Identification and Antifungal Susceptibility Testing of *Candida* Species: A Comparison of Vitek-2 System with Conventional and Molecular Methods

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**ABSTRACT**

**Background:** *Candida* infection is a major cause of morbidity and mortality in immunocompromised patients; an accurate and early identification is a prerequisite need to be taken as an effective measure for the management of patients. The purpose of this study was to compare the conventional identification of *Candida* species with identification by Vitek-2 system and the antifungal susceptibility testing (AST) by broth microdilution method with Vitek-2 AST system. **Materials and Methods:** A total of 172 *Candida* isolates were subjected for identification by the conventional methods, Vitek-2 system, restriction fragment length polymorphism, and random amplified polymorphic DNA analysis. AST was carried out as per the Clinical and Laboratory Standards Institute M27-A3 document and by Vitek-2 system. **Results:** *Candida albicans* (82.51%) was the most common *Candida* species followed by *Candida tropicalis* (6.29%), *Candida krusei* (4.89%), *Candida parapsilosis* (3.49%), and *Candida glabrata* (2.79%). With Vitek-2 system, of the 172 isolates, 155 *Candida* isolates were correctly identified, 13 were misidentified, and four were with low discrimination. Whereas with conventional methods, 171 *Candida* isolates were correctly identified and only a single isolate of *C. albicans* was misidentified as *C. tropicalis*. The average measurement of agreement between the Vitek-2 system and conventional methods was >94%. Most of the isolates were susceptible to fluconazole (88.95%) and amphotericin B (97.67%). The measurement of agreement between the methods of AST was >94% for fluconazole and >99% for amphotericin B, which was statistically significant (*P* < 0.01). **Conclusion:** The study confirmed the importance and reliability of conventional and molecular methods, and the acceptable agreements suggest Vitek-2 system an alternative method for speciation and sensitivity testing of *Candida* species infections.

**Key words:** Antifungal agents, antifungal resistance, *Candida* species, fungi, mycology, opportunistic infections

**INTRODUCTION**

*Candida* infections often associated with high morbidity and mortality have increased remarkably during the couple of decades.¹ The incidence of *Candida* infections is on the rise with the increase in number of immunosuppressed drugs as well as the use of medical and surgical interventions.² Although *Candida albicans* is the most prevalent species,³ an epidemiological shift in *Candida* pathogens has been recently noted by the increasing number of infections caused by non-*albicans* *Candida* species (NAC).¹³⁻⁸ The increased species diversity

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and incidence of infections have resulted in the need for an accurate and rapid identification of *Candida* isolates and have become important for proper patient management as various species respond differently to antifungals and for the prevention of emergence of drug resistance.[1,2,6]

**Conventional methods of identification are time-consuming.[6,7]** Commercially available biochemical and molecular methods, which allow identification within several hours, have been developed and evaluated.8 The Biomerieux Vitek-2 system includes the Vitek-2 cards that allow species identification by comparison of the biochemical profile with an extensive database. The system also incorporates the antifungal susceptibility testing (AST) cards, which is designed for AST.9 Recently, molecular genotyping methods have become more popular for epidemiological analysis.[10]

AST has been increasingly required in clinical practice. It is already well established that the outcome of invasive fungal infections could be improved by early initiation of appropriate antifungal agent based on the susceptibility profile of infecting *Candida* species.[11,12] Recently, Biomerieux Vitek-2 expanded its role in this area with a yeast susceptibility test that determines *Candida* growth spectrophotometrically using Vitek-2 microbiology systems, performing fully automated testing of susceptibility to fluconazole, amphotericin B, fluconazole, and voriconazole.[12]

The earliest possible identification and drug susceptibility profiling of *Candida* infections in immunocompromised patients allow for prompt optimization of antimicrobial therapy and diminished need for additional diagnostic studies, helping timely in saving the life of many patients. Hence, the objectives of this study were to compare the Vitek-2 yeast identification system with conventional and molecular methods of identification and Vitek-2 AST system with the broth microdilution method.

**MATERIALS AND METHODS**

The study was conducted at the Mycology Laboratory, Department of Microbiology, Maulana Azad Medical College and Associated Lok Nayak Hospitals, New Delhi, India, which is a 1500-bedded tertiary care hospital where patients came from all over the India. All the isolates of *Candida* spp. were recovered from various clinically available specimens, namely, oropharyngeal swab, blood culture, sputum, urine, cerebrospinal fluid, and stool.

The following strains were used as controls for the evaluation of various methods: *C. albicans* ATCC90028, *Candida parapsilosis* ATCC22019, *Candida krusei* ATCC6258, *Candida glabrata* ATCC90030, and *Candida tropicalis* ATCC750.

Identification and speciation of clinical isolates were done by conventional methods, Vitek-2 system, and the molecular methods. For the purpose of comparison, molecular methods were taken as gold standard method.

