. *T. rubrum* was reported to contain two types of mitochondrial DNA patterns, one of which was phylogenetically grouped with isolates of *T. mentagrophytes*, *T. mentagrophytes* var. *interdigitale*, *T. mentagrophytes* var. *goetzii*, *T. tonsurans* and *A. vanbreuseghemii* [7].

Typing methods applied to bacterial and yeast epidemiology include REA, hybridisation with DNA probes, polymerase chain reaction (PCR) and random amplification of polymorphic DNA (RAPD). We and others have achieved success with many of these methods in studies of *Candida albicans* [8–11] and *Malassezia* spp. [12, 13], and recently there have been reports of typing *Aspergillus* spp. by RAPD and REA [14]. Therefore, these techniques were used to examine the differentiation of dermatophyte strains and species from skin and nail infections.

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Materials and methods

Strains

The isolates described in this study were kindly identified and provided by Dr Y. Clayton of St John's Institute of Dermatology, London, Dr J. Reuter of the William Harvey Hospital, Ashford, Kent, Mr R. J. Barnard of the Queen Elizabeth Hospital, King's Lynn and Dr Humphreys of the Royal Infirmary, Edinburgh. The reported sites of isolation of these dermatophytes were given as follows: 30 *T. mentagrophytes* and 24 *T. rubrum* from skin or nail of the feet; seven *T. mentagrophytes* and three *T. rubrum* from nail; one *T. mentagrophytes* from fingernail; one *T. rubrum* from the shoulder; one *T. rubrum* from the leg and one *T. mentagrophytes* from the skin of the scalp.

Growth

Dermatophyte isolates were grown on Oxoid Sabouraud Dextrose Agar (Unipath, Basingstoke) and spore suspensions were prepared and stored in distilled water at room temperature. Isolates were grown in 10 ml of Sabouraud broth (Oxoid Mycoseptone 10 g/L, dextrose 40 g/L) in a 28-ml screw-capped bottle at 30°C in an orbital incubator for 3–4 days. The fungus culture was then vortex mixed with sterile glass beads to produce short mycelial fragments and this suspension was used to inoculate 100 ml of Sabouraud broth which was cultured with shaking overnight.

DNA extraction

The mycelial mass was harvested by centrifugation, washed with sterile distilled water and resuspended in 10 ml of SCS buffer (1 M sorbitol, 20 mM sodium citrate; Sigma); 200 µl of a solution containing zymolyase 20T (ICN Biomedicals) 3 mg and novozyme (Calbiochem) 3 mg was added followed by β-mercaptoethanol (Sigma) 175 µl, and the suspension was incubated at 30°C for 5 h. The mycelial mass was pelleted by centrifugation at 2000 *g*, suspended in 2 ml of GES reagent (5 M guanidium thiocyanate, 100 mM EDTA, lauroylsarcosine 0.5%) [15] and kept at room temperature for 30 min. After the addition of cold 5 M potassium acetate (Sigma) 400 µl and incubation for 30 min on ice, chloroform:pentanol (24:1; Sigma) 2 ml was added, mixed and then centrifuged at 2000 *g* for 5 min. The upper aqueous phase was removed and DNA was precipitated with an equal volume of cold absolute ethanol and placed at –20°C overnight. The DNA was pelleted by centrifugation at 4000 *g* for 10 min, dissolved in 0.75 ml TE buffer (10 mM Tris, 1 mM EDTA, pH 8) and 20 µl of RNAase 5 mg/ml were added and allowed to digest for 2 h at 37°C. The DNA solution was then purified by phenol-chloroform extraction and the DNA was precipitated with ethanol. Finally, after centrifugation at 10 000 *g* for 5 min the DNA pellet was dissolved in 50 µl of TE.

