REVIEW ARTICLE

Application of PCR to the identification of dermatophyte fungi

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Infection of the keratinised tissues (skin, hair and nails) in man and animals by keratinophilic fungi (dermatophytes) results in dermatophytosis (also known as tinea or ringworm). As conventional laboratory procedures for the identification of dermatophytes are either slow or lack specificity, improved diagnostic methods are required. The application of nucleic acid amplification technology has made rapid and precise identification of dermatophytes possible. Recent studies have shown that when one of the four random primers (OPAA11, OPD18, OPAA17 and OPU15) was used in arbitrarily primed PCR (AP-PCR), up to 20 of the 25 dermatophyte species or subspecies under investigation could be distinguished on the basis of characteristic band patterns detected in agarose gel electrophoresis. A combination of two random primers (OPD18 and OPAA17) used in separate reaction tubes identified 23 of the 25 dermatophyte species or subspecies examined. AP-PCR provides a rapid and practical tool for identification of dermatophyte isolates that is independent of morphological and biochemical characteristics and thus enhances laboratory diagnosis of dermatophytosis.

Introduction

Dermatophytes comprise three closely related keratinophilic fungal genera (*Epidermophyton*, *Microsporum* and *Trichophyton*) that colonise keratinised tissues (skin, hair and nails) of man and animals. The colonisation process is assisted by the release of various proteolytic and other enzymes by dermatophytes, which in turn provoke inflammatory responses in the host, resulting in dermatophytosis (also known as tinea or ringworm) [1].

Whereas the genus *Epidermophyton* is represented by a single species (*E. floccosum*), the genera *Microsporum* and *Trichophyton* are complex and made up of multiple species [2]. In particular, besides the presence of >15 recognised species in the genus *Trichophyton*, several different variants are also identified within the species of *T. mentagrophytes* [2]. Because many *Microsporum* and *Trichophyton* species are pathogenic for man and the dermatophytosis caused by various dermatophyte species cannot be easily differentiated on the basis of clinical manifestations, it has been necessary to develop improved laboratory diagnostic methods.

For many years, conventional laboratory methods based on the detection of phenotypic characteristics, such as microscopy and in-vitro culture, have played an essential role in dermatophyte identification. However, these procedures generally suffer from the drawbacks of being either slow or non-specific. Recent developments and applications of nucleic acid amplification technology have provided the opportunity to enhance the quality and speed of dermatophyte diagnosis. This review presents a brief update on the use of molecular detection methods, in particular, arbitrarily primed PCR (AP-PCR) for rapid identification of dermatophyte isolates.

Clinical background

Despite being highly infectious and extremely common, dermatophytes have frequently been regarded as insignificant human pathogens, because dermatophytosis is generally mild and not life-threatening. The fact that, historically, many dermatophyte species and strains had relatively restricted geographical distribution also reinforces this notion. However, significant demographic changes that have taken place in the past few decades have led to some noticeable modifications in dermatophyte profiles. For instance, in the USA, the most commonly isolated dermatophytes have shifted from *M. audouinii* in the 1940s to *T. rubrum* in the

1960s and *T. tonsurans* in the 1990s [3, 4]. Similarly, in Victoria, Australia, while *T. mentagrophytes* isolations showed a slight decline over the past three decades (from 29.0% in the 1960s to 26.5% in the 1990s), *T. rubrum* has become more prevalent (from 43.1% to 69.5%). However, *M. canis* has decreased dramatically during the same period (from 19.4% to 0.9%) [5].

Furthermore, with an increasingly aging population and the increasing occurrence of immunocompromised patients, many previously uncommon fungal infections, including dermatophytosis, have emerged as important causes of morbidity. The application of chemotherapy has also contributed to the occasional modification and alteration of the morphological characteristics of dermatophytes, resulting in atypical colonial growth and appearance and complicating laboratory identification procedures based on phenotypic features. Therefore, the availability of improved laboratory methods is essential for rapid and accurate detection and differentiation of the dermatophytes involved, so that appropriate treatment and preventative measures can be applied.

