Identification of Candida inconspicua clinical isolates and testing of fluconazole, amphotericin B, flucytosine and caspofungin susceptibility

Ph.D. theses

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Introduction

Diseases caused by yeasts range from transient mucosal to invasive, often fatal infections. These are often associated with invasive diagnostic and therapeutic methods, with the use of broad-spectrum antibiotics, and occur frequently in the immunocompromised host.

Until the early 1990's, practically only *Candida albicans* was isolated from different infections. With the introduction and routine use of the well-tolerated antifungal agent, the fluconazole in treatment and prevention of fungal disease altered this situation, and the importance of multiresistant *C. glabrata, C. krusei* and *C. tropicalis* has been increasing, mostly in invasive disease. The mortality caused by these species can reach 40-80%, depending on the patient group (malignant disease, prolonged neutropenia, young or old age).

Diagnosis of yeast infections

Diagnosing a yeast infection can be very difficult especially in case of invasive infections. Only 50% of hemocultures are positive in fungemias, while in case of other sample types it can be difficult to decide the pathogenic role of the isolated yeast and to distinguish colonization and infection.

In routine diagnostics, identification of yeasts can easily be performed using several simple tests. Production of germ tubes and chlamydospores are characteristic in case of *C. albicans* and *C. dubliniensis*. On the widely used CHROMagar Candida medium the four most important species (*C. albicans, C. tropicalis, C. krusei* and *C. glabrata*) can easily be distinguished. This medium facilitates the identification of mixed infections. Different commercial identification tests based on carbohydrate assimilation profiles (e.g. API ID32C) are available, but depending on the species misidentification can be frequent.

Serological diagnosis of invasive fungal infections demonstrates cell wall proteins and carbohydrates, or antibodies against them. Simultaneous demonstration of mannan and the specific antibody against it in blood showed 93% specificity and 80% sensitivity, thus similar tests may have a role in the diagnosis of invasive fungal infections.

In the past few years specific, sensitive and fast diagnostic methods utilizing DNA analysis are more and more widely used. In case of yeasts restriction fragment length polymorphism (RFLP) is especially suitable for routine identification. When RFLP is performed on amplified ribosomal DNA (rDNA), the method is referred to as ribotyping. The genetic basis of the method is that fungal rDNA is arranged in tandem repeats, and all repetitions include a copy of 18S, 5.8S and 26S rDNA. Between the coding regions are the internal transcribed spacer (ITS) and the intergenic spacer (IGS) regions. The rDNA regions are conservative in their base sequences, while spacer regions are relatively variable, showing high variability even within a genus. Conservative (rDNA) regions provide sequences suitable for primer annealing, but the co-amplified spacer regions will be variable, thus suitable for discriminative rDNA analysis, yielding species-specific restriction fragment patterns.

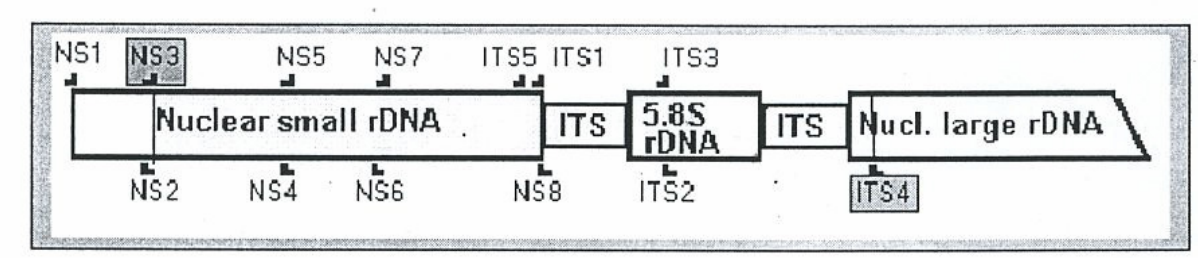

FIG. 1. Schematic representation of the fungal ribosomal genes.

Treatment of yeast infections

The number of available antifungal agents is low, especially in comparison to the wide palette of the antibacterial drugs. For treatment of invasive fungal infections only a few groups of antifungals are available: polyenes, azoles, 5-flucytosine and echinocandins.

Polyenes are pore-forming fungicidal drugs, but toxicity limits their use. Primary resistance against polyenes is relatively rare (some *C. lusitaniae* and *C. guilliermondii* strains and *Trichosporon beigelii*). Secondary resistance was detected in case of C. tropicalis, *C. krusei* and *C. glabrata*, which was due to the decreased ergosterol content in the cell membrane.

