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Use of variability of the fungal ITS2 intergenic region for the identification of medically important yeast species

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Abstract: Yeasts are common fungal opportunistic pathogens in humans playing a significant role in the morbidity and mortality of immunocompromised patients. Number of resistant yeast species is responsible for infections and consequent infectious complications, but the final microbiological diagnosis can be affected by variability of their phenotype and may result in incorrect identification. For the purposes of this study, advanced applications of molecular genetic methods based on certain up-to-date knowledge of fungal internal transcribed spacer (ITS) regions have been employed, which could support a possibility of universal application of such methods for identification of any pure yeast culture. In this study, the targeted DNA was amplified by a couple of primers, and the products of PCR reaction were divided by capillary electrophoresis. In the cases, in which the measured sizes of fragments did not correspond with the anticipated sizes, fragments were used for the sequencing analysis and compared to the nucleotide databases using the BLAST tool. Out of 208 isolates, 7.2% ($n = 16$) of cases occurred to be incorrectly determined, particularly in the group of *non-albicans Candida* species accounting for as many as 21.7%.

Key words: yeasts; ITS2 intergenic region; capillary electrophoresis.

Abbreviations: ITS, internal transcribed spacer.

Introduction

Yeasts are opportunistic pathogens which colonize mucous membranes and skin. They represent a source of serious infectious complications mostly in patients with immunesuppression (Jautová et al. 2001). Increase in the incidence of such infections is associated with several factors, in particular the underlying immune deficiency, the use of broad-spectrum antibiotics, cytotoxic agents (e.g. anticancer agents), immunosuppressive drugs, central venous catheters, extensive surgery, etc. (Fridkin et al. 1996).

From the clinical samples, different yeast species and yeast organisms may be isolated, including those less common, which until today have rarely or hardly ever been associated with human diseases. Various commercial systems that can identify these pathogens have been developed. Although correct identification of clinically relevant yeast strains may be achieved with these systems, incomplete or incorrect identification may occur (Guarro et al. 1999).

Differences in the internal transcribed spacer (ITS) 2 regions of fungi were reported to be useful for the rapid identification of clinically important species (White et al. 1990). The PCR with fungus-specific primers, targeting the conserved sequences of 5.8S and 28S rDNAs, as well as those of 18S and 28S rDNAs, results in the respective amplification of species-specific regions which vary in the amplicon length and sequence according to species (Henry et al. 2000). Using the variability in the ITS regions of fungi may achieve a specific identification method of yeast strains and promise more exact alternative to the conventional methods (Fujita et al. 2001).

In association with the foregoing results, in which antifungal susceptibility of clinical yeasts isolates was determined, our purpose was to prove their phenotypic identification considering some unusually resistant isolates.

Material and methods

The strains were isolated from patients with gynecologic infections ($n = 181$), leukaemia ($n = 79$), hospitalized in the intensive care unit ($n = 38$) and from stomatology departments ($n = 22$) with suspected or proven mycosis. Samples of oral smears, rectal swab, vaginal swab, and blood for haemocultivation were taken from patients. The overall number of 208 yeast isolates collected within the period 2005–2006 was retrospectively re-identified.

Cultivation of swabs was performed on Sabouraud's dextrose agar containing antibiotics (gentamicine and chloramphenicol) for 12 to 24 hours at the temperature of 35 °C. Blood samples of the volume of 5–10 mL were taken, placed in the bottles of Bactec Mycosis IC/F (Becton Dickson) and cultivated in the BACTEC incubator for at least 7 days.

Yeast species isolated from clinical samples were routinely identified by the Chromagar Candida (BBL) and AUXACOLOR 2 (Bio Rad) biochemical tests.

The DNA of the yeasts involved in the study was isolated by the method employed in the original study by Espenshade (Sambrook & Russell 2001).

For the amplification of the target sequence (part 5.8S and 26S rRNA gene and involved ITS2 region), the ITS3 (5'-GCATCGATGAAGAACGCAGC-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') primers were used. The amplification programme was started by the initial denaturation step at 94℃ for 4 min, followed by 30 cycles: denaturation at 94℃ (30 s), annealing at 55℃ (30 s), synthesis at 72℃ (1 min), up to final extension at 72℃ (4 min).

For the fragment analysis of PCR products, the primer ITS3 was labelled by the fluorescent dye 6-FAM (Sigma). The PCR products were isolated using capillary electrophoresis ABI PRISM 3100-Avant Genetic Analyser Biosystems along with GeneScan-500 LIZ Size Standard (Aplied Biosystems) according to the standard module. The collected data were analyzed by GeneMapper Software v.3.5 (ABI PRISM 3100-Avant Genetic Analyser).