**Identification by conventional methods**

Identification and speciation of *Candida* isolates were done on the basis of germ tube production, morphology on corn meal agar with Tween 80 (Hi Media, India), HiCrome *Candida* agar morphology (Hi Media, India), carbohydrate fermentation, and assimilation tests using yeast nitrogen base agar (Difco, Becton Dickinson, India) as per the standard recommended procedures[13–19] and using the above control strains.

**Identification by Vitek-2 system**

Pure subcultures suspended in aqueous 0.45% (wt/vol) NaCl to achieve a turbidity equivalent to a McFarland 2.0 standard were measured on the DensiChek turbidity meter (Biomerieux, India), an instrument designed to measure the optical density of an organism suspension. The reading range of the DensiChek turbidity meter is 0.0–4.0 McFarland. The Vitek-2 instrument was automatically filled, sealed, and incubated by individual test cards with prepared culture suspension. Cards were held at 35.5°C for 18 h, with optical density readings taken automatically at every 15 min. Based on these readings, an identification profile was established and interpreted according to a specific algorithm.[10]

**Identification by molecular methods**

Molecular identification was performed by the Southern blot hybridization and random amplified polymorphic DNA (RAPD) analysis. Before genotyping, chromosomal DNA was isolated from each isolate using Xu *et al.* method.[17] After evaluating the quality of DNA on agarose gel, the DNA concentrations of each sample were measured and were subsequently subjected for further tests as described below:

**DNA fingerprinting of the isolates by Southern blot hybridization**

For DNA fingerprinting, around 2 µg of chromosomal DNA from each isolate was digested with restriction enzyme EcoR1. Digested DNA was separated on agarose
Random amplified polymorphic DNA

For RAPD analysis, the DNA was purified by the method described by Makimura et al. with slight modifications. Twenty random oligonucleotides (Sigma) were used as a primer for the PCR reaction. Different polymerase chain reaction (PCR) parameters were standardized to optimize the conditions for achieving better results. Finally, PCR was carried out with 50 ng DNA; 200 μM (each) dATP, dCTP, dGTP, and dTTP; 50 pmol oligonucleotides; 0.25 U Taq polymerase; and PCR buffer. The final volume of the reaction mixture was 30 μl. The cycling conditions were 94°C for 2 min, then thirty cycles of denaturation at 94°C for 1 min, thirty cycles of annealing at 42°C for 1 min, and extension at 72°C for 2 min. Final extension was given for 5 min at 72°C. Amplified products (30 μl) were resolved by agarose gel electrophoresis (1%) at 100 V for 1.5 h. The gel was stained with ethidium bromide, visualized under UV light, and photographed.

Antifungal susceptibility testing

Clinical and laboratory standards institute broth microdilution method

Susceptibility of Candida isolates to antifungal fluconazole and amphotericin B was done by the broth microdilution method as per the Clinical and Laboratory Standards Institute (CLSI) M27-A3 document using Roswell Park Memorial Institute medium and morpholinepropanesulfonic acid buffer. The concentration range was tested between 0.125 and 128 μg/ml for fluconazole and 0.016–16 μg/ml for amphotericin B. Minimum inhibitory concentration (MIC) was recorded as the lowest concentration of the drug that produced a visible decrease in turbidity compared to drug-free growth control according to the CLSI standards. The MIC breakpoints recommended by CLSI guidelines were followed. For fluconazole, MIC breakpoints were as follows: Susceptible, MIC ≤ 8 μg/ml; susceptible dose-dependent, MIC 16–32 μg/ml; and resistant, MIC ≥ 64 μg/ml. For amphotericin B, isolates with MICs of ≥ 1 μg/ml were categorized as resistant.

Vitek-2 antifungal susceptibility testing method

Inoculum suspensions for Vitek-2 cards were obtained from the overnight cultures, with the turbidity being adjusted to a 1.8–2.2 McFarland standard according to the manufacturer’s recommendations. A standardized inoculum suspension was placed into a Vitek-2 cassette along with a sterile polystyrene test tube and an antifungal susceptibility test card for each organism. The loaded cassettes were placed into the Vitek-2 instrument, and the respective inoculum suspensions were diluted appropriately by the instrument, after which the cards were filled, incubated, and read automatically. The incubation time varied from 9.1 to 27.1 h based on the rate of growth in the drug-free control well. In accordance with the M27-A3 document, the results from the 48 h reading were used. Complete data (from the CLSI and Vitek-2 methods) for each fungal isolate were recorded.

Statistical analyses

The reproducibility of AST was assessed by nonparametric correlation coefficient, and AST was considered reproducible if the correlation coefficient was P < 0.05. All statistical analyses were done with the Statistical Package for the Social Sciences (version 17.0; SPSS S.L., Madrid, Spain). All tests of statistical significance were two tailed.