Conventional diagnostic methods

The current diagnosis of dermatophytosis is based upon microscopic identification of hyphae directly from lesion materials followed by in-vitro culture. Although rapid and economical, microscopical examination is not species-specific. Also, this technique is relatively insensitive, showing false negative results in up to 15% of cases [1]. Therefore, its role in dermatophyte diagnosis is limited to giving an initial, quick screen for hyphae and other mycotic elements, and further follow-up with in-vitro culture is invariably required irrespective of the microscopic observations, as effective treatment and prevention of dermatophytosis depend on knowledge of the specific dermatophyte involved. In-vitro culture is capable of providing a species-specific determination of dermatophytes on the basis of morphological and biochemical criteria in 10–15 days in >95% of cases. However, for some unusual and atypical isolates, identification may require a range of culture media such as Christensen's urea agar, Lactrimel agar, Littman Oxgall agar, 1% peptone agar, Sabouraud's dextrose agar with NaCl 5%, Trichophyton agars (no. 1–7) and the in-vitro hair perforation test. These tests are not only costly and time-consuming (some results may take a further 3–4 weeks after primary isolation), but also demand specialist skills. More importantly, because these conventional methods depend on measurement of the phenotypic characteristics of dermatophytes, they can be easily influenced by outside factors (such as temperature variations and chemotherapy) that may interfere with the metabolic process of the dermatophyte and affect the interpretation of in-vitro culture results.

Nucleic acid-based techniques

Nucleic acid-based techniques rely on the detection of genotypic differences in pathogenic organisms. They are intrinsically more specific and more precise than those based on phenotypic features [6], as genotypic characteristics are less likely to be affected by external influences such as temperature variations and chemotherapy. The recent development of nucleic acid amplification technology such as PCR, as well as arbitrarily primed PCR (AP-PCR) or random amplified polymorphic DNA (RAPD) [7–9], has further enhanced the sensitivity and speed of nucleic acid-based diagnostic procedures. Several groups have successfully applied PCR technology to the phylogeny and diagnosis of pathogenic fungi, including dermatophytes [10–18]. These studies have exploited the differences of dermatophytes and other fungi in their small (18S) [10–13] and large (25S) [14, 15] ribosomal RNA genes and mitochondrial DNA [16], as well as chitin synthase 1 gene [17, 18]. This has resulted in a more accurate assessment of phylogenetic relationships among dermatophytes and other fungi [10, 14, 18] and improved detection and identification of some common dermatophytes [11–13, 15–17]. Nevertheless, as these methods frequently require additional manipulation such as restriction endonuclease digestion, hybridisation or sequencing after amplification, they are not easily adopted for routine use in clinical laboratories. Moreover, many of these reported methods are capable of distinguishing only a limited number of dermatophyte species and, therefore, are more suited for genus-specific rather than species-specific identification.

AP-PCR

Random amplification and identification of DNA fragments unique to a certain strain or species in AP-PCR enables various micro-organisms to be differentiated rapidly and precisely from each other [7–9]. AP-PCR amplification is achieved through the use of short random primer(s) (usually 10 nucleotides long) at relatively low temperature (normally <40°C). Although the exact mechanisms behind AP-PCR are not properly understood, it has been hypothesised that by reducing the stringency of the primer annealing temperature, a random primer that shows no complete homology to a genome may have a perfect match of two-to-three nucleotides from the 3'-end of the primer to the template strand to allow annealing and priming complementary strand synthesis by the DNA polymerase, as a putative three-nucleotide sequence can be found in principle once in a 64-nucleotide sequence (4^3 permutations). When two such annealing and priming events occur within a certain distance of each other and in proper orientation, the sequence between the matching sites can be amplified effectively. Subsequent identification of characteristic DNA band patterns in agarose gel electrophoresis enables differentiation of

various micro-organisms. The application of AP-PCR obviates the necessity to know the detailed sequences of the gene regions concerned as well as the need for further manipulation after amplification.