For the sequencing reaction, ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction Kit in 3.1 (Applied Biosystems) was used. Products of sequencing reaction were separated by the ABI PRISM 3100–Avant Genetic Analyser according to the standard module. The data were analyzed by DNA Sequencing Analysis Software v.5.1 (ABI PRISM 3100–Avant Genetic Analyser). Through the Chromas Pro (v.1.34) and Clustal W (v.1.8) programmes, the sequences were analyzed and compared to the nucleotide database by employing the BLAST tool (Altschul et al. 1990).

Results

Using Chromagar Candida, species such as *C. albicans, C. tropicalis* and *C. krusei* were determined directly from the mixed cultures. The Auxacolor 2 biochemical test allowed identification of pure cultures in at least 31 yeast species.

For the purposes of molecular-genetic identification, the DNA was obtained by isolation from the pure yeast culture successfully amplified in a PCR reaction by universal primers. Differences in size of the amplified products of individual isolates were determined by capillary electrophoresis ABI Prism 3100 with high precision (theoretically 1 bp; the measured values are not real). In order to establish a size database of amplified products, collection strains were used (Table 1). The clinical isolates, in which the size of amplified products did not correspond with the size of amplified products in a database, were searched and consecutively re-identified on the basis of direct sequencing and comparing the alignments between the obtained and the BLAST sequences (Fig. 1).

Table 1. Sizes of PCR fragments for different yeast species.

Species	Fragments size
C. albicans	336–337
C. glabrata	413–414
C. krusei	337–339
S. cerevisiae	416–417
C. parapsilosis	306–307
C. lusitaniae	248–249
C. guilliermondii	374–375
Ch. tropicalis	324–325
C. norvegensis	318–319
C. kefyr	426–427
C. inconspicua	300–301
T. asahii	354–356

Having employed conventional methods, we identified 10 yeast species; most frequently *C. albicans* strains ($n = 151$), followed by *C. glabrata* ($n = 16$), *C. krusei* ($n = 11$), *S. cerevisiae* ($n = 8$), *C. parapsilosis* ($n = 7$), *C. zeylanoides* ($n = 5$), *C. tropicalis* ($n = 4$), *C. lusitaniae*, *C. guilliermondii* (both $n = 3$), and *C. tropicalis* ($n = 2$).

Except for three isolates, phenotypic identification in 148 cases of yeasts *C. albicans* was confirmed by the fragment analysis of the amplified products using PCR. Based on the sequence analysis of an amplified product and by comparing the DNA alignments to the BLAST results, three remaining isolates were identified as strains *C. norvegensis*, *C. tropicalis*, and *S. cerevisiae.*

According to the biochemical profile of Auxacolor 2, it was impossible to reliably distinguish species *C. glabrata* from *C. zeylanoides.* After having conducted several additional tests, five isolates were identified as *C. zeylanoides.* The respective sizes of the amplified products of such isolates were not correspondent with the species of *C. zeylanoides*, however, subsequently to these five isolates, four sequences of ITS2 region of *C. glabrata* were recognized, and the last one we recognized was the sequence of *C. inconspicua.*

As far as *C. krusei* strains are concerned, their typical growth, along with colour reaction on Chro-

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C. parapsilosis     CTCAAACCCTCG--GGTTTGGTGTTGAGC-G-A-------TACG--CTG-GGTTTGCTTG
C. tropicalis       CTCAAACCCCCG--GGTTTGGTGTTGAGC-A-A-------TACG--CTA-GGTTTGTTTG
C. lusitaniae       CTCTAACCCCCG--G-TTAGGCGTTGCTCCG-A-------AATA--TCA-ACCGCGCTGT
C. albicans         CTCAAACCGCTG--GGTTTGGTGTTGAGC-A-A-------TACGACTTG-GGTTTGCTTG
C. kefyr            CTCAAACCTTTG--GGTTTGGTAGTGAGTGATA-------CTCGTCTCG-GGTTAACTTG
C. krusei           CTTGCGCGTGCGCAGAGTTGGGGGAGCGGAGCG-------GACGACGTGTAAAGAGCGTC
C. norvegensis      CTTGCGCAAGCA--GAGTTGGGGTTGCCACG-G-------CCCG---TGCGGCCTGTGTG
C. inconspicua      CTTGCGCAAGCA--GAGTTGGGGTTGTCACC---------TTTG---GGTGGCTCCCCCG
C. glabrata         CTCAAACACGTT-GTGTTTGGTAGTGAGTGATACT-----CTCGTTTTTGAGTTAACTTG
S. cerevisiae       CTCAAACATTCT---GTTTGGTAGTGAGTGA---------TACTCTTTGGAGTTAACTTG
C. guilliermondii   CTCAAACCCCCG--GGTTTGGTATTGAGTGATACTCTTAGTCGGACTAGGCGTTTGCTTG
T. asahii           CTCAACCACTAG--GGTTTCCTAATGGATTGGA-------TTTGGGCGTCTGCGATTTC
                    **              *       *
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Fig. 1. Variability in partial nucleotide sequence of yeasts ITS2 region.