Restriction endonuclease analysis

DNA solution (25 µl) incubated at 37°C for 4 h with 10–15 U of restriction endonuclease in the buffer concentration recommended by the manufacturer. The endonucleases used were *Acc*I, *Bam*HI, *Bgl*II, *Cla*I, *Eco*RI, *Hae*III, *Hinc*II, *Hind*III, *Hinf*I, *Hpa*II, *Pst*I, *Pvu*II, *Xba*I or *Xho*I (Pharmacia, Herts, or BRL, Paisley). The DNA fragments were separated by electrophoresis in agarose 0.8% gels, submerged in TBE buffer (89 mM Trizma, 32 mM boric acid, 2.5 mM EDTA; Sigma), at 30 V for 16 h. The gels were stained with ethidium bromide and recorded on Polaroid 665 film or on a UV Product (Cambridge) video camera system.

Hybridisation with poly (dG-dT)

Restriction gels were Southern blotted [16] on to Stratagene (Cambridge) flash prime nylon membrane. Poly (dG-dT) (Pharmacia) was biotinylated with the photobiotin labelling kit from BRL according to the manufacturer's instructions. The blots were pre-hybridised for 4 h at 42°C in formamide 50%, 5 × SSC (1 × SSC is 150 mM NaCl, 15 mM sodium citrate, pH 7.0), 5 × Denhardt's solution (USB, Amersham International, Bucks) and fat-free milk 5% solution (dried fat-free UHT milk powder suspended in water to 5%). Hybridisation was performed at 42°C for 18 h in formamide 40%, 5 × SSC, 1 × Denhardt's solution, fat-free milk 5% solution, dextran sulphate 5% and poly (dG-dT) at a concentration of approximately 100 ng/ml.

The membranes were washed five times at room temperature in 2 × SSC and hybridisation was detected by the BRL streptavidin alkaline phosphatase, BluGENE detection system according to the manufacturer's instructions.

RAPD

Approximately 10 ng of DNA was placed into a 0.5-ml tube with a reaction mix containing 1 × magnesium-free reaction buffer (Promega, Hants), 2 mM MgCl₂, 3 U Taq polymerase (Promega), 0.1 mM deoxynucleotides (Sigma), 8–16 nM primer made up to a total volume of 50 µl with molecular biology grade water and overlaid with mineral oil (Sigma). Primers OPA 1–20 were obtained from Operon Technologies Products (Newcastle upon Tyne, Tyne and Wear) all with a 50 mol % G + C content. Primers 515FPL 5'-GCCAG-CAGCCGCGGTAA-3' [17], 806R 5'-GGACTACAG-GGTATCTAAT-3' [18] and 1492RPL 5'-GGTTACC-TTGTTACGACTT-3' [19] were a gift from Dr E. Power (St Thomas' Hospital, London), primers 213 5'-TCGGTGGCTGCTGCCCCACC-3' and 215 5'-GCG-TACCCCGGCGACCTCACC-3' [20], primers ERIC 2 5'-AAGTAAGTGACTGGGGTGAGCG-3' and ERIC

IR 5'-ATGTAAGCTCCTGGGGATTAC-3' [13] and HLWL85 5'-ACAAGTCTC-3' [21] were made by Pharmacia.

The PCR reactions were performed in a Hybaid thermocycler with conditions of 1 min at 94°C, 2 min at 34°C, 2 min at 72°C for 30 cycles, or a Perkin Elmer model 480 thermal cycler with conditions of 45 s at 94°C, 45 s at 34°C, 1.5 min at 72°C. The PCR products were separated on agarose 2% gels submerged in TBE at 70 V for 4 h. The DNA was stained with ethidium bromide and recorded photographically.

Amplification and restriction of the 5.8S rDNA and internal transcribed spacer regions

Primers ITS1 5' TCCGTAGGTGAACCTGCGG 3' and ITS4 5' TCCTCCGCTTATTGATATGC 3' [22] were used for amplification of dermatophyte rDNA. The reaction mix consisted of 1 × magnesium-free reaction buffer, 1.5 mM MgCl₂, 0.2 mM deoxynucleotides, 2 nM of each primer (Pharmacia), 3 U Taq polymerase and c. 10 ng DNA, made up to a total volume of 50 µl with molecular biology grade water and overlaid with mineral oil. This was placed into the Hybaid thermocycler for 30 cycles of 95°C for 1 min, 55°C for 30 s, 72°C for 1 min.

