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MOLECULAR-GENETIC APPROACHES TO IDENTIFICATION AND TYPING OF PATHOGENIC *CANDIDA* YEASTS

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Currently, invasive candidal infections represent an increasing cause of morbidity and mortality in seriously ill hospitalised patients. Because the accurate diagnosis of candidiasis remains difficult, a fast and reliable assay for characterization of fungal pathogens is critical for the early initiation of adequate antifungal therapy and/or for introduction of preventive measures. As novel molecular genetic techniques are continuously introduced, their role in the management of infectious diseases has also been growing. Today, molecular strategies complement conventional methods and provide more accurate and detailed insight. It can be expected that future technical development will improve their potential furthermore. In this article, we provide a critical review on the value and limitations of molecular tools in pathogenic *Candida* species identification and strain typing regarding their sensitivity, discriminatory power, reproducibility, cost and ease of performance.

INTRODUCTION

Candida yeast species belong to opportunistic pathogens; they are frequently found in samples from humans and from their environment and are capable of causing a variety of diseases. The mechanism of becoming pathogenic is still unknown in these common commensals, although impaired host defence mechanisms are considered to be fundamental[1]. Systemic infections caused by *Candida* spp. are becoming increasingly common in modern hospitals and frequently occur in patients with inherited and acquired immunodeficiencies, particularly in certain risk groups of patients, such as neutropenic patients, HIV-positive patients, and patients under intensive care. Since the number of these cases has risen in the last decades, the incidence of systemic candidiasis has been steadily increasing in parallel and it also has been associated with high rates of mortality of over 30 % despite appropriate treatment[2, 3]. *Candida* is now the fourth among microorganisms responsible for nosocomial bloodstream infections[4]. Survival of critically ill patients is dependent on prompt diagnosis and subsequent early initiation of antifungal therapy[5]. Therefore, rapid and reliable methods of detection are necessary to be developed and introduced.

Unfortunately, diagnosis of invasive candidiasis is still problematic, because clinical symptoms are non-specific and conventional assays are not satisfactorily precise and may take several days to obtain results. Blood cultures, which are assumed to be the most reliable marker of invasive candidiasis, are commonly negative, whereas positive cultures from other sites may represent colonization[6]. Also, blood culture requires 2–5 days for correct identification. Molecular-genetic approaches, which rely on rapid detection of *Candida* yeasts nucleic acids in clinical samples, offer a promising alternative.

In addition to detection of *Candida* yeasts, species identification is very much in demand in some situations, because susceptibility to antifungal agents, probability of resistance development, and ability to cause disease can vary among different species. The most often identified species has always been *Candida albicans*, however the incidence of non-*albicans Candida* species has been increasing with the changing spectrum of patients and these species have also been associated with higher mortality[7]. *C. glabrata* and *C. krusei* show lower sensitivity to fluconazole in contrast to *C. albicans*, and emergence of secondary resistance in *C. lusitaniae* to amphotericin B has also been observed rarely[8, 9]. *C. parapsilosis* persists in hospital environments, thereby enhancing the chance of nosocomial infection. It also commonly colonizes skin of patients and is able to form biofilms on plastics. This often results in candidaemia in patients with indwelling venous catheters.

Also, epidemiological investigations of infection outbreaks in hospitals are highly desirable to identify the source and route of infection to eradicate it. Such investigations require accurate strain typing, because in candidaemia it is problematic to distinguish between endogenous source of infection and infection transmitted exogenously from other infected patients or even health care workers[10]. Similarly to the species identification, phenotyping or genotyping can be applied in strain typing. Phenotyping methods characterize products of gene expression, thus reflecting genetic diversity. This is also

their main shortcoming, because even minor variation in growth conditions can influence gene expression[11], which leads to poor interlaboratory reproducibility. Phenotyping systems are also considered cumbersome, time-consuming and provide limited data for differentiation between epidemiologically unrelated isolates. In addition, some *Candida* species are capable of spontaneous switching between numbers of phenotypes[12], so their phenotypic characteristics may be unstable or variable. In contrast to phenotyping, genotyping techniques detect differences in genetic information directly. Therefore, they are less sensitive to variations in growth conditions and also possess several other advantages over phenotyping procedures, e.g. higher discriminatory power, speed and reproducibility.

This review focuses on the vigorously fermenting field of molecular genetic approaches to *Candida* yeast identification and typing. Not only are new techniques and their modifications published at a growing pace, but also commercially available kits and other initiatives continually contribute to their standardization and ease of use, thus facilitating implementation of molecular approaches in routine use. It seems, that molecular techniques will soon revolutionise the identification of *Candida* species.

A. DETECTION AND SPECIES IDENTIFICATION

Techniques that rely on amplification of target yeast DNA are used almost exclusively for detection purposes, because the amount of yeast DNA available in a clinical sample is typically very low. These techniques include PCR and NASBA. Alternatively, yeast cells can first be multiplied during cultivation in blood culture bottles, and *Candida* DNA can then be detected in positive bottles by FISH with species-specific probes. This approach requires more time and is most probably less sensitive than PCR or NASBA. On the other hand, it may be more economic, because only positive bottles are examined. Also, results of FISH detection can later be verified by phenotyping of subcultured isolates, which are in addition available for strain typing. For general overview of detection techniques see also Fig. 1.