Table 2. Summary results in booth methods.

Species	Phenotypic identification (n)	Genotypic identification (n)
C. albicans	151	150 (−3 +2)
C. glabrata	16	20 (+4)
C. krusei	11	11
S. cerevisiae	8	9 (−2 +3)
C. parapsilosis	7	6 (−2 +1)
C. lusitaniae	3	2 (−1)
C. guilliermondii	3	3
C. tropicalis	4	3 (−3 +2)
C. zeylanoides	5	0 (−5)
C. kefyr	2	3 (+1)
C. norvegensis	No identification	1 (+1)
C. inconspicua	No identification	1 (+1)
T. asahii	No identification	1 (+1)

magar Candida, enabled us to identify these species in a relatively reliable manner. Within all eleven isolates, the results of identification on Chromagar Candida were proved by Auxacolor 2 and consequently also by molecular-genetic analysis.

S. cerevisiae strains typically showed their high variable biochemical profile and various results from one isolate were usually gathered. From a practical point of view, it was interesting to observe some other *C. tropicalis* strains growing on Chromagar Candida which first produced green to blue colonies and the blue ones after 48-hour incubation. After 12 to 24 hour incubation, the same colouring was at same time produced by *C. albicans* strains. Therefore, in these cases interpretation of the results taken after a longer incubation was more reliable. Occasionally, the growth of colourful colonies of *S. cerevisiae* and *C. parapsilosis* (mostly purple) and, eventually, colourless growth of chromogeneous species was observed.

In total, eight *S. cerevisiae*, seven *C. parapsilosis*, and four *C. tropicalis* strains were identified on the basis of phenotype. The sizes of the respective fragments of the three *C. tropicalis* isolates, two *S. cerevisiae* and *C. parapsilosis* isolates did not correspond with the defined species, and following sequencing analysis, three of *C. tropicalis* isolates were re-identified as *C. albicans*, *C. parapsilosis* and *T. asahii*. Two *S. cerevisiae* isolates were re-identifined as *C. kefyr* and *C. albicans* and two *C. parapsilosis* isolates were re-identified as *C. tropicalis* and *S. cerevisiae*.

Similarly, out of three isolates of *C. lusitaniae,* there was one isolate subsequently re-identified as *C. parapsilosis*. *C. guilliermondii* and *C. kefyr* species were correctly identified in all cases (Tables 2, 3).

In total, 16 isolates were re-identified. Selection of misidentified yeasts based on fragment analysis of PCR products was in all cases correct. By the Chromagar Candida and Auxacolor 2 biochemical test, total of 92.4% out of clinical yeast isolates were identified identically, compared to DNA applications. Of the remained isolates, 7.6% (in the group of *non-albicans Candida* yeasts up to 21.7%) were inaccurately identified.

Discussion

Taxonomy and identification of pathogenic fungi is established mainly on the evaluation of morphological

Table 3. Alignment scores.[a]

Species	n	ITS2 sequence-based identification via BLAST				
		Accession No.	*S*(bits)	*I*(%)	*G*(%)	*E* value
C. glabrata	4	Y939793.1	685	99	0	0.0
		AY198398.1	635	93	1	4e-179
		DQ347494.1	616	93	1	3e-173
		AY939793.1	466	89	1	4e-128
S. cerevisiae	3	EF042285.1	631	98	1	8e-178
		EU649672.1	620	96	1	1e-174
		EU649672.1	508	92	1	8e-141
C. albicans	2	AB305093.1	601	99	0	5e-169
		AB305093.1	580	99	0	1e-162
C. tropicalis	2	AB305099.1	649	93	0	0.0
		EU589208.1	592	99	0	3e-166
C. parapsilosis	1	EF191046.1	463	98	0	2e-127
C. norvegensis	1	AB278170.1	499	97	0	3e-138
C. kefyr	1	AY939806.1	789	99	0	0.0
C. inconspicua	1	AB179767.1	311	87	0	1e-81
T. asahii	1	AM900369.1	883	98	0	0.0

[a] n = number of isolates selected based on fragment analysis, S – score; I – identity; G – gaps; E – expect value.

and biochemical criteria. Results may be affected by phenotype variability, however, the final microbiological diagnosis should always be based on a correct identification of the pathogen (Guarro et al. 1996; Vaughan-Martini 2003). Based on the results of this study, by employing a conventional procedure, most of the isolated yeasts were identified correctly. Incorrect identification is more evident among species other than *C. albicans*, however, the frequency of occurrence of antifungal resistance among these species is higher. The colourless growth on the chromogenic media and variable biochemical profiles made it harder to distinguish the isolated yeasts. These results are comparable with the observations published by Campbel et al. (1999), Odds & Bernaerts (1994) and Pfaller et al. (2003).

