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Identification of Different Candida Species Isolated in Various Hospitals in Ankara by Fungichrom Test Kit and Their Differentiation by SDS-PAGE

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Abstract : Identification of 78 *Candida* isolates was carried out by a commercial Fungichrom test kit, the reliability of which was further confirmed by conventional biochemical tests and the API 20C kit. Differentiation between different *Candida* species was also observed when sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) of whole cell proteins was used. The most frequently isolated species of yeast from vaginal swabs obtained from patients was *Candida albicans*, which accounted for 39 of 78 (50.00%) of the isolates from women in Ankara. The next most frequent *Candida species* were *Candida glabrata* (*Torulopsis glabrata*) and *Candida krusei*, which were isolated from 26.92 and 11.53% of the women respectively. Other species of *Candida* were cultured less often: 7 *Candida kefyr* (8.97%); 1 *Candida tropicalis* (1.28%) and 1 *Candida parapsilosis* (1.28%). The results obtained in this study strongly indicate that as an alternative to classical identification methods, the Fungichrom test can also be used for rapid and accurate identification of all *Candida* sp.

Key Words: *Candida* spp., isolation, identification, Fungichrom test kit, SDS-PAGE

Introduction

The number of fungal infections caused by yeasts has dramatically increased over the past several decades. Among them, the imperfect yeast *Candida albicans* and several related *Candida* species are of foremost importance as opportunistic pathogens in immunocompromised hosts and may cause life-threatening infections. Their incidence has greatly increased with the introduction of broad-spectrum antibiotics, immunosuppressive corticosteroids, and antitumor agents (1, 2). *Candida albicans* is the yeast pathogen most frequently isolated from patients with vaginitis (3, 4, 5). Recently, an increase in other species, including *C. tropicalis*, *C. glabrata*, *C. krusei*, and *C. parapsilosis*, which are the cause of the opportunistic infection oropharyngeal candidiasis (OPC), has been observed (4). *C. glabrata* has become a prominent pathogen in some institutions (6). It is therefore of the utmost importance to rapidly and reliably identify *C. albicans* as well as other *Candida species* in routine clinical microbiology practice (7). The conventional methods of identifying yeasts to the species level in the clinical microbiology laboratory rely on criteria such as morphology, growth characteristics and carbon source assimilation or fermentation, as well as appearance on differential isolation media (8, 9). Isolates of *C. albicans* are typically identified by their ability to form germ tubes (GT) or chlamydospores under the appropriate conditions (10). It has also been demonstrated that the MUREX *C. albicans* (MC) (Murex Diagnostics), Albicans-Sure (AS) (Clinical Standards Laboratories), and BactiCard *Candida* (BC) (Remel) test kits can be used for the rapid, presumptive identification of *Candida albicans* (11). To identify other species of *Candida*, commercial carbohydrate assimilation systems, such as the ID 32C system and API 20C test kit, are widely available (12).

The present study was undertaken to identify all *Candida species* isolated from vaginal specimens of patients suffering from vaginitis in Ankara hospitals by the commercial Fungichrom test kit (International Mycoplasma, Signes, France) as an alternative to previously described methods and test kits.

Materials and Methods

Collection of specimens: A total of 78 isolates, which consisted of 6 different species of yeasts, were analyzed in this study. All of the strains were isolated from specimens in the microbiology and bacteriology laboratories of seven different hospitals in Ankara, Turkey. Specimens were collected from patients all symptomatic with vaginitis by passing a sterile cotton swab several times across the vaginal surface, including vaginal discharge materials. Immediately after sampling, each swab was replaced in its sterile tube and transported within 30 minutes of sampling from the place of collection to the laboratory. All test individuals were sampled within a 4-week time period. All isolates were stored at -70°C in skim milk and then subcultured and grown for 24 to 48 h on Sabouraud dextrose media before testing.

Isolation of *Candida* sp.: The cotton end of each swab was inserted into 0.5 ml of sterile water in a microcentrifuge tube, the tube was rigorously mixed for 30s with a laboratory tabletop vortex mixer and 0.15 ml of the wash was spread onto plates containing Sabouraud dextrose agar, and plates were incubated at 37°C for 48 h.

Conventional methods for identification of *Candida* sp.: Yeast-like growing colonies on Sabouraud dextrose agar were routinely Gram-stained and, if found to be yeast cells microscopically, were identified with the standard carbohydrate assimilation or fermentation tests (13) and with the API 20C system (bioMerieux Vitek, Inc., Hazelwood, Mo.) (14). The API 20C yeast identification system was inoculated with the samples, and the results were interpreted by following the manufacturer's instructions.