The rDNA fragments were digested with endonuclease by placing 6 µl of the amplified DNA solution into a reaction tube and adding 0.5 µl of 10 × restriction enzyme buffer, 0.5 µl restriction endonuclease and 4 µl water. This was incubated at 37°C for 4 h and the fragments were separated in polyacrylamide 6% gels (acrylamide:bis-acrylamide ratio 29:1, Sigma), submerged in TBE at 6–7 V/cm. The gels were stained with ethidium bromide and photographed. The restriction endonucleases used were: *Alu*I, *Eco*RI, *Hae*III, *Hinf*I, *Hpa*II, *Mbo*I, *Msp*I, *Rsa*I and *Taq*I from Pharmacia or BRL.

Results and discussion

Restriction digests were difficult to interpret because of background genomic smearing and the large numbers of bands produced by many of the endonucleases. Patterns from 23 *T. mentagrophytes* isolates were very similar with *Eco*RI, demonstrating minimal band variation in the 4-kb region of the gel. Fig. 1a and b shows typical patterns from isolates of *T. mentagrophytes* and *T. rubrum*. Generally, it was more difficult to obtain interpretable RFLP patterns from *T. rubrum* and *Eco*RI digests of 13 isolates showed minimal band variation in the 5–6 kb region (data not shown). The endonuclease of choice was *Eco*RI, although several other endonucleases produced some bright bands, including *Xba*I, *Xho*I and *Pst*I. *Hpa*II, *Hinf*I and *Mbo*I were not suitable, as they produced patterns in

the 5-kb region and less. However, for a typing tool to be useful the results need to be clearly distinguishable and a number of different types must be recognised for the method to have wide applicability. Few differences in the REA patterns were detected, the results were often difficult to see and these factors combined with gel-to-gel variation made interpretation difficult. Therefore a DNA probe was selected to enhance the interpretation of the results and to provide better discrimination.

Poly (dG-dT) has been used successfully to differentiate strains of *C. albicans* [23], *C. glabrata* [24] and *Malassezia pachydermatis* [12] and appeared to be an ideal probe for the epidemiology of the dermatophytes. Hybridisation to blots of *Eco*RI-digested DNA demonstrated minimal variation in 28 isolates of *T. mentagrophytes* around 9 kb, and in 12 isolates of *T. rubrum* between 3 and 4 kb (Fig. 2). On this basis, only two or three types of each species could be detected. The choice of restriction endonuclease can affect the discrimination of strains [12, 24, 25] and hence the discrimination of the probe. Therefore the panel of restriction endonucleases was used to digest DNA from three isolates of each species and the blots of these gels were probed with poly (dG-dT). As so few variations between isolates had been detected by *Eco*RI, and to prevent the possibility of comparing a common type with each enzyme, at least one of the isolates was varied in each digest. All the endonucleases tested produced almost identical poly (dG-dT) patterns for isolates of *T. mentagrophytes* (Figs 2 and 3) and of *T. rubrum* (Figs 2 and 4), and although minor differences of one band were seen these would not be sufficient for the discrimination of large numbers of isolates. Micro-organisms have been shown to exhibit restriction fragment length polymorphisms due to point mutations [25], and the *C. albicans*-specific 27A probe has been shown to give small differences in the patterns of the same isolate after several subcultures [26]. Therefore, the small differences described from the dermatophyte typing by REA and poly (dG-dT) would be of doubtful significance for epidemiological purposes.