1. PCR-based Methods

The invention of PCR was a landmark in the progress of molecular microbiology and has had a substantial impact on the diagnosis of infectious diseases. The key strongpoint of these techniques consists in the amplification and detection of minute amounts of microbial nucleic acid in the background of host DNA. PCR-based methods can be appreciable especially when conventional methods are not available, are insensitive or slow.

1.1. Target and primer selection

Generally, two strategies of PCR target selection can be adopted. If species-specific sequences are selected as primer-annealing sites, PCR will enable highly specific detection of just one pathogenic yeast species. On the other hand, when universal panfungal sequences are targeted, PCR will result in amplicons in case any fungal DNA is present in samples. *Candida*-genus specific sequences can also be targeted to detect all *Candida* yeast species. If a broader spectrum of species is targeted, post-PCR analysis is necessary for subsequent species identification (see below). To ensure high sensitivity of PCR detection, primers should preferentially target multicopy genes. Also, high specificity should be secured by targeting sequences specifically found only in the pathogen of interest. The ribosomal RNA (rRNA) gene appears to meet both of these criteria. A tandem array of 50 to 100 copies of the rRNA gene can be found in the haploid genome of all fungi. This consists of the small subunit rRNA gene (18S), the 5.8S gene and the large subunit rRNA (25S) gene, separated by the internal transcribed spacer regions, ITS1 and ITS2. While rRNA genes are highly conserved in fungi, ITS regions involve both highly variable and highly conserved areas[13], thus allowing the generation of species, genus or fungus specific primer. The gene family of secreted aspartyl proteinases represents another group of sequences found in several copies in the yeast genome, which can serve as a target for PCR-amplification[14]. In addition, there are many examples of single-copy genes that can be caught by PCR and seem to be highly fungal specific, e.g. the P450 lanosterol-14α demethylase gene[15], actin gene[16] and heat shock protein 90 gene[17].

1.2. Nested PCR

Nested PCR can be used to increase both the sensitivity and specificity of PCR detection. In this approach, two rounds of PCR are performed. In the first round, outer primers target a larger region for amplification. Amplicons from this round are then added as template into the second round reaction mixture, where inner primers target a fragment of the first round amplicon. Specificity of the assay is increased, because four primers have to anneal in an arranged fashion instead of just two in a single PCR. Sensitivity is increased, because addition of fresh reagents and dilution of first round amplicons in the second round mixture enables additional amplification of a fragment of the amplicons from the first round mixture. Two-rounds setting of nested PCR can also be used to combine the advantages of broad-range and species-specific targeting of yeast sequences. The outer primers can target universal sequences resulting in amplicons in a broad range of yeast species, followed by several parallel second round reactions with species-specific inner primer pairs. When second round primers are carefully designed to prevent interference, primer mixes can be used in a common reaction mixture to reduce costs, in an approach called multiplex PCR. Nested PCR approach was adapted for use in *Candida* species detection by Kanbe et al.[18], and Bougnoux et al.[19] On the other hand, the extreme sensitivity of nested PCR results in its major drawback – the occurrence of false-positive results mainly due to the cross-contamination with previously amplified products[20], and also due to contamination with environmental microorganisms, or even contaminated commercially available reagents[21]. To

