MOLECULAR DETECTION AND IDENTIFICATION OF PATHOGENIC FUNGI IN CLINICAL SAMPLES

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ABSTRACT

Objective: To compare the conventional identification with the molecular methods, based on fungal DNA markers.

Methods: 95 strains were isolated at SVB between Jan-Apr 2009. Their isolation was made on BHI sheep blood/agar, Sabouraud agar, ChromID Candida agar. Yeasts were identified according to Germ tube test, India ink examination, rapid urease test, detection of soluble Cryptococcus neoformans antigens, API ID 32 C and VITEK 2C. At the CTMA-UCL 30 DNA extractions were made with NucliSENS® lysis magnetic extraction reagents and NucliSENS® miniMAG. A real time PCR amplification was performed using the ITS3 and ITS4 primers. Data were recorded on a Roche LightCycler® 480 System. Aspergillus fumigatus strain DSM 63359 was used as a positive control. The amplicons were sequenced on an automated 3130 Genetic Analyser.

Results: The strains identified by conventional methods in SVB were: C. albicans, C. glabrata, C. krusei, Sacharomices cerevisiae, C. kefyr, C. tropicalis, C. guillermondii, C. holmii, Aspergillus fumigatus, Aspergillus flavus, Aspergillus niger, Aspergillus spp, Cryptococcus neoformans. Correlation between the melting genotype and the phenotypic identification was perfect for C. albicans, C. glabrata, C. krusei, C. guillermondii and C. neoformans.

Conclusions: SVB place a high priority on maximizing their capabilities for the early diagnosis of opportunistic fungal infections.

Key words: molecular diagnosis, pathogenic fungi, quantitative detection, Real-Time PCR.

BACKROUND

The infectious fungi constitute one of the most important threats to the survival of immunocompromised hosts. A vast array of fungi, previously considered to be nonpathogenic, may serve as significant human pathogens. Conventional laboratory methods for diagnosis of fungal infections remain useful, but are often slow and lack sensitivity.

OBJECTIVE

The aim of this study was to compare the conventional identification with the molecular methods, based on fungal DNA extraction and selection of DNA markers for identification of fungi.

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METHODS

Isolation and identification of clinical isolates

A number of 95 strains from 665 patients were isolated at "Dr. V. Babes" Hospital on BHI agar +10% sheep blood, Sabouraud dextrose agar (pH 5,6) with chloramphenicol and gentamicine, chromID Candida agar (BioMerieux) at 37°C /24 h and room temperature/4 days, between Jan-Apr 2009.

- Yeasts were identified at "Dr. V. Babes" Hospital according to Germ tube test (Candida albicans), India ink examination (Cryptococcus neoformans), rapid urease test (Cryptococcus spp and Rhodotorula spp), API ID 32 C BioMerieux (yeasts), VITEK 2C (yeasts), Detection of soluble Cryptococcus neoformans antigens in biological fluids (serum, CSF, BAL, urine) (PASTOREX CRYPTOPLUS 61747/ BIO-RAD).
- Filamentous fungi were identified according to the morphology of the conidial state.

Safe transportation and storage protocols for medical yeasts and fungi

Fresh subcultures on Sabouraud slants were send to the Laboratory of Applied Molecular Technology, Université Catholique de Louvain, Brussels, Belgium (CTMA-UCL).

The hospital SVB contracted an internationally specialized company in infectious substances transporting ("TEEN TRANS"). The transport of the samples to the CTMA was made in accord with "Guidance on Regulations for the Transport of Infectious Substances", applicable as from 1 Jan 2009/2009-2010 (WHO) www.iata.org. Packages are marked to provide information about the contents of the package, the nature of the hazard, and the packaging standards applied.

Cultures were maintained at 4°C before manipulation.

On the first day, strains were sub-cultured on Sabouraud at 37°C. All strains but 1 (94) were growing after 5 days. 0.2 ml of concentrated spores or yeasts suspensions in water were then transferred in cryo-tubes containing 0.8 ml of a 20% solution of skim milk and thoroughly mixed. Duplicated tubes were stored at minus 80°C at two different locations.

DNA extraction from fungi and yeasts

On the second day, one loop of the cultures number 64 to 67 and 70 to 95 (Table 1) were resuspended in 500 µL NucliSENS® lysis buffer (NucliSENS® lysis magnetic extraction reagents, NucliSENS® miniMAG System, Biomérieux bv, Boxtel NL) in a tube containing the FastPrep® Lysing Matrix A. The tubes were then shaked for 40 seconds at 6 m/s in FastPrep®-24 MP™ homogenizer (FastPrep®, MP Biomedicals Europe sa, Brussels, BE). The swabs were directly resuspended in the NucliSENS® lysis buffer. Total DNA was released from the homogenized suspension using the NucliSENS® miniMAG system and reagents according to the manufacturer's instructions. DNA was eluted in 50 µL.