Preparation of inoculum to be used in Fungichrom test: Yeast colonies growing on Sabouraud dextrose agar plate were carefully collected by either a sterile wire loop or Pasteur pipette. Collected colonies were discharged into a sterile vial and mixed well by gentle shaking. The standardization of the inoculum can be performed with a densitometer. As verified by densitometer, the turbidity of the inoculum for identification was equal to #2 McFarland standard. The commercially available rapid enzymatic Fungichrom test kit was used according to the manufacturer's instructions. The Fungichrom test detects the enzymes L-prolin-aminopeptidase, ß-galactosaminidase, ortho nitrophenyl ß-galactosidase, glycine amidase, and phenol oxidase in yeast cells grown on culture media.

Inoculation of the test tray: The test tray was marked carefully for proper identification, and the adhesive tape covering the test tray was lifted. By using one of the pipettes supplied in the test kit, each well was inoculated with 2 drops of the inoculation medium for identification and incubated at 37°C for 24 to 48 h. The test tray was read when the color of the positive control well turned from violet to yellow or colorless. Following incubation at 37°C for 24 h, 1 drop of 0.1N NaOH was added to well GAL and the test tray was read according to the test procedure system. Interpretation of the Fungichrom tray was performed using the identification table.

Preparation of whole cell proteins (WCPs) for SDS-PAGE. For each culture, a loopful of overnight growth from Sabouraud dextrose agar plate was suspended in 2 ml Sabouraud dextrose broth and incubated in a rotated incubator for 48 h (at 37°C, 150 rpm). Samples were then transferred into eppendorf tubes and centrifuged for 3 minutes at 12500 rpm and the collected cells were washed three times with sterile distilled water. The washed cells were stirred after adding 25µl SDS-sample buffer (0.06 M Tris, 25% glycerol, 0.5% SDS, 1.25% β-mercaptoethanol, and 0.001% bromophenol blue; pH 6.8), and the proteins were denatured in boiling water for 5 minutes. The supernatant was then centrifuged again for 3 minutes at 12100 rpm, collected in an eppendorf tube and kept at -50°C until electrophoresis was carried out.

Electrophoresis. Whole cell proteins were analyzed by SDS-PAGE as described previously by Laemmli (15). This method used 2 cm layer of 4% acrylamide stacking gel and a 10 cm layer of 10% acrylamide separating gel. Electrophoresis was performed with a discontinuous buffer system in a BRL gel apparatus, model V16-2BRL, Gaithersburg MD, USA. The gel was run at a constant current of 20mA through stacking gel and 35 mA through separating gel, or until the bromophenol blue marker had reached the bottom. Gels were then stained with Coomassie Brilliant Blue R 250 (15).

Results

A total of 78 isolates, which consisted of 6 different species of yeast, were analyzed in this study. To our knowledge, this is the first description of the utilization of the Fungichrom test kit for the rapid and accurate identification of different *Candida species* collected from

the vagina. These organisms were fresh clinical isolates, all of which were cultured from specimens received in 7 different hospitals in Ankara, Turkey. The isolates tested were from vaginal secretions of patients suffering from gynecological problems. According to the results obtained with the Fungichrom test kit, *C. albicans* was the most frequently isolated species in the vaginal specimens, accounting for 39 out of 78 (50.00 %) isolates. The next most frequent *Candida species* were *Candida glabrata* (*Torulopsis glabrata*) and *Candida krusei*, which were isolated from 26.92 and 11.53% of the women respectively. Other species of *Candida* were cultured less often: 7 *Candida kefyr* (8.97 %); 1 *Candida tropicalis* (1.28%) and 1 *Candida parapsilosis* (1.28 %). The Fungichrom test detected the enzymes L-prolin-aminopeptidase, ß-galactosaminidase, ortho nitrophenyl ß-galactosidase, peptidase, glycine amidase, and phenol oxidase in yeast cells grown on culture media. The identification of yeast was based on the presence or absence of various enzymes, visualized by colorimetric reactions. For a reaction to be considered positive, it was necessary for the color of the test tray to change from violet (negative reaction) to yellow to colorless (positive reaction). The interpretation of the Fungichrom test kit was carried out either by a coding system or by the identification table supplied in the test kit. In the Fungichrom test kit, enzymatic activities were revealed by three kinds of reaction: hydrolysis of chromogenic substrate, assimilation of natural substrates, and oxidation of synthetic substrates. The reliability of the Fungichrom test kit in identifying *Candida species* was further confirmed by conventional biochemical tests and the API 20C system. The results of the Fungichrom test kit were in good agreement with the results obtained from API 20C and conventional biochemical tests.