Azole-type antifungal agents are fungistatic drugs targeting the biosynthesis of ergosterol. Since the beginning of the 1990's newer triazole drugs have been developed

and marketed with the aim of broadening the antifungal spectrum (fluconazole, itraconazole, voriconazole, posaconazole, ravuconazole).

The most widely used antifungal drug both in treatment and in chemoprophylaxis is the fluconazole, which is effective against yeasts, but not against molds. It can safely be used for antifungal prophylaxis in neutropenic patients, even for long periods. Established primary resistance is exhibited by *C. krusei* and some *C. glabrata* strains. In the past few years, two less frequently isolated species, *C. inconspicua* and C. *norvegensis* were also suspected to possess primary decreased susceptibility to fluconazole. Secondary resistance develops mostly during prolonged treatment in *C. albicans*, *C. dubliniensis* and *C. glabrata*.

5-flucytosine targets nucleotide synthesis. Secondary resistance is developed easily during treatment, therefore it is used less frequently and mostly combined with amphotericin B.

The newest antifungal agent group is the echinocandins. Caspofungin and micafungin are available for treatment of invasive and therapy-resistant Candida-infections. These fungicidal drugs target cell wall biosynthesis and seem to be effective against both yeasts and molds. Primary resistance is found in *Cryptococci,* but secondary resistance is rare and was observed only after prolonged treatment. *C. parapsilosis*, *C. guilliermondii* and *C. lusitaniae* show relatively high caspofungin MIC values.

Though therapeutic success depends only partly on the susceptibility of the fungus, in case of invasive infection the status of the host is at least equally important, therapy failure is frequently caused by primary or secondary resistance. In case of susceptible isolates, therapy resistance of the fungus can be caused by biofilm formation or tolerance to the antifungal used.

Susceptibility testing of yeasts

According to recent guidelines, susceptibility testing is not always necessary, as antifungal susceptibility of several species is predictable. *C. albicans* and *C. parapsilosis* are susceptible to most antifungals, while *C. krusei* tends to be multiresistant. Susceptibility testing should always be performed in case of invasive and therapy-resistant infections. The less frequent, but increasingly prevalent species represent a problem from several points of view. For the clinician the problem is that their susceptibility to the gold

standard amphotericin B and/or to the most widely used fluconazole can be decreased. As the susceptibility testing methods are not validated on these species, an inappropriate method can lead to false susceptibility results in the laboratory.

The most exact and reproducible method in the *in vitro* susceptibility testing of yeasts is the NCCLS M27A reference method, which was accepted in 1997. This method employs a predetermined medium, starting inoculum, preparation procedure and incubation time, but its labor intensiveness precludes its use in routine diagnostics. The incubation time is 48 or 72 hours depending on the species. Interpretation guidelines are available in the document M27A only in case of fluconazole, itraconazole and 5-flucytosine. In case of the recently introduced caspofungin neither the optimal incubation time nor the evaluation criteria is available.

Several commercial tests alternative to the standard method can be found, but only a few shows good (or at least acceptable) correlation with the standard method. Out of these Etest is the most popular, which utilizes a strip containing the antifungal in a preformed concentration gradient. This method is not only a good alternative to the standard method, but according to several authors, in case of amphotericin B it is even better in detection of resistant isolates.

Panels based on breakpoint detection are also available, which examines the antifungal effect of two concentrations. Fungitest allows the testing of six antifungal agents simultaneously, but suffers from the inappropriate breakpoint concentrations and the inadequate sensitivity of the indicator used.

In critically ill patients (endocarditis, prolonged neutropenia) besides MIC determination it can be necessary to determine a better predictor of clinical efficacy, i.e. fungicidal effect. This can be studied through determination of the minimal fungicidal concentration or drawing time-kill curves. In contrast to bacteria, in case of fungi standardized testing protocol is not available. Recently Cantón et al. described a methodology, which due to the higher starting inoculum (10^4cells/ml) is suitable to measure at least 99,9% killing rate, if the whole content of the wells not exhibiting growth is transferred to agar plates. This method revealed that a proportion of *C. parapsilosis and C. dubliniensis* strains is tolerant to amphotericin B, i.e. drug concentrations 32 times MIC are not capable of killing the yeasts. This method is recommended by the authors for routine use in case of invasive candidiases not reacting to therapy, as MFC results showed

good correlation to time-kill curve determination. This method was not applied to study any other antifungal besides amphotericin B.