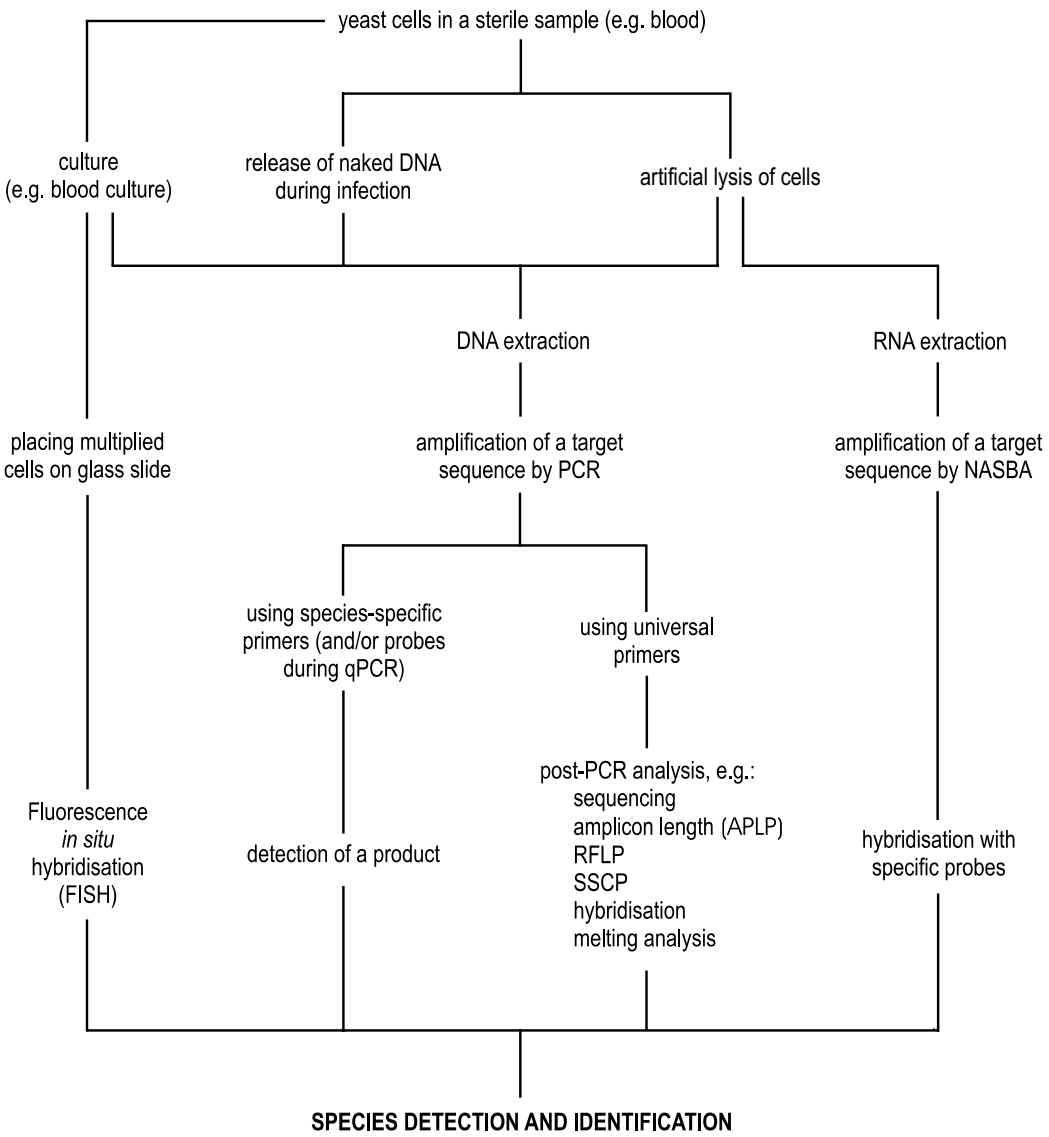

Fig. 1. General overview of detection and identification techniques.

avoid this pitfall, laboratories must follow stringent precautions such as establishing separate rooms and equipment for each step of the PCR and other procedures[22].

1.3. Real-time PCR

The real-time polymerase chain reaction uses fluorescent reporter molecules to visualize the production of amplicons during each cycle of the PCR reaction. This is in contrast to endpoint detection in conventional PCR, where the amplicon is detected after completed amplification only. Real-time monitoring of amplification based on increase of fluorescence of reporter molecules enables quantification of the target DNA, because the timepoint at which the amplicons reach a specific fluorescence level during cycling corresponds with the starting amount of target DNA. This correlation is impossible in the case of conventional PCR, where the final amount of amplicons always reaches a uniform level due to inhibition of further amplification in the plateau phase of the reaction. The process of amplification can be monitored either using labelled probes which specifically hybridise to the newly formed amplicon molecules, or by staining newly formed double-stranded DNA molecules with non-specific dsDNA binding dyes (e.g. SYBR Green I, BEBO, LC Green or BOXTO). The use of probes increases the specificity of PCR, because an additional sequence homology between the amplicon and probe is necessary for successful reporting of amplification. When a dsDNA binding dye is used instead of a specific probe, melting analysis of the amplicon has to be performed subsequently to verify the identity of the amplicon. Sometimes, unambiguous differentiation between specific and non-specific products can be problematic. On the other hand, melting analysis can provide additional useful information about the amplified sequence. Traditionally, one has to choose between the use of a sequence-specific probe and a non-specific dsDNA binding dye, because of spectra overlap in fluorescent dyes used for labelling of probes and for dsDNA staining. Recently, however, the use of BOXTO dsDNA binding dye has been reported as compatible with the use of probes labelled by FAM[23].

The use of an integrated thermocycler/fluorimeter with highly efficient heat exchange mechanism has significantly shortened the turnaround time of real-time PCR. Both amplification and detection take place in the same closed vessel, reducing post-amplification manipulation steps and dramatically decreasing the risk of false-positive results. Despite greater start-up expense and the lack of standardization, the oncoming explosion of new chemistries and instrumentation, sensitivity, reproducibility and potential for high-throughput, will nevertheless make the real-time PCR attractive and indispensable for future diagnostic mycology.

Several studies have reported the identification of *Candida* species by targeting the rRNA gene complex using real-time PCR[24-28]. The wide variety of fluorescent dyes available makes it possible to amplify multiple templates in a single tube, as fluorescent dyes with different emission spectra may be attached to the different probes, providing the dyes are compatible with the excitation and detection criteria of the real-time cycler used[29]. Bu et al.[30] described the detection and quantification of five fungal species in a multiplex real-time PCR assay.

1.4. Post-PCR analysis

Whether conventional or real-time PCR is used, several options for post-PCR analysis are available to characterize the amplicon and conclusions can be drawn on its species-specificity, especially if universal sequences are targeted for amplification. Obviously, the only ultimate and most accurate way of post-PCR analysis is direct sequencing[31]. Although commercial systems are available[32], this option is still too expensive and laborious for routine use. However, alternative sequencing techniques, e.g. pyrosequencing, are under continuous development and promise further reduction of costs in the future. All the other techniques of amplicon post-PCR analysis rely in some way on characterization of its sequence-related variability. The length of the amplicon can be roughly estimated by agarose gel electrophoresis, which represents the most simple and traditional technique of post-PCR analysis, also called Amplified product length polymorphism (APLP). More accurate length characterization of amplicons can be achieved by polyacrylamide gel electrophoresis, which can be automated in a capillary-based analyser[33, 34]. (Marino 1994, Chen 2000). Restriction analysis of amplicons represents a rather cheap and elegant but laborious and more time-consuming technique[35, 36]. Similarly, single-strand conformational polymorphism (SSCP) can be employed to evaluate sequence-based characteristics of amplicons[37, 38], but it is not widely used because of special expertise and labour needed for correct performance.