By assessing >90 individual random primers in AP-PCR it was possible to select several with potential for identification and differentiation of dermatophytes. These included OPAA11 (5'-ACCCGACCTG-3') [19, 20], OPD18 (5'-GAGAGCCAAC-3') [20], OPAA17 (5'-GAGCCCGACT-3') [21] and OPU15 (5'-ACGGGCCAGT-3') [22]. By employing a rapid DNA purification protocol [23] and, more recently, a further improved mini-preparation procedure [24], 230 isolates from 25 dermatophyte species and subspecies (varieties), comprising 16 *Trichophyton* species and subspecies (varieties), eight *Microsporum* species and *E. floccosum*, were examined with one of these four primers in AP-PCR [19–22] (Table 1). The results indicated that of the 25 dermatophyte species and subspecies examined, 16 (except between *T. equinum* var. *autotrophicum* and *T. tonsurans*; among *T. gourvillii*, *T. rubrum*, *T. soudanense* and *T. violaceum*; and among *T. mentagrophytes* var. *interdigitale*, var. *mentagrophytes* and var. *nodulare*) displayed distinct DNA band patterns after amplification with random primer OPAA11 [19, 20] (Tables 1 and 2). Similarly, 19 of the 25 dermatophyte species or subspecies (no differentiation between *T. equinum* var. *autotrophicum* and *T. mentagrophytes* var. *nodulare*; between *T. gourvillii* and *T. rubrum*; and between *T. mentagrophytes* var. *interdigitale* and var. *mentagrophytes*) could

be identified with random primer OPD18 [20] (Tables 1 and 2); 20 (except among *T. gourvillii*, *T. rubrum* and *T. soudanense*; and between *T. mentagrophytes* var. *interdigitale* and var. *nodulare*) could be identified with random primer OPAA17 [21] (Tables 1 and 2); and 19 (except among *T. gourvillii*, *T. rubrum* and *T. soudanense* and among *T. mentagrophytes* var. *interdigitale*, var. *mentagrophytes* and var. *nodulare*) could be distinguished with random primer OPU15 [22] (Tables 1 and 2). It appears that the usefulness of AP-PCR for the identification of dermatophyte species and subspecies is further enhanced by a combination of two random primers with PCR performed in separate reaction tubes [20]. For instance, the use of OPAA11 and OPD18 in AP-PCR would result in the differentiation of 21 (except between *T. gourvillii* and *T. rubrum*; and between *T. mentagrophytes* var. *interdigitale* and var. *mentagrophytes*) among the 25 dermatophyte species or subspecies examined [20] (Tables 1 and 2). More importantly, the use of OPD18 and OPAA17 would help identify 23 (except between *T. gourvillii* and *T. rubrum*) of the 25 dermatophyte species or subspecies examined (Tables 1 and 2). It is of interest to note that although DNA from non-dermatophyte fungi such as *Scytalidium*, *Fusarium*, *Aspergillus* and *Candida* spp. also generated DNA products in AP-PCR with these random primers, the band patterns formed by these fungi were distinct from those formed by dermatophytes. Therefore, they could be differentiated from dermatophyte species and subspecies [25].

