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PCR identification of *Trichophyton mentagrophytes* var. *interdigitale* and *T. mentagrophytes* var. *mentagrophytes* dermatophytes with a random primer

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Dermatophytes are a group of keratinophilic fungi falling within the genera of *Epidermophyton*, *Microsporum* and *Trichophyton*. The genus *Trichophyton* is particularly important and complex; it comprises at least 15 recognised species. In addition, there are several different variants in the species *T. mentagrophytes*, which occur both in man and animals. The current methods of determining *T. mentagrophytes* varieties may require several different culture media and time-consuming procedures, as well as specialist skills. This study used a random primer, 5'-GAGCCCGACT-3', in the arbitrarily primed polymerase chain reaction (AP-PCR) and showed that the two common *T. mentagrophytes* varieties (var. *interdigitale* and var. *mentagrophytes*) can be clearly identified on the basis of their characteristic DNA band patterns. The relative reproducibility, ease of use and precision of this method make the AP-PCR a valuable tool in the laboratory diagnosis of human dermatophytosis.

Introduction

Tinea (or ringworm) is a common disease of man and animals resulting from the infection of skin, hair and nails by keratinophilic fungi – dermatophytes. Taxonomically dermatophytes are divided into three genera: *Epidermophyton*, *Microsporum* and *Trichophyton*. While *Epidermophyton* is represented by a single species (*E. floccosum*), *Microsporum* and *Trichophyton* contain multiple species, many of which are pathogenic to man [1].

The genus *Trichophyton* is particularly important, and represents the most frequently isolated of all dermatophytes from human patients. According to recent surveys, *T. rubrum* makes up c. 55% and *T. tonsurans* 31% of the clinical dermatophyte isolations in the USA [2–3]. Similarly, *T. rubrum* accounts for 65% and *T. mentagrophytes* 32% of the clinical isolations in Victoria, Australia (unpublished data). The genus *Trichophyton* is also extremely complex. Apart from the existence of at least 15 recognised species in the genus [1], there are several different variants within the species of *T. mentagrophytes*, occurring both in man and animals [4]. While *T. mentagrophytes* var.

interdigitale is anthropophilic and frequently found in man, *T. mentagrophytes* var. *mentagrophytes* is zoophilic and isolated mainly from animals. Furthermore, *T. mentagrophytes* var. *quinckeanum* is seen in rodents, and *T. mentagrophytes* var. *erinacei* is associated with hedgehogs [4]. Interestingly, some authors refer to the different varieties of *T. mentagrophytes* as separate species, e.g., *T. interdigitale* rather than *T. mentagrophytes* var. *interdigitale*. Although some zoophilic varieties of *T. mentagrophytes* produce only minor or subclinical infections in animals, they can initiate a severe inflammatory response in man [4]. Therefore, from an epidemiological point of view, it is important to determine the variety of *T. mentagrophytes* and its origin in order to prevent spread of infection.

At present, the laboratory identification of *Trichophyton* species and varieties is based on microscopic examination and in-vitro culture. The current methods of determining *T. mentagrophytes* varieties may involve several different culture media such as Littman Oxgall agar, Lactritmel agar, Sabouraud's dextrose agar with NaCl 5%, peptone agar 1%, hydrolysis of urea, vitamin-free agar (*Trichophyton* no. 1 agar) and in-vitro hair perforation test. These tests are costly and time-consuming and also demand specialist skills. Therefore, it is no surprise that some results may take weeks to produce.