One of the newest techniques for large-scale strain differentiation is RAPD. After selection of a primer that confers the best discrimination, RAPD has been shown to differentiate strains of *C. albicans*, *Aspergillus fumigatus* and several bacterial species [8, 10, 14, 27, 28]. A total of 26 primers was tested for the discrimination of up to eight isolates of *T. rubrum* and eight of *T. mentagrophytes*. The majority of primers tested were 10-mers with GC contents of 50 mol%; however, other primers of 17–21-mer lengths and with GC contents of 42–76 mol% were tried. All the primers enabled the two species to be recognised, but none could distinguish between the isolates of *T. rubrum*. From the eight *T. mentagrophytes* isolates

Fig. 1. *Eco*RI digests of DNA from isolates of *T. mentagrophytes* (Tm) and *T. rubrum* (Tr). (a) lane 1, λ *Hind*III marker; 2, Tm 204; 3, Tm H211; 4, Tr H192; 5, Tm H155; 6, Tm UID; 7, Tm L128; 8, Tr H271; 9, Tr H296; 10, Tm H167. (b) lane 1, λ *Hind*III marker; 2, Tm H118; 3, Tr H153; 4, Tm H155; 5, Tm H156; 6, Tm H164; 7, Tm H167; 8 Tm H186. The figure shows the similarity of the *T. mentagrophytes* isolates with variation only detectable around the 4-kb region of the gels.

Fig. 2. Poly (dG-dT) blot of *Eco*RI-digested DNA from isolates of *T. mentagrophytes* and *T. rubrum* demonstrating species differences, but only minor strain differences. Lane 1, λ Hind III; 2, Tr 035; 3, Tr H391; 4, Tr 011; 5, Tm 138; 6, Tm 349; 7, Tm 439.

Fig. 3. Poly (dG-dT) blots of *T. mentagrophytes* DNA digested with *Xba*I, *Pst*I or *Cla*I. Lane 1, λ Hind III; 2, 009 *Xba*I; 3, 143 *Xba*I; 4, 012 *Pst*I; 5, 201 *Pst*I; 6, 157 *Cla*I; 7, 138 *Cla*I.

examined, primer OPA13 produced differences of two bands with isolate M348 and three bands with isolate 93M236 (Fig. 5). Four patterns were produced by primer HLWL85: isolates H78, 143, 349, 012 and H211

showed one pattern; isolates L83 and M348 differed from each other and the other pattern by one band in each case; isolate 93M236 differed from the other isolates in at least six bands. When the sites of isolation of these fungi are considered, isolate M348 was from the skin of the scalp, isolate 93M236 was grown from fingernail and the six other isolates originated from skin or nail from the feet. The variations in the number of bands could indicate some genetic differences between the isolates, or perhaps the variety of *T. mentagrophytes*. The latter can be determined by mating tests with the telomorphs of *T. mentagrophytes*. Liu *et al.* [29] have demonstrated that this may be achieved by RAPD and differentiated *T. mentagrophytes* var. *interdigitale* from *T. mentagrophytes* var. *mentagrophytes* – although the patterns were very similar and differed by only one band.

Three of the primers screened had been applied to RAPD studies of other fungi. Primers ERIC 2 and ERIC IR were used for studies of *C. albicans* [30] and *Malassezia* species [13], but unfortunately these primers afforded no subspecies division of *T. rubrum* and only distinguished isolate 93M236 from the other *T. mentagrophytes*. Primer HLW85 was used to discriminate isolates from oral *C. albicans* infections [10], but failed to separate the *T. rubrum* isolates, although subtle differences were detected amongst *T. mentagrophytes*. However, all these primers permitted differentiation of the two dermatophyte species and concurred with the results of Liu *et al.* [31], who

Fig. 4. Poly (dG-dT) blots of *T. rubrum* DNA digested with *Xba*I, *Pst*I or *Cla*I. Lane 1, λ *Hind*III; 2, 108 *Xba*I; 3, 038 *Xba*I; 4, 145 *Pst*I; 5, 171 *Pst*I; 6, 149 *Cla*I; 7, 93M161 *Cla*I.

differentiated the species *T. mentagrophytes*, *T. rubrum* and *T. tonsurans* with a 10-mer oligonucleotide.