Characteristics of C. inconspicua and C. norvegensis

The five most frequently isolated yeast species are *C. albicans, C. glabrata, C. tropicalis, C. krusei and C. parapsilosis* both in Hungary and abroad, but other species are also encountered rarely but regularly. We noticed during routine diagnostic work in 2001-2002 that the frequency of isolation of *C. norvegensis* or *C. inconspicua* had increased. As these two species are thought to be fluconazole resistant, we started to study them.

Differentiation of the two species with traditional microbiology methods is difficult. According to the widely accepted API ID 32C panel the two species differ only in their esculin hydrolysis, thus the panel differentiates them by means of supplemental tests, i.e. pseudohypha production. *C. norvegensis* produces pseudohypha on rice extract agar and hydrolyses esculin, while *C. inconspicua* is negative in all two tests.

Due to the difficulties of differentiation, earlier authors employed molecular biology techniques for identification of the three fluconazole-resistant *Candida species C. krusei, C. norvegensis and C. inconspicua*. Nho et al. identified 10-10 strains of the three species obtained from the strain collection of the BioMerieux using traditional (API ID32C, pseudohypha production) and molecular biology methods (rDNA-RFLP and RAPD). For RFLP they amplified the rDNA region ITS1-ITS4 with PCR and restricted the amplicons with the enzyme HhaI. The species differentiation was done by high resolution agarose gel electrophoresis and sequencing of the amplicons. Their results were in accordance with that of the API ID32C panel, though none of the examined *C. norvegensis* strains produced pseudohyphae, despite pseudohypha production is characteristic to this species.

Fluconazol susceptibility of *C. inconspicua* and *C. norvegensis* shows a diverse pattern in the literature. Though both species are considered fluconazole resistant, authors frequently find some susceptible strains depending on the method used. Similarly, susceptibility to amphotericin B and 5-fluorocytosine was found to depend on the testing method.

Aims of the study

1. We investigated the exact identity and species distribution of yeasts diagnosed as *C. norvegensis* or *C. inconspicua* during the routine work using PCR-RFLP of ribosomal DNA.
2. We tested the fluconazole susceptibility of *C. inconspicua* clinical isolates by the standard broth microdilution method (NCCLS M27A-2) in order to prove the putative primary fluconazole resistance of this species. We also tested the influence of higher starting inoculum and shorter (24 hours) incubation time on the MIC values obtained compared to the standard method.
3. In search for an effective therapeutic option, we determined the susceptibility of the strains to amphotericin B, 5-fluorocytosine and caspofungin using the standard method, and, in case of amphotericin B and caspofungin using Etest.
4. We also tested the fluconazole, amphotericin B and 5-fluorocytosine susceptibility using Fungitest. The results were compared to the categorization obtained with the standard method.
5. We determined the minimal fungicidal concentrations of amphotericin B, 5-fluorocytosine and caspofungin using higher starting inoculum.
6. Based on the MFC results, we standardized the caspofungin MIC determination, determining the correct incubation time and end-point criterion.

Materials and methods

Isolation of yeasts from clinical specimen

We collected isolates from 01.01.2001. to 31.12.2003. We used 29 isolates of 22 patients identified as *C. inconspicua* or *C. norvegensis* using API ID32C for studying species identification and 48 C. inconspicua isolates from 48 different patients for susceptibility studies. Eleven patients received fluconazole prior to isolation of the fungus, but none of them received amphotericin B, 5-fluorocytosine or caspofungin. The most frequent specimen types were the lower (24 isolates) and upper (16 isolates) airway samples, but isolates from sterile sites (blood, peritoneum) were also encountered.

Identification of yeasts

Cultures grown on Sabouraud dextrose agar were incubated in fetal calf serum for two hours in order to detect germ tube formation. Subcultures were prepared on CHROMagar Candida and rice extract agar. Assimilation profiles were detected by API ID32C.

For definitive identification we used rDNA-RFLP. DNA was extracted following Hoffman and Winston. We amplified an approximately 1900 bp long fragment of rDNA, using the primers NS3 and ITS4.The amplified fragment consisted of a part of the 18S and 26S rRNA genes, the whole 5.8S rRNA gene and the two ITS regions. The amplified product was restricted with the enzymes MspI, RsaI, ScrFI and HaeIII. Fragments were separated with conventional gel electrophoresis, stained with ethidium bromide and visualized under UV light. Evaluation of the patterns was performed with the software Molecular Analyst. *C. krusei* ATCC 6258*, C. inconspicua* ATCC 16783, *C. norvegensis* ATCC 22977 strains were used as reference strains.