To avoid the time-consuming and laborious electrophoresis step, used traditionally in the above-mentioned techniques, two alternative approaches can be applied. Microtitration plate enzyme immunoassay (PCR-EIA) can be utilized as a user-friendly alternative, which also improves detection sensitivity[39]. Amplicons hybridise to two oligonucleotide probes, a biotin-labelled genus-specific probe and a digoxigenin-labelled species-specific probe, and the hybridisation complex is added into streptavidine-coated wells of a microtiter plate. The positive reaction is caught by peroxidase-conjugated anti-digoxigenin antibodies. Melting analysis of amplicons represents another recent promising choice, because of its simple, rapid and economic performance. It relies on staining of double-stranded DNA (amplicon) with a fluorescent dye, which is released from the structure once DNA strands are separated during denaturation (melting) with increasing temperature. The concomitant decrease of fluorescence can be followed by a fluorimeter, either embedded into a real-time PCR instrument or used as a separate melter instrument[40]. Characteristic melting curves are recorded, because the process of melting depends both upon the length of the amplicon and upon its sequence, where AT-rich regions melt at lower temperatures, whereas GC-rich ones at higher temperatures. Use of melting analysis in post-PCR analysis of pathogenic yeasts' amplicons has not been reported yet. However, there is no doubt about its potential in this area, particularly since high resolution melting analysis (HRMA) and saturating dyes are now available[41]. The first use of HRMA in diagnostic microbiology was reported in mycobacteria recently[42].

2. Nucleic Acid Sequence Based Amplification (NASBA)

NASBA is a specific and very sensitive RNA amplification technique, which exploits the action of three enzymes, i.e. reverse transcriptase, RNase H and T7 RNA polymerase, in an isothermal amplification process with cDNA as an intermediate[43, 44]. For schematic overview of its reaction steps, see Fig. 2. In medically important fungi, conserved regions of the 18S rRNA gene can be targeted by the amplification[45]. Labelled oligonucleotide probes are then hybridised to an internal specific sequence of the *Candida* yeast species. Amplification and detection can be completed within few hours and the analysis has shown a detection limit of 1 CFU. NASBA has been evaluated to detect six various *Candida* species[46]. The main benefits of NASBA compared to PCR are no need of thermal cycling instrument and specific detection of living yeast cells, because RNA unlike DNA is rapidly degraded outside cells. The main disadvantage, which prevents more widespread use of NASBA, is the high price of the three enzymes mixture.

3. Identification by Fluorescence in situ hybridisation (FISH)

Fluorescent *in situ* hybridisation (FISH) with fluorescein-labelled oligonucleotide probes is a convenient way to detect yeasts without the need of pure culture. The employment of novel PNA (peptide nucleic acid) probes combines their high-affinity with advantages of targeting highly structured rRNA region, which has extended the potential of this method. Briefly, probes are hybridised to smears made directly from the contents of blood culture bottles on a slide, non-hybridised probes are washed out and slides are examined by fluorescence microscopy to reveal the presence of the organism. The sensitivity of the method has been estimated at least as similar to most results obtained by PCR-based assays[47]. Due to a simple