In terms of the time required for testing, AP-PCR has a

Table 1. Examination of DNA products from dermatophyte fungi by AP-PCR

Dermatophyte species and varieties	Number tested	DNA products (kb)* obtained with			
		OPAA11	OPD18	OPAA17	OPU15
<i>T. ajelloi</i>	1	1.7, 0.8	2.8, 1.9	1.9, 0.8, 0.5	2.2, 1.4, 1.0, 0.7
<i>T. concentricum</i>	1	1.5, 0.9	3.4, 2.1, 2.0, 1.2	2.5, 0.4	2.7, 1.1
<i>T. equinum</i> var. <i>autotrophicum</i>	1	2.5, 1.5, 0.9	3.3, 2.1, 1.0	3.5, 2.9, 1.0	0.9
<i>T. gourvillii</i>	1	1.9, 1.7, 0.5	3.1, 1.2	3.4, 2.5, 0.6, 0.4	2.6, 2.0, 1.8, 0.5
<i>T. mentagrophytes</i> var. <i>erinacei</i>	2	1.8, 1.0	3.0, 1.9, 1.3	1.5, 0.4	2.4, 1.4
<i>T. mentagrophytes</i> var. <i>interdigitale</i>	50	2.5, 1.9, 0.9	3.2, 2.0, 0.9	3.5, 2.8, 1.3	2.5, 2.0, 1.4, 0.8
<i>T. mentagrophytes</i> var. <i>mentagrophytes</i>	7	2.5, 1.9, 0.9	3.2, 2.0, 0.9	3.5, 2.8	2.5, 2.0, 1.4, 0.8
<i>T. mentagrophytes</i> var. <i>nodulare</i>	4	2.5, 1.9, 0.9	3.3, 2.1, 1.0	3.5, 2.8, 1.3	2.5, 2.0, 1.4, 0.8
<i>T. mentagrophytes</i> var. <i>quinckeanum</i>	9	1.8, 1.2	3.2, 2.1, 1.1, 0.8	3.5, 1.0	2.7, 1.8, 1.2, 0.7
<i>T. rubrum</i>	76	1.9, 1.7, 0.5	3.1, 1.2	3.4, 2.5, 0.6, 0.4	2.6, 2.0, 1.8, 0.5
<i>T. schoenleinii</i>	1	2.0, 1.3	3.2, 2.0	3.4, 0.9	2.4, 1.8, 1.2, 0.6
<i>T. soudanense</i>	7	1.9, 1.7, 0.5	3.2, 1.2	3.4, 2.5, 0.6, 0.4	2.6, 2.0, 1.8, 0.5
<i>T. tonsurans</i>	21	2.5, 1.5, 0.9	3.2, 1.8, 1.2	3.4, 2.8, 0.9	2.6, 0.8
<i>T. terrestre</i>	2	2.6	3.4, 2.8, 1.5	2.8, 1.2, 0.9, 0.7	2.0, 1.4, 0.8, 0.5
<i>T. verrucosum</i>	5	2.7, 1.9, 1.5, 0.7	3.3, 1.9, 1.2, 0.5	0.5	2.5, 1.9, 1.0
<i>T. violaceum</i>	11	1.9, 1.7, 0.5	3.2, 2.5, 1.9, 1.2	3.5, 2.6, 1.1, 0.4	2.7, 2.2, 1.9, 0.5
<i>M. audouinii</i>	3	2.8, 1.3, 0.2	2.8, 1.6, 1.4, 1.1	3.0, 2.0, 1.3	2.3, 1.3, 0.8
<i>M. canis</i>	10	2.6, 1.2, 0.8, 0.4	1.2	3.0, 1.2, 0.7	2.2, 1.0, 0.5
<i>M. cookei</i>	2	1.4, 1.2, 0.7, 0.3	2.5, 1.5, 0.9, 0.7	3.0, 2.7, 1.3	1.5, 1.1, 0.8
<i>M. ferrugineum</i>	2	2.6, 1.2, 0.4	1.5	3.0, 1.3	2.3, 1.0, 0.6
<i>M. fulvum</i>	1	1.9, 1.1, 0.8, 0.6	3.3, 2.1, 0.8, 0.3	2.7, 1.8	2.3, 1.8, 0.9, 0.4
<i>M. gypseum</i>	3	1.8, 0.8	3.2, 2.1, 1.6, 1.0	3.0, 2.7, 1.1, 0.9	2.6, 1.5, 0.8
<i>M. nanum</i>	1	2.0, 1.1, 0.7, 0.2	0.9	1.2, 0.9	2.2, 0.6
<i>M. persicolor</i>	2	1.8, 1.0, 0.5	3.0, 1.9, 1.0, 0.3	2.6, 1.3, 1.0	1.3, 0.9
<i>E. floccosum</i>	7	2.8, 2.2, 1.7, 0.9	1.8, 1.0	0.8	3.0, 2.5, 1.3
Total	230				

*The DNA products were amplified in AP-PCR from each of the 25 dermatophyte species and varieties with one of the four random primers (OPAA11, OPD18, OPAA17 and OPU15), and their sizes (in kb) determined by agarose 1.5% gel electrophoresis with λ HindIII + EcoRI as molecular markers [18–21].