Antifungal susceptibility testing

NCCLS M27A-2 document was used as reference method. Fluconazole (Pfizer), flucytosine (Sigma) and caspofungin (Merck) were dissolved in sterile distilled water, while amphotericin B (Sigma) was dissolved in dimethylsulfoxide. Concentrations ranges were 0.25-128 μg/ml, 0.12-64 μg/ml, 0.015-8 μg/ml and 0.015-8 μg/ml in case of

fluconazole, flucytosine, amphotericin B and caspofungin, respectively. Suspensions of 0.5 McFarland density were prepared from fresh (20-24 hour) cultures on Sabouraud dextrose agar. These suspensions were diluted to the desired concentration in RPMI 1640 broth and this final suspension was used to inoculate the microplates containing the serial dilutions of antifungal agents. Plates were incubated at 35 °C and were evaluated both after 24 and 48 hours. Results after 48 hours were regarded as reference in case of amphotericin B, fluconazole and flucytosine , while in case of caspofungin reference results were read after 24 hours of incubation.

Etest and Fungitest were performed according to the instructions of the manufacturer. Incubation temperature was 35 °C, results were read after 24 and 48 hours in case of Etest and after 48 hours only in case of Fungitest.

C. krusei ATCC 6258 and *C. parapsilosis* ATCC 22019 were used as quality control strains during susceptibility testing.

Determination of minimal fungicidal concentration

MFC determination was performed according to Cantón et al with modifications. MFCs of flucytosine, amphotericin B and caspofungin were determined. Preparation of the plates was performed as in case of NCCLS M27A-2. In case of flucytosine and amphotericin B final inoculum was 10^4 cells/ml verified by quantitative culturing. Incubation time was 48 hours at 35 °C. After 48 hours, the whole content of clear wells were mixed by pipetting and 100-100 µl was transferred to Sabouraud dextrose agar. The suspension was allowed to dry, then with streaking cells were removed from the drug. Plates were incubated for another 48 hours at 35 °C.

In case of caspofungin we used the modified method of Cantón et al. using 10^5 cells/ml inoculum. The whole content of clear wells and wells showing partial inhibition were transferred to drug-free Sabouraud agar plates as described above. Plating was performed both after 24 and 48 hour incubation.

Interpretation of results

Interpretation of fluconazole and flucytosine MIC values followed the NCCLS guidelines. In case of amphotericin B we used the presently most widely used <1 µg/ml as

the resistant breakpoint. In case of caspofungin we used the <=1 μg/ml as the resistant breakpoint according to Stone et al.

We compared the MIC values obtained with different methods to that obtained with the standard method, except for caspofungin, where a standard method is not yet published. Discrepancies between MICs of no more than ± one dilutions were used to calculate the percentage of agreement. Categorical agreement was defined as the same category result compared to the reference method category. MFC was defined as the concentration capable of killing 99,9% of the starting inoculum.

Results

Identification of C. inconspicua

Germ tubes were not produced by any reference or clinical isolates. API ID32C panel identified 27 isolates as *C. inconspicua/C. norvegensis* and two isolates as *C. inconspicua/C. norvegensis/C. krusei*. Reference strains were identified correctly. For further discrimination the panel recommends esculin hydrolysis, which was positive in case of 28 out of the 29 isolates. Thus, 28 strains were identified as *C. norvegensis* based on the assimilation profiles. One of 29 clinical isolates produced pseudohyphae on rice extract agar, thus, only this one was found to be *C. norvegensis* based on pseudohypha production.

The rDNA-RFLP patterns of the three reference strains were easily distinguishable. Patterns of clinical isolates were uniformly identical to that of C. inconspicua ATCC 167873. Based on these results, all of our esculin-hydrolyzing, pseudohypha negative isolates proved to be C. inconspicua.

Fluconazol susceptibility testing

Results of the standard broth microdilution method showed that the majority of the strains exhibits dose-dependent susceptibility to fluconazole (MIC_{90}=32 μg/ml), none of the strains was susceptible. Using normal and elevated inoculum and evaluation after 24 hours, three and one isolates, respectively, showed susceptibility and out of the five resistant strains, three and two strains, respectively, were dose-dependent susceptible. The agreement between the modified broth microdilution methods and the standard method was excellent (93.7-100%). Categorical agreement was between 66.7 and 89.5%. The categorical discrepancies were mostly due to classification of dose-dependent strains as resistant.