Real-time PCR amplification and presumptive identification of the basis of melting curve genotyping

A real-time PCR amplification of the internal transcribed spacer (ITS) 2 region was performed using previously described primers ITS3 5'–gcatcgatgaagaacgcagc– 3' and ITS4 5'–tcctccgcttattgatatgc– 3'. The real-time PCR assay was performed using 5 µL of each DNA solution, 330 nM of each primer, 10 µL SYBR® Green I Master (Roche Diagnostics, Mannheim, Germany) in a total reaction volume of 20 µL. The reaction was initiated at 95°C for 5 min followed by 40 cycles of denaturation at 95°C for 10 sec, annealing at 60°C for 15 sec and extension at 72°C for 10 sec, followed by 1 cycle at 95°C for 5 sec, 65°C for 1 min, and 97°C with a continuous acquisition mode as requested for the analysis of the melting curves. Data were recorded as crossing points (Cps) on a Roche LightCycler® 480 System, using the analytical software LCS480 1.2.9.11 from the same manufacturer. Five ng of DNA from Aspergillus fumigatus strain DSM 63359 (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany) was used as a positive control.

All DNA manipulations pre- and post-PCR were performed in separate designated rooms with separate pipetting devices to avoid contamination of the samples with foreign DNA. Furthermore, all equipment used in the preamplification steps was DNA-free and further irradiated by ultraviolet (UV) light to prevent contaminating DNA from causing false-positive results. Master-mixture water controls

Table 1. *Medical yeasts and fungi isolated at "Dr. V. Babes" Hospital and submitted to DNA identifications at CTMA-UCL*

Strain number	Source	Phenotypical Identification	Strain number	Source	Phenotypical identification
64	oropharynx	*Candida albicans*	81	vagina	*Saccharomyces cerevisiae*
65	oropharynx	*Candida albicans*	82	vagina	*Saccharomyces cerevisiae*
66	oropharynx	*Candida albicans*	83	vagina	*Saccharomyces cerevisiae*
67	oropharynx	*Candida albicans*	84	oropharynx	*Candida kefyr*
70	vagina	*Candida glabrata*	85	oropharynx	*C. guilliermondii*
71	vagina	*Candida glabrata*	86	BAA	*Aspergillus fumigatus*
72	stool	*Candida glabrata*	87	sputum	*Aspergillus flavus*
73	vagina	*Candida glabrata*	88	BAA	*Aspergillus niger*
74	vagina	*Candida glabrata*	89	BAA	*Aspergillus spp*
75	vagina	*Candida glabrata*	90	ear	*Aspergillus spp*
76	Vagina/HIV (+)	*Candida krusei*	91	sputum	*Aspergillus spp*
77	vagina	*Candida krusei*	92	LCR/HIV(+)	*Cryptococcus neoformans*
78	vagina	*Candida krusei*	93	LCR/HIV(+)	*Cryptococcus neoformans*
79	Stool/ HIV(+)	*Candida kefyr*	94	Blood/ HIV(+)	*Cryptococcus neoformans*
80	vagina	*Candida tropicalis*	95	Superficial wound	*Candida holmii*

BAA: Broncho-alveolar aspirate

and DNA extraction controls were used for every batch of samples processed.

RESULTS

All tested DNA were positive except 94 which is the strain that was not growing at 37°C. PCR products were classified in 9 different genotypes according to the melting profile of their respective PCR products. Correlation between the melting genotype and the phenotypic identification performed at "Dr. V. Babes" Hospital was perfect for C. albicans, C. glabrata, C. krusei, C. guillermondii, and C. neoformans (table 2). Ambiguous results were obtained for the Aspergillus species and some non albicans Candida. These results were discussed further according to the DNA sequence results.

DNA sequencing of purified PCR products

The amplicons were sequenced on both strands on an automated 3130 Genetic Analyser apparatus (Applied Biosystems, Nieuwekerk, NL), using the Taq Dye Deoxy Terminator Cycle Sequencing kit from the same manufacturer.

Consensus sequences were prepared with the help of the freeware BioEdit available at http://www.mbio.ncsu.edu/BioEdit/bioedit.html

High quality consensus sequences were obtained for most PCR products but VB83 and VB95. For VB83, saturation of peaks due to high DNA concentration was observed. For VB95 two different sequences were superimposed. This observation is coherent with the presence of a double peak in the melting profile (Table 2).