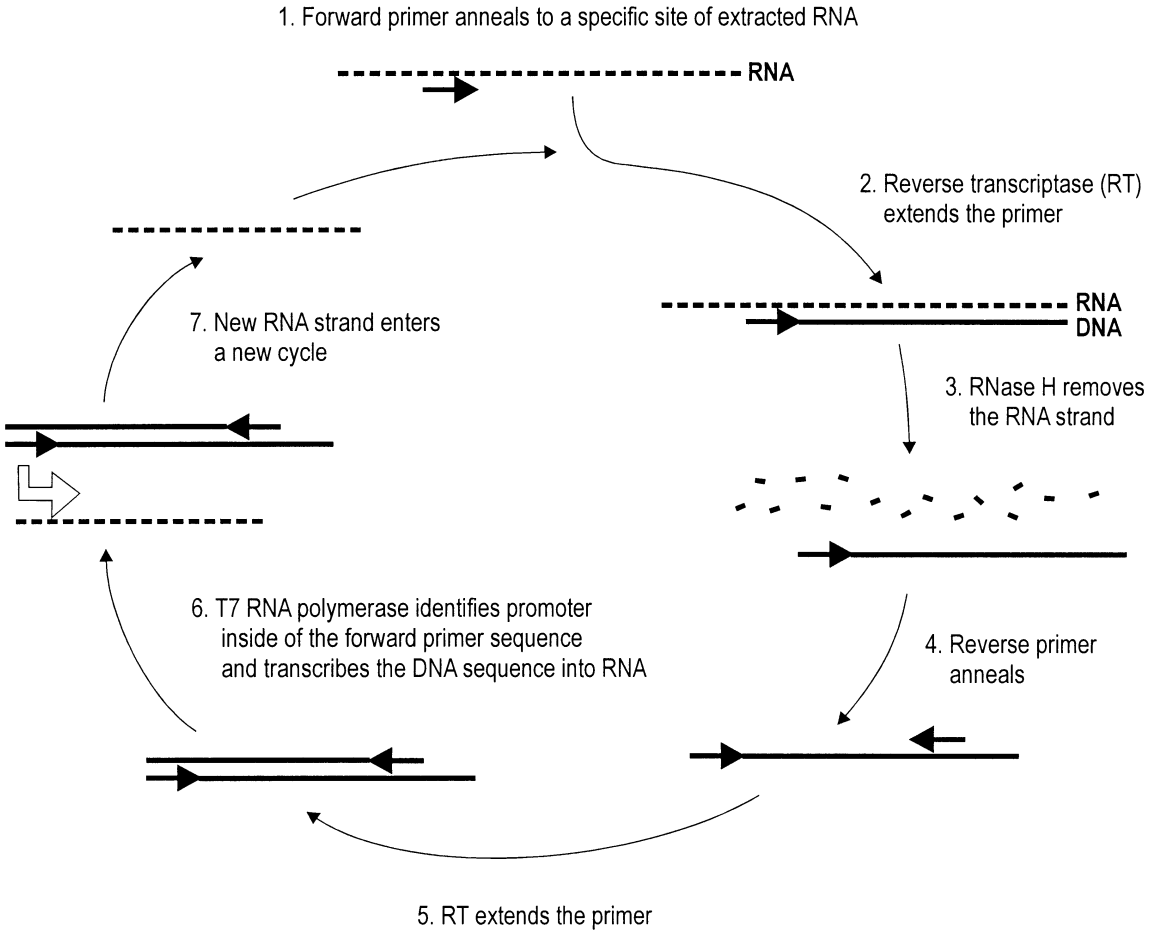

Fig. 2. Nucleic Acid Sequence Based Amplification (NASBA) reaction steps.

technical protocol with the exclusion of DNA extraction, the entire PNA FISH requires only 2.5 hours after a blood culture is designated positive by an automated blood culture system, the whole procedure is suitable for automation. FISH including probes specific for *Candida* species has been demonstrated to be a reasonable diagnostic tool for species identification[48]. PNA FISH has been developed to differentiate *C. albicans* from non-*albicans Candida* species[49], evaluated in a multicenter study[50] and its implementation in hospital reduced antifungal drug expenses[51]. The same group also conducted a PNA FISH assay to differentiate *C. albicans* from *C. dubliniensis*[52].

B. STRAIN TYPING

In contrast to detection techniques, which target universal or species-specific sequences, strain typing aims to differentiate specific strains or clones of a given species among clinical isolates. Therefore, pure culture of each isolate has to be available. Again, a number of techniques seek to address sequence polymorphisms of strains or clones. As more such polymorphisms are included into the comparison, as higher discriminatory power can be achieved. Therefore, it is sometimes recommended to apply at least two independent techniques in a given array of isolates to verify the results and increase the discriminatory power.

1. Electrophoretic Karyotyping (EK)

Most fungal species display chromosome-length polymorphism, which results from unequal chromosomal rearrangements or from chromosome breakage and healing[53]. Sequences that cause these unequal rearrangements are mainly transposable elements and other dispersed repeats. Chromosome-length polymorphisms can be assessed by electrophoretic karyotyping, exploiting a modified type of electrophoresis. During conventional electrophoresis, fragments longer than 50 kb show the same mobility and remain unseparated. Pulse Field Gel Electrophoresis (PFGE) allows separation of extremely large DNA fragments or even whole chromosomes in agarose gel using an alternating electric field. In order to avoid mechanical breakage of DNA molecules during common extraction procedures, intact cells are first embedded into an agarose block and lysis is carried out in a plug of agarose excised from the initial block. The agarose matrix keeps the DNA molecules intact, in place, while allowing the reagents to diffuse freely. A plug can be incubated with detergents and enzymes to remove cellular components from the DNA. Then the plug is inserted into the gel and intact chromosomes migrate from the plug into the gel during

electrophoresis. After each change of polarity of the current during pulsed-field gel electrophoresis, DNA coils reorient and move in a different direction. This helps them to pass through the gel. With each reorientation of the electric field, smaller sized DNA molecules realign and move in the new direction more quickly than the larger ones. Thus, the larger DNA molecules lag behind which contributes to size-separation of individual chromosomes. Chromosome sizes can vary greatly between different *Candida* strains, thus resulting in various banding patterns known as electrophoretic karyotypes. Evaluation of differences in banding patterns can be performed visually or can be computer-assisted. If chromosomal length polymorphism is not satisfactorily discriminatory, chromosomes can be digested by rare-cutting restriction endonucleases prior to electrophoresis, to get several variable large DNA fragments. Although the equipment needed for PFGE is rather expensive and sample preparation is labour-intensive, tedious and not suitable for analysis of large numbers of samples, EK is a well-established method in *Candida* spp. typing[54]. It has been successfully used to differentiate strains of *C. albicans*, *C. lusitaniae*, *C. parapsilosis*, *C. tropicalis* and *C. glabrata*[55-59], and to distinguish *C. albicans* from the phenotypically close species *C. dubliniensis*[60]. It also performed well when compared to other typing techniques[61].