Using Etest we found that only about half of the strains was dose-dependent susceptible, the number of resistant isolates were higher. After 24 hours three strains showed false susceptibility. MIC values were especially high after 48 hours (MIC_{90}=128 μg/ml). Regardless of incubation time, Etest was able to detect strains proved resistant with the standard method correctly.

Fungitest results found the majority of the strains (87,4%), besides five susceptible and one resistant isolates, to be dose-dependent susceptible. Categorical agreement with the standard method was good (81.1%), but five dose-dependent susceptible strains were classified as susceptible and four out of five resistant strains were classified as dose-dependent susceptible.

In vitro activity of amphotericin B against C. inconspicua

Using the standard method, more than half (27/48, 56.3%) of the strains proved to be resistant to amphotericin B. By average, Etest MICs were two and one dilutions lower than MIC obtained with the standard method after 24 and 48 hours of incubation, respectively (MIC_{90}=0.5 μg/ml in both cases). Categorical agreement with the standard method was poor after 24 and 48 hours (41.7 and 45.8%). Fungitest found all strains susceptible to amphotericin B, thus all strains resistant with the standard method were found to be susceptible.

Determination of flucytosine susceptibility

The standard method found all strains susceptible to flucytosine (MIC_{90}=2 μg/ml). Using Fungitest only seven isolates proved to be susceptible, all other isolates (41/48) were intermediate. Agreement of Fungitest with the standard method was only 14.4%.

Determination of caspofungin susceptibility

Using the broth microdilution method, caspofungin showed excellent in vitro activity against C. inconspicua, especially when we used partial inhibition as end-point criterion (MIC_{90}=0.25 μg/ml both after 24 and 48 hours). When total inhibition was the end-point criterion, MIC values were two dilutions higher by average (MIC_{90}=1 μg/ml both after 24 and 48 hours). Correlation of Etest MICs (after 24 and 48 hours) was good only compared to the broth microdilution method read at 24 hours with partial inhibition as end-point criterion (85.7% vs. 62.5%).

Determination of minimal fungicidal concentration

MFCs of amphotericin B were two or four times MIC in case of the majority of the strains, tolerance to amphotericin B is not characteristic to *C. inconspicua*.

Flucytosine MFCs fell into a wide range (1-32 times MIC) and 50% of the isolates were tolerant to the drug.

Caspofungin MFCs were between 0.12-1 and 0.12-0.25 μg/ml after 24 and 48 hours of incubation, respectively, and never exceeded the value four times the MIC. In only two cases reached the MFC values after 24 and 48 hours of incubation the MIC found with total inhibition end-point after 48 hours of incubation.

Discussion

Identification of yeasts with traditional and molecular methods

Results of our first experiment confirm that biochemical activity and morphological examinations are not sufficient to exact species identification in case of *C. inconspicua* and *C. norvegensis*. The contradiction between the traditional methods can only be solved using molecular identification methods. Nho et al. addressed the same problem using the strain collection of BioMerieux. They used one restriction enzyme (HhaI), and as the fragment sizes were very similar, only sequencing of the fragments was able to differentiate *C. inconspicua* and *C. norvegensis* reliably. Our method, using four enzymes, is capable of distinguishing the two species without sequencing.

Fluconazol susceptibility

Our results confirm that *C. inconspicua* possess primary decreased susceptibility to fluconazole, thus fluconazole should not be used in treatment of infections caused by *C. inconspicua*. Modified methods proved to be prone to misdiagnosing resistant strains as dose-dependent susceptible, thus should not be considered as alternatives to the standard method in case of *C. inconspicua*.

Etest showed a number strains to be falsely resistant, but all resistant strains were detected correctly, regardless of incubation time. This is of therapeutic significance, as prior to exact species identification we can safely detect resistance avoiding ineffective therapy. Consequently, Etest is a good choice to screen for fluconazole resistance, but not for exact MIC determination.

Fungitest can detect resistance with only low accuracy, though the categorical agreement with the standard method was good (81.1%). Acceptance of Fungitest results without doubt can lead to serious therapeutic problem, as resistant isolates, when even the maximum dose of fluconazole is ineffective, can be categorized as dose-dependent susceptible. As *C. inconspicua* is isolated mostly from seriously ill patients, this issue is of critical importance for effective therapy.