Molecular identification on the basis of DNA sequence comparison

Sequences were compared against those in Genbank-EMBL using the blast algorithm available at http://www.ncbi.nlm.nih.gov/blast/Blast.cgi.

DNA identification is based on the position of the test sequence according to published sequence in the tree view generated from the resulting DNA sequence alignment.

Table 3 is showing the conclusion of the Blast analysis as the name of the taxon presenting highest score of similarity with the query sequence. No ambiguous identification (more than 1 taxon with highest level similarity).

Phenotypical identification performed at "Dr. V. Babes" Hospital was confirmed by ITS2 DNA sequence comparison for 19 out of 22 yeast isolates that were tested by both methods. The added value of molecular identification is more visible for the 6 filamentous fungi. The molecular method allows identifying isolates VB 89, 90 and 91 at the species level and suggests a misidentification of isolate VB88. The best performance obtained for identification of yeasts.

Table 2. *Comparison between melting genotype and phenotypical identification*

Strain number	Melting genotype	Phenotypical Identification	Strain number	Melting genotype	Phenotypical identification
64	*85.23 ± 0.05*	*Candida albicans*	81	*84.05 ± 0.06*	*Saccharomyces cerevisiae*
65	*85.23 ± 0.05*	*Candida albicans*	82	*82.48 ± 0.06*	*Saccharomyces cerevisiae*
66	*85.23 ± 0.05*	*Candida albicans*	83	*82.48 ± 0.06*	*Saccharomyces cerevisiae*
67	*85.23 ± 0.05*	*Candida albicans*	84	*85.23 ± 0.06*	*Candida kefyr*
70	*84.07 ± 0.06*	*Candida glabrata*	85	*84.98 ± 0.09*	*C. guilliermondii*
71	*84.07 ± 0.06*	*Candida glabrata*	86	*92.18 ± 0.08*	*Aspergillus fumigatus*
72	*84.07 ± 0.06*	*Candida glabrata*	87	*91.34 ± 0.05*	*Aspergillus flavus*
73	*84.07 ± 0.06*	*Candida glabrata*	88	*92.18 ± 0.08*	*Aspergillus niger*
74	*84.07 ± 0.06*	*Candida glabrata*	89	*91.34 ± 0.05*	*Aspergillus spp*
75	*84.07 ± 0.06*	*Candida glabrata*	90	*91.34 ± 0.05*	*Aspergillus spp*
76	*89.37 ± 0.27*	*Candida krusei*	91	*91.34 ± 0.05*	*Aspergillus spp*
77	*89.37 ± 0.27*	*Candida krusei*	92	*84.86 ± 0.07*	*Cryptococcus neoformans*
78	*89.37 ± 0.27*	*Candida krusei*	93	*84.86 ± 0.07*	*Cryptococcus neoformans*
79	*85.28 ± 0.08*	*Candida kefyr*	94	*No amplicon*	*Cryptococcus neoformans*
80	*85.28 ± 0.08*	*Candida tropicalis*	95	*83.52 ± 0.06* *89.94 ± 0.02* double peak profile	*Candida holmii*