2. Restriction analysis (REA)

In this technique, genomic DNA is typically cleaved by a frequently cutting restriction endonuclease to result in sequence-dependent restriction fragment length polymorphism (RFLP). This can be visualised by separating the fragments yielded using common agarose gel electrophoresis. The resulting banding patterns show interstrain variations as a result of the polymorphic nature of restriction site sequences or as a result of deletions and insertions in the DNA stretches between cleaving sites. Restriction analysis is rapid, easy, inexpensive, but restriction patterns are very complex and therefore difficult to compare. The majority of intense bands in an RFLP pattern represents rDNA sequences and mitochondrial DNA sequences; these fragments do not provide enough information to assess the relatedness of moderately related isolates. For easier comparison, separated fragments can be transferred onto a membrane and hybridised with a labelled fingerprinting probe that will recognize relatively few fragments of restricted DNA. Multi-copy probes designed to bind repeat sequences dispersed throughout the genome (e.g. RNA genes, mitochondrial DNA sequences and repetitive sequences) were applied[62]. In contrast to bacteria, endonuclease digestion of ribosomal cistrons generates fragments of similar relative size in yeasts, resulting in a simple Southern blot hybridisation pattern, which is low in resolution for strain discrimination.[63] Fungal rRNA and mitochondrial probes therefore have not been generally used in broad epidemiological studies[62]. Use of fragments containing repetitive genomic sequences as probes, e.g. the Ca3 probe, has been more successful[64]. Synthetic sequences derived from microsatellites have also been used to hybridise to hypervariable loci of fungal DNA cut with restriction endonuclease[65]. The locus of interest can also be amplified using gene-specific primers and then subjected to REA[66]. Trost et al.[67] evaluated identification and strain characterization of clinically relevant *Candida* species by amplification of intergenic spacers ITS1 and ITS2 followed by evaluation of RFLP of PCR products after sequence-specific enzymatic cleavage.

3. Random Amplified Polymorphic DNA (RAPD) or Arbitrarily primed PCR (AP-PCR)

Two groups originally developed this technique independently[68, 69]. Although the term AP-PCR may better fit the principle of the technique, the term RAPD (pronounced *rapid*) is more widely used because of its simplicity. RAPD is a PCR-fingerprinting technique, which employs a single short primer (typically 10 bases in length), sequence of which is chosen arbitrarily rather than based on knowledge of the targeted genomic sequence. Therefore, RAPD requires no prior knowledge of sequence of the examined organism and can be applied universally. At the beginning, a set of oligonucleotide primers of random sequences has to be screened; those with optimal performance are chosen for further analysis. Because of its short sequence, a RAPD primer is able to anneal to many locuses throughout the genome. In addition, when RAPD is performed for typing purposes, annealing occurs under low stringency conditions (typically 35–40 °C, 2.5 mM $MgCl_2$). Then, a primer likewise hybridises with many additional imperfectly matched sequences with sufficient affinity. If two molecules of the same primer anneal in a proper orientation close enough for the PCR to proceed efficiently, an amplicon is generated. Several amplicons are typically generated in a complex genomic DNA, which can be visualised as a banding pattern by gel electrophoresis. Because of competition for annealing of the primer, minor inter-strain polymorphisms in annealing sites result in strain-specific variations of banding patterns. For schematic overview of the technique see Fig. 3. The majority of amplified fragments originate from unique sequences rather than from repetitive elements[70]. When targeting the entire genome, RAPD generally results in more complex patterns than standard PCR or RFLP and may increase the likelihood of detecting interstrain differences[71]. RAPD is easily designed, technically simple, economic and also faster than other typing methods. However, special emphasis should be laid on the performance of this technique, because minor differences in experimental conditions can result in different profiles, which compromises intra- and inter-laboratory reproducibility of RAPD. There are many factors, that can influence the appearance or disappearance of bands, including Mg^{2+} concentration, primer/template concentration ratio, *Taq* polymerase concentration and source, the model of thermal cycler etc.[72-74] RAPD applied to eukaryotes seems to be more reproducible due to the higher stability of their genomes[72]. To overcome reproducibility obstacles, PCR can be also targeted to specific sequences, for example microsatellite sequences, and performed at higher stringency[75]. RAPD combines the advantage of operational simplicity with no need of

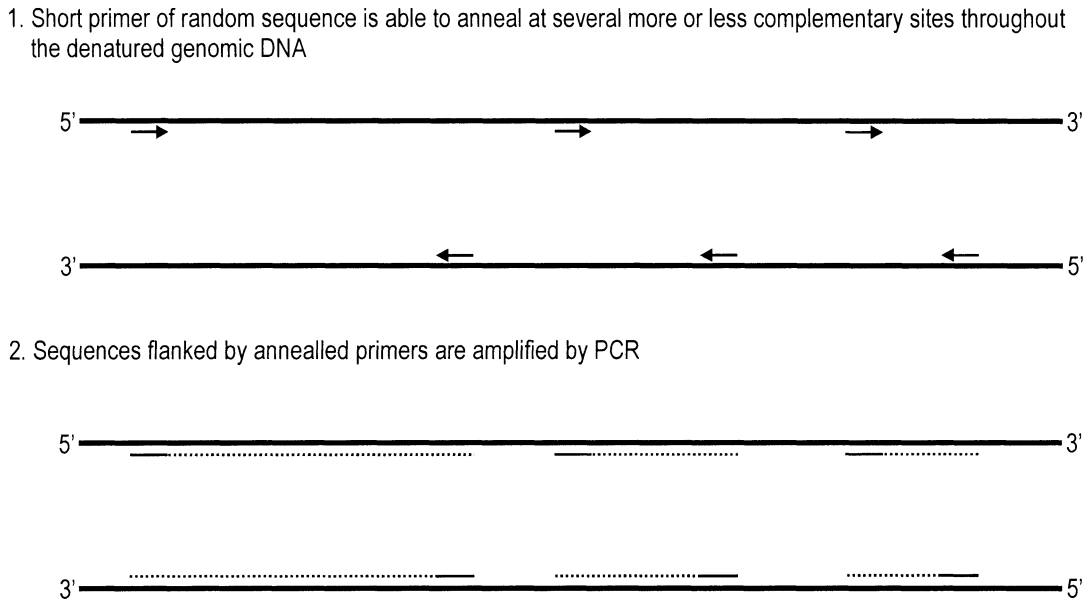

Fig. 3. Essential steps of Random Amplified Polymorphic DNA (RAPD) technique.