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With the development of polymerase chain reaction (PCR) technology, in particular the application of the arbitrarily primed PCR (AP-PCR) or random amplified polymorphic DNA (RAPD) technique [5–7], the potential to accelerate and improve the laboratory diagnosis of dermatophytes has been greatly enhanced. The PCR technology enables rapid detection and determination of various dermatophytes from minute amounts of starting materials, and the AP-PCR has the additional advantage of obviating the need to know the detailed sequences of the gene regions concerned as well as the necessity to further manipulate after amplification. A recent study demonstrated that the use of a random primer (5'-ACCCGACCTG-3') in the AP-PCR can rapidly differentiate several common *Trichophyton* dermatophytes including *T. rubrum*, *T. mentagrophytes* and *T. tonsurans* [8]. However, *T. mentagrophytes* var. *interdigitale* and var. *mentagrophytes* formed identical DNA band patterns, and could not be distinguished with the random decamer 5'-ACCCGACCTG-3' in the AP-PCR [8]. This prompted the search for additional random primers that could be used in the AP-PCR for improved determination of *T. mentagrophytes* varieties, especially between the two common varieties, *T. mentagrophytes* var. *interdigitale* and var. *mentagrophytes*. This study provides evidence that *T. mentagrophytes* var. *interdigitale* and var. *mentagrophytes* are distinguishable on the basis of their characteristic DNA bands amplified through the AP-PCR with a different random primer, 5'-GAGCCCGACT-3'. Future examination of the nucleotide base sequences of the specific bands observed will provide further insights into the genetic structures and possible functions of the gene regions involved. This in turn will lead to improved understanding of the genetic relationship among different *T. mentagrophytes* varieties, as well as enhanced diagnosis of human dermatophytosis.

Materials and methods

Dermatophytes

Dermatophytes were routinely isolated in this laboratory from infected skin, nails and hair of human patients. Initially, a small proportion of the samples was suspended in a drop of examination buffer – 0.5 ml of Blankophor BA 267 (Bayer) 0.1% w/v, 0.5 ml of KOH 10% and glycerol 50 µl – for detection by fluorescent microscopy. The samples were then inoculated on to Dermasel Agar (Oxoid) and Sabouraud Dextrose Agar (Oxoid) for incubation at 26°C for up to 4 weeks. The resultant dermatophyte isolates were identified by microscopic and culture techniques. Lactritmel agar, Christensens urea, Sabouraud dextrose agar with NaCl 5%, peptone agar 1% and Littman Oxgall agar were used for identification of *T. mentagrophytes* var. *mentagrophytes* and Lactritmel agar, Christensens urea, and occasionally Littman Oxgall agar for *T. mentagrophytes* var. *interdigitale*.

In the present study, 64 dermatophyte isolates were examined by the AP-PCR (Table 1). These included *Trichophyton mentagrophytes* var. *interdigitale* (20 isolates), *T. mentagrophytes* var. *mentagrophytes* (four), *T. mentagrophytes* var. *quinckeanum* (seven), *T. mentagrophytes* var. *erinacei* (one), *T. rubrum* (10), *T. violaceum* (three), *T. ajelloi* (one), *T. equinum* var. *autotrophicum* (one), *T. schoenleinii* (one), *T. terrestre* (one), *T. tonsurans* (six), *Microsporum canis* (three), *M. persicolor* (one), *M. cookei* (one) and *Epidermophyton floccosum* (four) (Table 1). Except for *T. mentagrophytes* var. *quinckeanum*, *T. ajelloi*, *T. equinum* var. *autotrophicum*, *T. schoenleinii* and *T. terrestre* isolates, which were obtained from reference collections in Australia and New Zealand, all dermatophytes under investigation originated from clinical samples in this laboratory. It is of interest to note that while *T. mentagrophytes* var. *interdigitale* was generally isolated from feet (including interdigital skins and toe nails), three of the four *T. mentagrophytes* var. *mentagrophytes* isolates were from the skin scrapings of the forearms of animal handlers who had dealt with an infected kangaroo, and the other from the chin of a child who had been playing with an infected pet.

Purification of fungal genomic DNA

Genomic DNA from dermatophyte isolates was purified as described previously [8]. Briefly, a dermatophyte isolate grown on a primary culture slope was collected with a sterile spatula into a 15-ml tube with 4 ml of SET buffer (75 mM NaCl, 25 mM EDTA, 20 mM Tris-HCl, pH 8). The mycelia were disrupted by mechanical shearing several times through an 18-gauge needle. Following the addition of 1 ml of freshly made lysozyme solution (Boehringer) 2.5 mg/ml, the tubes were incubated at room temperature for 1 h, 25 µl of RNAase (free of DNAase) 10 mg/ml were added, and the tubes were incubated at 37°C for 30 min. Next, 500 µl of SDS 10% and 50 µl of proteinase K 10 mg/ml were added and the tubes were incubated at 50°C for 1 h. Then 2 ml of 6 M NaCl and 7.5 ml of chloroform were added, mixed gently in a rotating shaker for 10 min and centrifuged at 3500 rpm for 20 min. The supernatant was transferred to a new 15-ml tube, and the DNA was precipitated with isopropanol 7.5 ml by gentle inversion and subsequent centrifugation at 3500 rpm for 20 min at 4°C. After washing with ethanol 70%, the DNA was vacuum-dried and dissolved in 1 ml of 1 × TE (10 mM Tris-HCl, pH 8.0, and 1 mM EDTA, pH 8.0). The DNA concentration was determined by absorption at UV 260 nm in a spectrophotometer, and a sample of the purified DNA was diluted to c. 10 ng/µl in sterile water which was stored at –20°C until used.