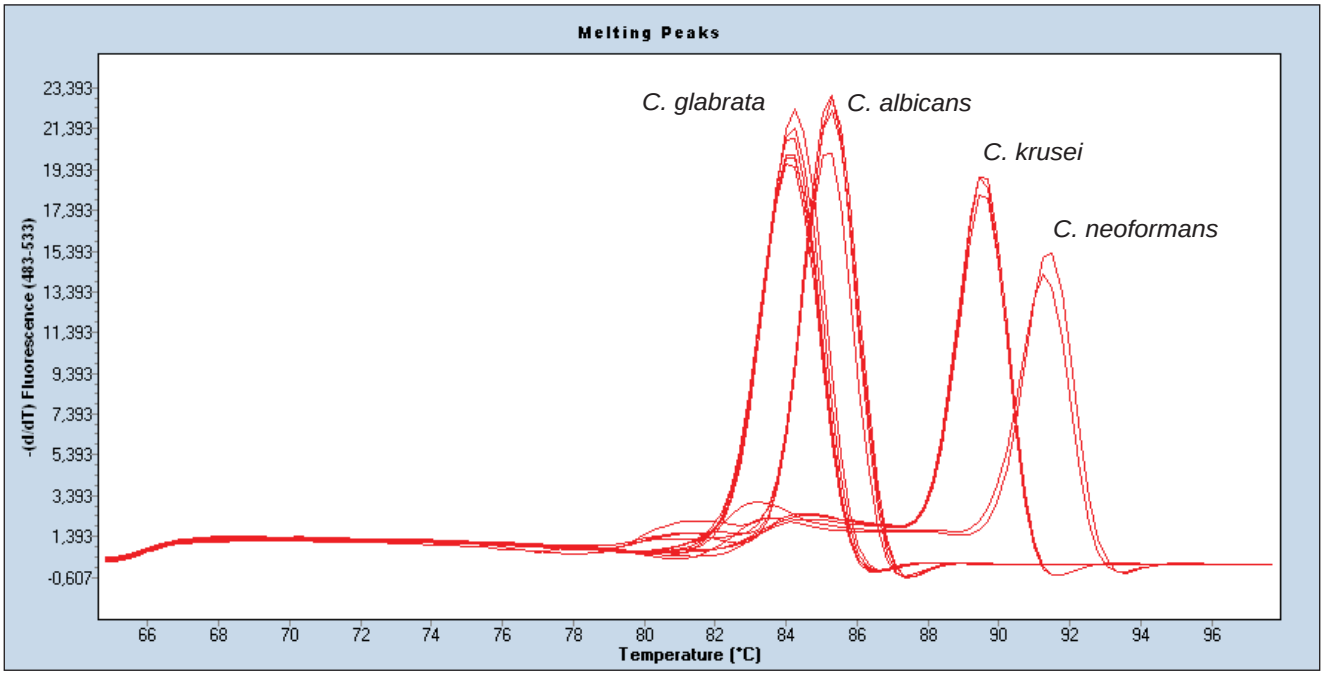

Figure 1. *Comparison between ITS-2 melting curves obtained for 4 different yeast species.*

CONCLUSIONS

Comparison between DNA identification at CTMA-UCL and phenotypical identification at "Dr. V. Babes" Hospital confirms the good quality of yeast identification protocols used in Bucharest. For filamentous fungi, acquisition of reference textbooks, participation of members of the staff in international course and blind exchange of strains between the CTMA-UCL and "Dr. V. Babes" Hospital will contribute to improve the expertise at "Dr. V. Babes".

The best performance obteined for identification of yeasts compare to filamentous fungi. Comparison between DNA identification at CTMA-UCL and phenotypical identification at SVB confirms the good quality of yeasts identification protocols. It is absolutely essential that SVB who caring out for

Table 3. *Results from ITS2 - DNA sequencing and identification according to DNA sequence comparison*

Strain number	ITS2-DNA sequence*	ITS2 Identification	Strain number	ITS2-DNA sequence	ITS2 Identification
64	341bp 0N	*C. albicans*	81	402 bp 0N	*C. glabrata*
65	340 bp 0N	*C. albicans*	82	264 bp 1Y	*S. cerevisiae*
66	341 bp 1S	*C. albicans*	83	245 bp 9N	*S. cerevisiae*
67	340 bp 0N	*C. albicans*	84	406 bp 0N	*Kluyveromyces marxianus*
70	416 bp 1N	*C. glabrata*	85	380bp 0N	*C. albicans*
71	416 bp 1N	*C. glabrata*	86	356 bp 0N	*A. fumigatus*
72	418 bp 0N	*C. glabrata*	87	356 bp 0N	*A. flavus*
73	416 bp 1N	*C. glabrata*	88	355 bp 0N	*A. fumigatus*
74	417 bp 1N	*C. glabrata*	89	357 bp 0N	*A. flavus*
75	416 bp 1N	*C. glabrata*	90	354 bp 0N	*A. flavus*
76	348 bp 0N	*Issatchenkia orientalis*	91	354 bp 0N	*A. flavus*
77	350 bp 1R	*Issatchenkia orientalis*	92	289 bp 0N	*C. neoformans*
78	348 bp 0N	*Issatchenkia orientalis*	93	376 bp 0N	*C. neoformans*
79	408bp 1W1M	*Kluyveromyces marxianus*	94	No amplicon	*Cryptococcus neoformans*
80	341 bp 0N	*Kluyveromyces marxianus*	95*	Mixed DNA sequence	*C. glabrata*

N, R, S,W, Y ambiguous bases according to IUAPC taxonomy
Issatchenkia orientalis is the teleomorph name for the species C. krusei
Kluyveromyces marxianus is the teleomorph name for the species C. kefyr

high-risk immunocompromised patients place a high priority on maximizing their diagnostic capabilities for the early detection of opportunistic fungal infections: just implementation of Real Time PCR at Microbiology Laboratory.