Differentiation between different *Candida* species was also observed when SDS-PAGE of whole cell proteins was used. Identification of different *Candida* species by conventional tests, which was further confirmed by the Fungichrom test kit, was examined further by the use of their whole cell proteins in SDS-PAGE, in which case 6 different protein band patterns representing 6 different species of *Candida* were observed (Figure 1). Each *Candida* sp. has its own characteristic band patterns as seen in SDS-PAGE. This may not be enough to identify each *Candida* sp. by referring to its electrophoretic pattern, but it is certainly clear that what can be achieved is not identification but differentiation between different

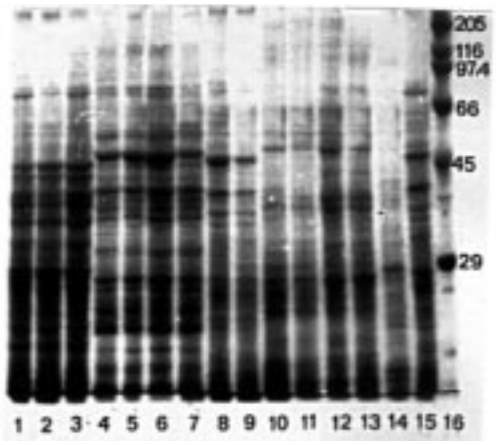

Figure 1. SDS-PAGE of whole-cell protein profiles of different *Candida* species. Lines 1-3: *Candida albicans*, 4-7: *Candida glabrata*, 8-9: *Candida crusei*, 10-13: *Candida kefyr*, 14: *Candida tropicalis*, 15: *Candida parapsilosis* and 16: molecular weight marker of SDS-PAGE in kD (SIGMA wide range marker).

Candida sp. We can conclude that analysis of different *Candida* sp. by SDS-PAGE gave reproducible whole-cell polypeptide profiles, and each species of *Candida* showed its own characteristic band pattern differentiating it from other *Candida* species.

Discussion

The observation of germ tube (GT) production as a method for presumptive identification of *C. albicans* has been in use for many years. This method is based on the fact that *C. albicans* produces short, slender, tube-like structures (germ tubes) when incubated at 35°C to 37°C in serum for 2 to 4 h (10, 16, 17). This method, although simple, takes several hours and requires a microscope and a skilled microscopist (18). Furthermore, up to 5% of the strains of *C. albicans* may be germ-tube negative and other yeasts may produce germ tube-like structures, e.g., pseudohyphae (19, 20). However, it is well-known that a germ-tube can be distinguished from a pseudohyphae by an experienced mycologist. Although the recently identified species *Candida dubliniensis* has been shown to produce both germ tubes and chlamydospores, efforts to differentiate this species from *Candida albicans* are still investigational. Recently, rapid enzymatic test kits (nonmicroscopic methods) such as MUREX *C. albicans* (MC) (Murex Diagnostics), Albicans-

Sure (AS) (Clinical Standard Laboratories), and BactiCard *Candida* (BC) (Remel) have been developed as alternatives to the GT test for the rapid screening of *C. albicans*. These enzymatic test kits detect the production of the enzymes L-proline aminopeptidase and ß-galactosaminidase by yeast isolates grown in culture, and fail to identify *Candida* spp. other than *C. albicans* because *C. albicans* is the only one that produces both L-proline aminopeptidase and ß-galactosaminidase. The other yeasts produce only one or neither of the enzymes. All these tests are able to detect only *C. albicans* and are not used for the identification of other species of *Candida*. To identify non-albicans *Candida species*, as well as to confirm their identification, commercial carbohydrate assimilation systems, such as the API 20C system, are widely available. The identification of *C. dubliniensis*, on the other hand, requires the development of more practical methods, and is still a research topic. As a result of this study, we have shown that the Fungichrom test kit can also be used as an alternative to previously described methods, conventional biochemical tests and kits for the rapid and accurate identification of both *C. albicans* and non-albicans *Candida* species, although for routine procedures, the germ tube test is still the method of choice in developing countries for the identification of *C. albicans*.

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