Arbitrarily primed PCR (AP-PCR)

The arbitrarily primed PCR was performed in a reaction mixture volume of 30 µl. The reaction mixture

was made up of *c.* 10 ng of genomic DNA, 50 mM KCl, 10 mM TRis-HCl (pH 9.0), 10 mM NaCl, 0.5 mM DTT, 2.5 mM MgCl₂, Triton X-100 0.1%, 200 μM of each of dNTPs (dATP, dCTP, dGTP and dTTP), 0.7 U of Taq DNA polymerase (Life Technologies) and 15 pmol of one of the 60 random decamers (Operon Technologies, USA). The reaction mixture was incubated in a GeneAmp™ PCR System 9600 (Perkin Elmer, Roche, USA) following the following protocol: three cycles of 94°C for 60 s, 36°C for 45 s, 72°C for 90 s and 32 cycles of 94°C for 30 s, 36°C for 45 s, 72°C for 90 s. A tube with no template DNA was included as a negative control. Upon completion, 5 μl of 10 × DNA sample buffer containing bromophenol and glycerol were added to each tube, from which *c.* 10 μl was electrophoresed (at 5 V/cm) in an agarose 1.5% gel in 1 × TAE buffer in the presence of ethidium bromide, and subsequently detected under UV light.

Results

Most *T. mentagrophytes* var. *interdigitale* and var. *mentagrophytes* isolates could be distinguished on the basis of their gross colonial and microscopic morphology, as the former tended to have powdery to downy

colonies and the latter powdery to granular colonies. Microscopically, both varieties possessed septate hyphae with single-celled hyaline microconidia, of which *T. mentagrophytes* var. *interdigitale* were subspherical to pyriform and var. *mentagrophytes* were spherical to subspherical. Nevertheless, *T. mentagrophytes* var. *mentagrophytes* isolates generally had more smooth, thin-walled, clavate multiseptate macroconidia than var. *interdigitale*. For some isolates, the cultural characteristics were inconsistent, and further testing was required to confirm their identity. Although both varieties hydrolysed urea, *T. mentagrophytes* var. *mentagrophytes* isolates often produced a yellowish diffusible pigment on Littman Oxgall agar, which was absent from cultures of var. *interdigitale*.

The results of the arbitrarily primed PCR appeared to be more discriminatory. Of the 60 random decamers examined in the AP-PCR, one primer (5'-GAGCCC-GACT-3') produced distinct band patterns from genomic DNA of different *Trichophyton* species and varieties (Fig. 1 and Table 1). It was particularly noticeable that while *T. mentagrophytes* var. *interdigitale* isolates generated DNA bands of 3.5, 2.8 and 1.3 kb (Fig. 1b–g), *T. mentagrophytes* var. *mentagrophytes* isolates had bands of 3.5 and 2.8 kb only (Fig. 1h–k) in AP-PCR with this primer. Furthermore, the

Fig. 1. Agarose gel electrophoresis of dermatophyte DNA products generated through the arbitrarily primed PCR with the random primer 5'-GAGCCCGACT-3'. Lane **a**, DNA mol.wt marker (λ HindIII + EcoRI) in kb; **b–g**, DNA products amplified from *T. mentagrophytes* var. *interdigitale* isolates; **h–k**, *T. mentagrophytes* var. *mentagrophytes* isolates; **l**, *T. mentagrophytes* var. *quinckeanum*; **m**, *T. mentagrophytes* var. *erinacei*; **n**, *T. rubrum*; **o**, *T. violaceum*; **p**, *T. ajelloi*; **q**, *T. equinum* var. *autotrophicum*; **r**, *T. schoenleinii*; **s**, *T. terrestre*; **t**, *T. tonsurans*; **u**, *M. canis*; **v**, *M. persicolor*; **w**, *M. cookei*; **x**, *E. floccosum*; **y**, negative control with no template DNA.