Table 2. Summary of dermatophyte identification by AP-PCR

Dermatophyte species and varieties	Identifiable by			
	OPAA11	OPD18	OPAA17	OPU15
<i>T. ajelloi</i>	Yes	Yes	Yes	Yes
<i>T. concentricum</i>	Yes	Yes	Yes	Yes
<i>T. equinum</i> var. <i>autotrophicum</i>	No (1)	No (4)	Yes	Yes
<i>T. gourvillii</i>	No (2)	No (5)	No (7)	No (9)
<i>T. mentagrophytes</i> var. <i>erinacei</i>	Yes	Yes	Yes	Yes
<i>T. mentagrophytes</i> var. <i>interdigitale</i>	No (3)	No (6)	No (8)	No (10)
<i>T. mentagrophytes</i> var. <i>mentagrophytes</i>	No (3)	No (6)	Yes	No (10)
<i>T. mentagrophytes</i> var. <i>nodulare</i>	No (3)	No (4)	No (8)	No (10)
<i>T. mentagrophytes</i> var. <i>quinckeanum</i>	Yes	Yes	Yes	Yes
<i>T. rubrum</i>	No (2)	No (5)	No (7)	No (9)
<i>T. schoenleinii</i>	Yes	Yes	Yes	Yes
<i>T. soudanense</i>	No (2)	Yes	No (7)	No (9)
<i>T. tonsurans</i>	No (1)	Yes	Yes	Yes
<i>T. terrestre</i>	Yes	Yes	Yes	Yes
<i>T. verrucosum</i>	Yes	Yes	Yes	Yes
<i>T. violaceum</i>	No (2)	Yes	Yes	Yes
<i>M. audouinii</i>	Yes	Yes	Yes	Yes
<i>M. canis</i>	Yes	Yes	Yes	Yes
<i>M. cookei</i>	Yes	Yes	Yes	Yes
<i>M. ferrugineum</i>	Yes	Yes	Yes	Yes
<i>M. fulvum</i>	Yes	Yes	Yes	Yes
<i>M. gypseum</i>	Yes	Yes	Yes	Yes
<i>M. nanum</i>	Yes	Yes	Yes	Yes
<i>M. persicolor</i>	Yes	Yes	Yes	Yes
<i>E. floccosum</i>	Yes	Yes	Yes	Yes

(1)–(10) indicate that dermatophyte species or varieties with the same number in the brackets displayed identical band patterns and therefore were not separable with the random primers in AP-PCR.

clear advantage over conventional techniques based on morphological and biochemical criteria (such as *in-vitro* culture). Although most dermatophytes can be identified after primary isolation (10–15 days), a few may require secondary culture on specialised media (10–15 days) (with some atypical, unusual or slow-growing isolates, the identification process may take even longer). With a rapid DNA mini-preparation method [24], genomic DNA from dermatophytes can be obtained from dermatophyte cultures within 1 h. AP-PCR amplification and product detection are completed within 4 h. Therefore, a definitive result can be generated easily from primary dermatophyte cultures within 1 day by AP-PCR. Besides being rapid, AP-PCR is also more sensitive, as the dermatophyte DNA sequence is amplified to one billion-fold within 30 cycles of denaturing, annealing and extension processes. Furthermore, AP-PCR is inherently more precise than the conventional methods, as it measures the genotypic rather than phenotypic characteristics of dermatophytes. In fact, of the 230 dermatophyte isolates examined, some isolates of *T. rubrum*, *T. mentagrophytes* var. *interdigitale*, *T. tonsurans* and *T. violaceum* displayed unusual colony morphology, yet they formed identical band patterns to those with typical cultural characteristics. For example, the identification by AP-PCR in this laboratory of two non-pigmented (i.e., white colony) *T. violaceum* isolates, which were later confirmed by an external reference laboratory, highlights the usefulness of the technique. Moreover, AP-PCR is also robust and reproducible, and major bands can be obtained

consistently between different runs [26–28]. A further advantage of AP-PCR is that it does not require viable organisms for testing. Indeed, this laboratory has been able to confirm the identities of 20-year-old non-viable dermatophyte isolates by AP-PCR.

To summarise, AP-PCR provides a practical solution to the difficulties encountered in the identification of dermatophyte isolates with varied morphological characteristics in conventional procedures, and represents a technological advance in the laboratory diagnosis of dermatophytosis. The future development of a procedure for the isolation of DNA from clinical materials such as skin, hair and nails would further enhance the potential of AP-PCR for identification of dermatophytes.

Future perspectives

The fact that various DNA fragments can be amplified from dermatophytes and other fungi with one of four random primers in AP-PCR suggests that the gene regions recognised by these primers are probably evolutionarily variable. The elucidation of the precise structures and functions of the gene regions involved will require nucleotide sequence analysis of the characteristic bands amplified. A detailed knowledge of these gene regions will provide insight into the molecular basis and genetic relationships of various dermatophytes. Moreover, the availability of nucleotide sequence data of the distinct DNA fragments obtained

will help design primers specific for individual dermatophyte species and subspecies. The subsequent application of these specific primers in colorimetric detection systems together with an optimised protocol for DNA preparation from nails, skin and hair will vastly improve the efficiency and specificity of laboratory detection and identification of dermatophyte fungi. However, it will be quite some time before species-specific primers for each individual dermatophyte are identified and tested. Until then, AP-PCR will continue to play an important role in determining the taxonomic status of the dermatophytes concerned.

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