Efficacy of amphotericin B against C. inconspicua

The majority of the strains was found to be resistant using standard method, thus amphotericin B does not seem a good choice against *C. inconspicua*. Surprisingly and contrasting to published data, categorical agreement between the standard method and Etest was poor. Our data show that in case of *C. inconspicua* the applicability of Etest in screening amphotericin B resistance is limited.

Fungitest generated a number of false susceptible data, which is due to the inadequate break-point (2 and 8 versus the presently accepted 0.5 μg/ml) set in the test. Due to this problem Fungitest is unable to detect resistant strains safely, thus in case of amphotericin B its use is insecure.

Determination of flucytosine susceptibility

As all isolates were susceptible, *C. inconspicua* does not exhibit primary resistance to flucytosine. Using Fungitest the proportion of false results is high, again due to the inadequate break-points (2 versus 4 μg/ml).

Determination of caspofungin susceptibility

Caspofungin showed excellent in vitro activity against *C. inconspicua*, regardless of the susceptibility testing method used. In accordance with the published data, MIC values obtained with total inhibition end-point criterion are one-two dilutions higher than that found using partial inhibition. Only two comparative studies of broth microdilution and Etest methods in caspofungin MIC determination were published up to now. Both groups used total inhibition as end-point criterion. Good correlation was found only when less strict agreement definition (±2 versus ±1 dilution) was used. Our results suggest that MIC values obtained with partial inhibition show good correlation within ±1 dilution.

Minimal fungicidal concentration

Amphotericin B tolerance is not characteristic to *C. inconspicua*. It is noteworthy that MFC values always remained below the maximum attainable serum concentration (2 μg/ml). This means that administering a daily dose of 1 mg/kg can lead to serum

concentrations sufficient to kill the fungus. Nguyen et al. found that MFCs higher than 1 μg/ml after 48 hours of incubation frequently correspond to therapeutic failure. Present results and that of Nguyen et al. warrants further study to decide whether amphotericin B is appropriate against *C. inconspicua*.

Half of the strains were found to be tolerant to flucytosine, showing that flucytosine is mostly fungistatic against *C. inconspicua*. Clinical importance of this phenomenon is unknown. Our data show that MFCs of flucytosine do not reach the safely attainable serum concentration (100 μg/ml), thus flucytosine may have a role in treatment of *C. inconspicua*, but as resistance develops easily, flucytosine should only be used as part of combination therapy.

Caspofungin MFCs correspond well to MICs obtained after 24 hours of incubation with partial inhibition as end-point criterion, suggesting that differences between MICs obtained with partial and total inhibition end-point criteria are mostly due to dead cells and debris. Klepser et al. used another echinocandin, anidulafungin (LY303366) in their experiments and found that at concentrations above MIC_{80} fungal cells were unviable as revealed by scanning electron microscopy. Following Klepser et al., we used transmission electron microscopy to confirm that growth in wells showing partial inhibition was due to dead cells rather than viable growth. Thus, using total inhibition as end-point criterion leads to falsely high MICs, and for correct MIC determination partial inhibition should be used, at least in case of *C. inconspicua*. In case of other yeasts of clinical importance, further studies are required to investigate the relationship between different MICs and MFCs.

Summary

We performed a comparative study of traditional and molecular methods for correct identification of the easily confused *C. inconspicua* and *C. norvegensis*. By means of PCR amplification and four-enzyme restriction analysis of a rDNA fragment we could reliably distinguish the two species.

We determined the fluconazole, amphotericin B, flucytosine and caspofungin susceptibilities of 48 *C. inconspicua* clinical isolates using standard broth microdilution. We proved that *C. inconspicua* exhibits primary decreased susceptibility to fluconazole, and the proportion of amphotericin B resistant strains is high. Flucytosine and caspofungin showed good in vitro activity against *C. inconspicua*. Out of the alternative susceptibility testing methods, only caspofungin Etest is capable of correct MIC determination, though fluconazole Etest helps in recognition of fluconazole resistance prior to exact species identification avoiding therapy failure.

Due to the inappropriate break-points, Fungitest is not recommended for susceptibility testing of *C. inconspicua*.

Based on the MFC values obtained, further, preferentially in vivo studies are required to decide the applicability of amphotericin B and flucytosine in treatment of *C. inconspicua* infections. Caspofungin showed good fungicidal activity against *C. inconspicua*. Using our MFC results we proved that using total inhibition as end-point criterion during MIC determination leads to falsely high MIC values, caused by ostensible growth due to dead cells and debris. Consequently, MIC value should be read as the first well showing prominent growth inhibition as compared to the control, rather than the first clear well (total inhibition).

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