Table 1. Summary of dermatophyte species and varieties examined in arbitrarily primed PCR with the random primer 5'-GAGCCCGACT-3'

Dermatophyte species and varieties	PCR bands (kb)	Number of isolates tested
<i>T. mentagrophytes</i> var. <i>interdigitale</i>	3.5, 2.8, 1.3	20
<i>T. mentagrophytes</i> var. <i>mentagrophytes</i>	3.5, 2.8	4
<i>T. mentagrophytes</i> var. <i>quinckeanum</i>	3.5, 1.0	7
<i>T. mentagrophytes</i> var. <i>erinacei</i>	1.5, 0.4	1
<i>T. rubrum</i>	3.4, 2.5, 0.6, 0.3	10
<i>T. violaceum</i>	3.5, 2.6, 1.1, 0.4	3
<i>T. ajelloi</i>	1.9, 0.7, 0.5	1
<i>T. equinum</i> var. <i>autotrophicum</i>	3.4, 2.8, 1.0	1
<i>T. schoenleinii</i>	1.0	1
<i>T. terrestre</i>	3.5, 1.2, 0.9, 0.7	1
<i>T. tonsurans</i>	3.4, 2.8, 0.9	6
<i>Microsporum canis</i>	3.0, 1.2	3
<i>M. persicolor</i>	2.6, 1.3, 1.0	1
<i>M. cookei</i>	2.7, 1.2	1
<i>Epidermophyton floccosum</i>	0.7	4

other two *T. mentagrophytes* varieties examined (var. *quinckeanum* and var. *erinacei*) also demonstrated distinct band patterns (bands of 3.5 and 1.0 kb for the former and bands of 1.5 and 0.4 kb for the latter) (Fig. 1l–m). It was significant that all other dermatophyte species produced characteristic band patterns as well in the AP-PCR with the random primer (Fig. 1n–x and Table 1). The negative (no template DNA) control displayed no band at all in the test (Fig. 1y).

Discussion

The results obtained suggest that although *T. mentagrophytes* varieties are taxonomically closely related, they possess certain unique genetic structures, which are detectable by DNA amplification technology. The observation that *T. mentagrophytes* var. *interdigitale* isolates formed DNA bands of 3.5, 2.8 and 1.3 kb and var. *mentagrophytes* isolates produced bands of 3.5 and 2.8 kb only in the AP-PCR with the random primer 5'-GAGCCCGACT-3' makes their genetic differentiation possible. In addition, the other two *T. mentagrophytes* varieties, i.e., var. *quinckeanum* and var. *erinacei*, appeared to be distinguishable from *T. var. interdigitale* and var. *mentagrophytes*, and also from each other, as they showed characteristic bands in the AP-PCR with the random primer. Furthermore, it would be interesting to examine other *T. mentagrophytes* varieties such as *T. mentagrophytes* var. *nodulare*, and see if they can also be distinguished in the AP-PCR with this random primer.

Despite its reliance on random priming events, the AP-PCR is remarkably reproducible. Similar to the earlier report [8], AP-PCR with the random primer 5'-GAGCCCGACT-3' is also reasonably robust in that all major bands can be repeatedly obtained from run to run (data not shown). As the AP-PCR involves DNA amplification, it is highly sensitive and rapid compared to conventional methods. Moreover, because of its ability to detect differences at the genetic level, the

AP-PCR is intrinsically precise, and is not subject to the phenotypic variations which are commonly shown by dermatophytes, frequently affecting the interpretation of in-vitro culture results. In fact, of the *T. mentagrophytes*, *T. rubrum* and *T. tonsurans* isolates examined, some demonstrated considerable variations in terms of colony colour and morphology, and yet gave the same band patterns as those with typical cultural characteristics (data not shown).

The exact genetic structures and functions of the characteristic bands amplified from *Trichophyton* dermatophytes with the random primer 5'-GAGCCCGACT-3' are not clear at the moment; however, it is anticipated that the ongoing analysis of the nucleotide base sequences of these bands will shed further light into the molecular basis of and genetic relationship among various *Trichophyton* species and varieties, and result in further improvement in PCR-based assays for the diagnosis of human dermatophytosis in terms of specificity and direct application to clinical specimens.

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