prior sequence data, which makes it suitable especially for typing of a collection of isolates of less studied microorganisms, when interlaboratory comparison is not required. Therefore, RAPD is the most widely used typing technique in clinical mycology. It has been repeatedly shown, that RAPD can be employed as a convenient tool for species identification as well as strain typing[76-79]. On the other hand, problems with possible bias during complicated comparison of banding patterns consisting of bands of variable intensity should not be denied. To overcome this, a new McRAPD approach, which takes advantage of melting analysis of RAPD amplicons, has been introduced recently[80]. It omits both gel electrophoresis and tedious analysis of banding patterns and directly yields numerical data, which can be subjected to automated unbiased analysis. It was first employed for rapid and accurate species identification in five pathogenic yeast species. However, when high-resolution melting device and saturating dye are used to increase its discriminatory power, it seems to be well suitable for strain typing as well (unpublished data).

4. Amplified fragment length polymorphism (AFLP)

Amplified fragment length polymorphism is a PCR-based DNA fingerprinting technique[81]. In short, the AFLP procedure usually starts by digestion of genomic DNA using two restriction enzymes (generally a hexa-cutter and a tetra-cutter) followed by ligation of double-stranded adapters to the ends of the restriction fragments. Afterwards, amplification of the restriction fragments by PCR using two primers complementary to the adapter- and restriction site sequences takes place. To reduce the amount of resulting amplicons, one to three selective nucleotides are usually added to the 3'-ends of primers. Then, a subset of restriction fragments, harbouring complementary sequence to the primer extension adjacent to the restriction site, is amplified. Finally, amplified restriction fragments are separated by gel electrophoresis and analysed. Primers may be labelled by fluorescent tags enabling a computer-based automated sequence analyser to read the polyacrylamide gel electrophoretic patterns (Fluorescent Amplified Fragment Length Polymorphism, FAFLP). For schematic overview of the technique see Fig. 4. A typical AFLP fingerprint represents between 50-100 bands, which greatly increases the discriminatory power of AFLP and this facilitates its use in epidemiological studies. On the other hand, the expense and expertise needed discourage its routine use. Like RAPD and RFLP, AFLP requires no prior sequence information. However, compared to RAPD, specific primers and stringent annealing temperature ensure that AFLP is a highly reproducible and robust method, whereas compared to RFLP, fingerprints obtained with AFLP are more informative, easier to read and reading can be automated. At first, AFLP was used for bacteria typing in microbiology[82]. More recently, pathogenic *Candida* species identification[83] and typing[84] have also been reported.

5. DNA sequencing

Obviously, the most accurate way to compare two individuals, strains or clones is to sequence their entire genomes. Of course, such an approach is impracticable for routine use. Instead, parts of a selected fungal gene can be amplified and resulting sequences can be compared[85]. However, focusing on just one variable fragment can hardly provide enough data for strain typing and is usually