Recently, primers have been designed for the PCR detection of fungal DNA selecting a region of rDNA as the target [32]. Amplification of the 18S rDNA region followed by REA provided a method of speciating candidal yeasts [33]. A similar approach, this time analysing the 5.8S and internal transcribed spacer (ITS) regions of the rDNA, separated a selection of basidiomycetous yeasts [34]. Comparisons of these DNA sequences revealed similarity between species in the 5.8S gene, but indicated that there was some variation in the ITS regions. When the 5.8S gene and ITS regions were amplified from the rDNA of seven *T. mentagrophytes* and four *T. rubrum* isolates and digested with up to nine restriction endonucleases, only one isolate of *T. mentagrophytes* (93M236) could be distinguished – although again the two species could be recognised easily (Table 1).

Variation in the intergenic spacer regions of rDNA has been reported between isolates of other fungi. For instance, part of an intergenic spacer region from the rDNA of *Asp. nidulans* was used as a probe against *Eco*RI-digested DNA from 11 isolates of *Asp. fumigatus* and three types were detected [35]. Poly-

morphisms have been identified also in the spacer regions between the 16S and 23S rDNA of *Cryptococcus neoformans* isolates [36].

A number of molecular techniques have been applied to the differentiation of dermatophytes and most have indicated these to be a closely related group of fungi [3–5, 7, 37, 38]. Isolates of *T. tonsurans* were also examined by REA, poly(dG-dT), RAPD, REA of the 5.8S-ITS region (S. A. Howell and P. Johnson, data not shown) and these were found to be identical to each other and to resemble *T. mentagrophytes*, an observation described for comparisons of mitochondrial DNA patterns [7]. Other typing methods have been employed for dermatophyte epidemiology including polyacrylamide gel electrophoresis of whole-cell proteins from *Microsporum canis* [2]. This technique demonstrated inconsistent differences between clinical isolates which all resembled the (–) mating type of *Arthroderma otae*. However, mating studies of reference (+) and (–) type strains revealed that their progeny could be distinguished, showing that the PAGE technique was capable of distinguishing between genetically different forms.

All the techniques reported here successfully distinguish between the species *T. mentagrophytes* and *T. rubrum* and would provide rapid methods for recogni-

Fig. 5. RAPD screening of *T. mentagrophytes* isolates with primers OPA 13 and HLWL85. Isolates 93M236 and M348 were distinguished by OPA 13, and primer HLWL85 distinguished isolates L83, 93M236 and M348. Lane **kb**, 1-kb ladder. Samples amplified with primer OPA 13: lane 1, L83; 2, H78; 3, 143; 4, 012; 5, H211; 6, 349; 7, 93M236; 8, M348. Samples amplified with primer HLWL85: lane 9, L83; 10, H78; 11, 143; 12, 012; 13, H211; 14, 349; 15, 93M236; 16, M348.

Table 1. *Taq*I digestion of a PCR-amplified segment of rDNA from *T. mentagrophytes* and *T. rubrum*

Species	Isolate no.	Isolation site	Approximate fragment size (bp)
<i>T. mentagrophytes</i>	12	Nail	310, 260, 90, 80
	143	Skin, foot	310, 260, 90, 80
	349	Skin, foot	310, 260, 90, 80
	L83	Nail, foot	310, 260, 90, 80
	H78	Nail, foot	310, 260, 90, 80
	93M236	Nail, finger	300, 270, 90, 80
	M348	Skin, scalp	310, 260, 90, 80
<i>T. rubrum</i>	244	Skin, foot	270, 200, 150, 90, 80
	326	Skin, foot	270, 200, 150, 90, 80
	H396	Nail, foot	270, 200, 150, 90, 80
	AJ2	Nail, foot	270, 200, 150, 90, 80

tion of isolates reluctant to spore on laboratory media. However, further detailed study is required to find a suitable typing tool for the epidemiology of wild-type clinical dermatophyte isolates.

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