The benefit resulting from the diagnosis of pathogenic microbes on the gene level is well-known. In practice, commercially available PCR applications for the identification of some frequently occurring fungal species are now available, but a more universal approach is required. Consequently, other steps were included in addition to the basic procedure (generally the PCR reaction), e.g. PCR-RFLP (restriction fragment length polymorphism) analysis for species-specific fungal detection is widely used for experimental purposes (Esteve-Zarzoso et al. 1999; Dendis et al. 2003; Machouart et al. 2006).

In our study, in the basic initial stage, according to Fujita et al. (2001), the target DNA sequence of the pure yeast culture was amplified in a PCR reaction through a couple of universal primers, and the fragment, parts 5.8S and 26S, along with the ITS2 region of rRNA fungal gene, was obtained. In the ITS fungal regions, the presence of highly conservative and variable areas was proved, whereas it is possible to make use of interspecies differences in size and sequence of such areas for the species-specific identification of yeasts and moulds (White et al. 1990; Turenne et al. 2000).

Within the second step, yeast species were differentiated using capillary electrophoresis. This method allows for a more precise determination of the DNA fragment sizes, and in some cases these have been applied to species specific identification of pathogenic fungi through size polymorphism of ITS regions (Henry et al. 2000; Chen et al. 2001). Compared to the procedure, within which common restricting enzymes are being used, the respective analysis was conducted at an incomparably higher speed, whereas during a single run it was possible to analyze a few samples simultaneously.

Certain risks have arisen by using the above technique. Intraspecies diversity could alter the size of the target sequence (Henry et al. 2000; Chen et al. 2001). De Baere et al. (2002, 2005) suggested the influence of the sequence-dependent migration.

In order to minimize the risks in question, control fragments (reference strains) were added to the runs and run-to-run deviations were calculated. However, the sizes of individual fragments of particular species ranged in the typical interval, and the species identification of isolates was, based on such data, possible. Discrimination of isolates with a highly identical or very close fragment size might have been limited, consequently, based on the discrepancies between the results of conventional identification and those of consecutive fragment analysis, most of the incorrectly identified yeasts were reliably recognized.

As mentioned above, the results of fragment analysis could be unambiguous; in these cases it is possible to use sequence variability in the same area (ITS2 of rRNA gene) and downright to identify the isolate based on its comparison to a reference sequence. According to the actual knowledge, the ITS2 region is to show a lower intra-species sequence divergence in comparison to the ITS1 region, and it seems to be more appropriate for such types of analysis. The GenBank database (Benson et al. 2008) currently lacks any ITS sequence entries for some of yeast species. However, the number of ITS sequences available in public databases has increased rapidly in recent years and an expanding database may improve the quality and accuracy of fungal identification (Hinrikson et al. 2005; Ciardo et al. 2006; Leaw et al. 2006).

The ITS regions promise correct discrimination of yeasts species because of their high sequence variability. However, some other diagnostic loci are frequently used as a target of amplification. D1/D2 hypervariable region of the 26S rRNA gene is well-known and sequences published in databases are available not only for a limited number of yeasts species. In the cases of closely related yeast (fungal) species, particularly those that contained identical DNA sequences in the D1/D2 variable domain of the 26S r RNA gene, the analysis of ITS sequence polymorphisms can be useful and reliable for identifying the majority of clinically important yeast (fungal) species (Gaskell et al. 1997; Chen et al. 2001; Hinrikson et al. 2005; Leaw et al. 2006). Unfortunately, in the same cases, the lower alignment scores could be induced, depending of the quality of obtained sequences. We assume an anticipation to exist that the re-identified isolates with a lower alignment score could be linked to some different species; in these cases there probably exist closely related species of the same length of the ITS2 regions. Anyway, in these cases the results of conventional identification were declined, since too low alignment scores for these events were obtained.

Conclusion

In association with the foregoing results of the research, in which antifungal susceptibility of clinical yeasts isolates was determined, our purpose was to prove their phenotypic identification. Some of the isolates, within which occurrence of resistance was observed, were subsequently re-identified as species classified to be naturally resistant. It is assumed that correct identification is of great importance not only in respect with antifungal therapy or prophylaxis, but it may also be beneficial for correct determination of the nature and development of antifungal resistance of yeasts.

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