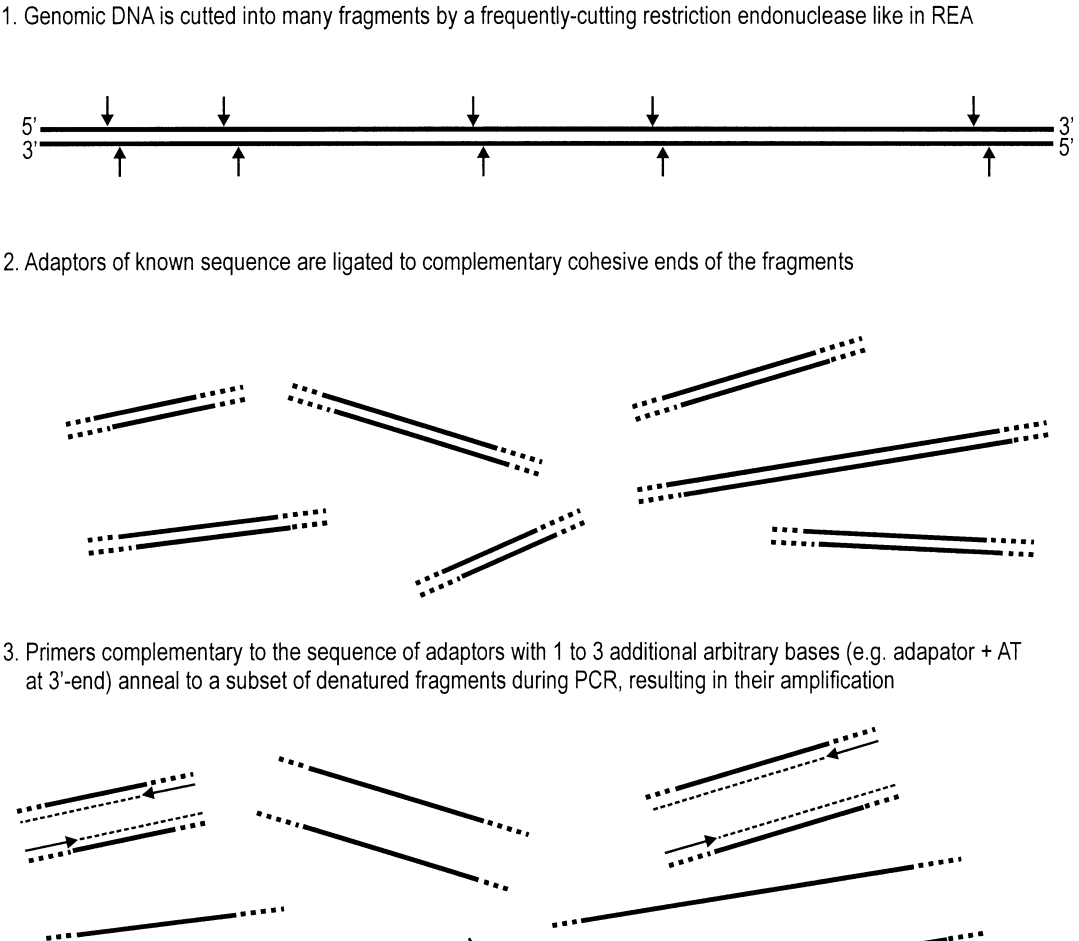

Fig. 4. Essential steps of Amplified Fragment Length Polymorphism (AFLP) technique.

suitable for species identification only. Coleman et al.[86] studied a fragment of the V3 variable region of the large ribosomal subunit genes from *C. dubliniensis* isolates and found out that it was significantly different from the other species analysed. Including more locuses into sequencing and consecutive comparison can increase the discriminatory power. Botterel et al.[87] used sequencing of three polymorphic microsatellite markers to *C. albicans* typing. To date, the most complex and promising use of sequencing for typing purposes is represented by the Multi Locus Sequence Typing (MLST) approach. MLST focuses on the nucleotide polymorphism of internal fragments of several housekeeping genes, where each unique allele combination determines a sequence type of strain. Fragments of the housekeeping genes are first amplified by PCR and then sequenced. This approach has many advantages – it provides unambiguous, portable and easy to standardize results. Absolute interlaboratory reproducibility can be achieved, enabling global epidemiological studies. The MLST approach is developed continuously as an open platform (http://www.mlst.net) (ref.[88]). Bougnoux et al.[89] investigated the potential value of MLST for characterization of clinical isolates of *C. albicans* and found it a highly resolving and stable method. MLST was also compared to other fingerprinting methods already established for *C. albicans* by Robles et al.[90] MLST system for strain differentiation has been already set up for *C. tropicalis*, *C. glabrata* and *C. albicans*[91]. Although the technique is currently not feasible for most laboratories, increasingly user-friendly automation together with cutting of costs due to newly emerging technologies (e.g. pyrosequencing) promise broader availability in the future.

6. DNA-microarrays

Microarray-based systems offer an attractive outlook not only for the future of strain typing. They a offer high level of sensitivity, specificity and throughput capacity, without requiring *a priori* knowledge of specific sequences. Chips or microarrays are high-density microscopic sets of oligonucleotide probes immobilized on solid surface, to which nucleic acid samples are hybridised. Perfectly matched sequences from the sample hybridise more efficiently to the corresponding oligomers on the array and give stronger signal than mismatched bound sequences. The final signal is detected by high-resolution fluorescent scanning and analysed by computer software, thus enabling automation and standardization[92]. Easier management of the vast data generated and reduction of the costs of DNA-chips are only a matter of time. Then, microarrays surely will move from the research area to clinical practice. For typing purposes, microarrays can be directed to identify the presence and quantity of different sequence variants of specific genes or regions, e.g. rRNA genes, internal transcribed spacers (ITSs) in particular. Ongoing sequencing projects in pathogenic yeasts will also soon enable quite straightforward designing of whole-genome DNA microarrays[93]. The use of microarrays for microbial fingerprinting has been already reported for *Salmonella enterica* isolates[94], for closely related *Xanthomonas* pathovars[95] and *Mycobacterium* species[96]. Rapid automated performance of tens of thousands of hybridisation assays on a tiny chip represents the strongest point of this technology.

CONCLUSION

To conclude, both yeast species identification and strain typing mainly relies on several modifications of two basic technologies – amplification of a DNA fragment followed by its analysis by different means, or, hybridisation of the total genomic DNA to a set of probes. It is not easy to foresee, which of these approaches will ultimately prevail. Most probably, the repertoire of techniques applied will be reduced only partly, because different techniques can best match different particular needs. Both the costs, easy operation and the reproducibility and resolving power will be considered for particular applications of different techniques. Also, both a trend towards better performance of less reproducible or less discriminatory but cost-effective techniques and a trend towards lower costs of highly reproducible and discriminatory but rather expensive techniques can be clearly observed. There is no doubt that PCR will be continued as the core of rapid detection and identification techniques. Due to its low costs and potential for automation, high resolution melting analysis (HRMA) promises to bring outstanding progress in post-PCR analysis in the near future. Melting analysis has already been applied in yeast species identification[80] and our preliminary data show, that it is also suitable for improving the potential of RAPD typing. Furthermore, the potential of HRMA to at least partly substitute for sequencing in MLST can be envisaged, possibly establishing a new Multi Locus Melting Typing (MLMT) approach. This should bring the merits of these technologies even closer to all routine laboratories in near future. In addition, the DNA-microarrays technology surely has the potential to revolutionize DNA-based diagnostics also in the field of clinical mycology in the more distant future.

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