RESULTS

With Vitek-2 ID system, 155 of 172 Candida isolates including C. albicans (n = 126), C. tropicalis (n = 12), C. krusei (n = 10), C. glabrata (n = 4), and C. parapsilosis (n = 3) were correctly identified. Eleven C. albicans were misidentified (6.39%) as C. famata (n = 6), C. tropicalis (n = 1), C. glabrata (n = 1), C. krusei (n = 1), C. capitata (n = 1), and K. ohmeri (n = 1). One each isolate of C. tropicalis and C. parapsilosis was misidentified as K. ohmeri and C. famata, respectively. Four isolates (2.32%) such as C. albicans (n = 2), C. parapsilosis (n = 1), and C. tropicalis (n = 1) were identified with low discrimination [Table 1].

On the other hand by conventional methods, 171 of 172 Candida isolates including C. albicans 138 (80.23%), C. tropicalis 14 (8.14%), C. krusei 10 (5.8%), C. parapsilosis...
5 (2.9%), and C. glabrata 4 (2.3%) were correctly identified. Only one C. albicans was misidentified as C. tropicalis.

All the isolates tested by Vitek-2 ID system and conventional methods were subjected to DNA fingerprinting analysis by using a widely used C. albicans specific probe, the CARE-2 probe. Two low discriminated and 11 misidentified isolates by Vitek-2 ID system and one misidentified isolate by conventional methods were identified as C. albicans by restriction fragment length polymorphism (RFLP) analysis with CARE-2 probe hybridization [Figure 1]. However, NAC isolates did not show any fingerprinting pattern when probed with CARE-2.

The isolates of NAC were subjected to RAPD analysis which produced Candida species-specific RAPD patterns distinct for individual ATCC standard strains. All the isolates tested to be NAC by conventional methods (except one C. albicans isolate which was misidentified as C. tropicalis) exhibited similar results by showing similar typical RAPD patterns to their respective ATCC strains [Figure 2]. Misidentified (n = 2) and low discriminated isolates (n = 2) with Vitek-2 ID system were identified as C. parapsilosis (n = 2) and C. tropicalis (n = 2) by RAPD patterns.

Measurement of percentage agreement between the Vitek-2 ID identification system and conventional methods of identification was >94% for all Candida isolates. Measurement of percentage agreement between the Vitek-2 ID system and conventional methods by Kappa was 70% for C. albicans, 97.8% for C. tropicalis, 97% for C. krusei, 96.7% for C. glabrata, and 98% for C. parapsilosis.

In AST for fluconazole, Vitek-2 AST system showed that 92.4% isolates of the Candida species were susceptible. All the isolates of C. albicans were susceptible while among NAC, 66.6% isolates were susceptible and remaining 33.4% were resistant. While by the CLSI broth microdilution method, 88.95% of Candida species isolates were susceptible with 97.1% C. albicans and 54.5% NAC were susceptible for fluconazole. All the isolates of C. parapsilosis were found to be susceptible while all the C. krusei (100%) were resistant to fluconazole by both the methods [Table 2]. The measurement of percentage agreement between the Vitek-2 AST system and CLSI broth microdilution method by Kappa was 94% for fluconazole.

For amphotericin B drug, Vitek-2 AST system showed that 98.3% isolates of Candida species were susceptible while 1.7% were resistant. All the isolates of C. albicans (100%) and 90.9% of NAC were susceptible [Table 2]. While when tested by CLSI broth micro-dilution method, 97.7% isolates of Candida species were susceptible to amphotericin B. All the isolates of C. albicans (100%), C. tropicalis (100%), C. glabrata (100%), C. parapsilosis (100%), and 60% isolates of C. krusei were susceptible with remaining 40% isolates of C. krusei being resistant. However, using the Vitek-2 AST system, 70% isolates of C. krusei were found to be susceptible. Except C. krusei, all the other Candida species isolates were susceptible to amphotericin B by both methods. The MIC of the two quality control strains was within the range of expected values and showed reproducibility by both methods. For amphotericin B drug susceptibility testing, the measurement of percentage agreement was >94%.

| C. albicans | 139 | 126 | 11 (C. Famata and s each of C. tropicalis, C. glabrata, C. krusei, G. capitatum, K. ohmeri) |
| C. tropicalis | 14 | 12 | 1 (K. ohmeri) |
| C. krusei | 10 | 10 | 0 |
| C. parapsilosis | 5 | 3 | 1 (C. Famata) |
| C. glabrata | 4 | 4 | 0 |
| Total | 172 | 155 | 13 |

**Table 1: Comparison of Vitek-2 ID system with conventional and molecular methods of identification (n=172)**

| Molecular methods (gold standard) | Correctly identified by Vitek-2 system | Misidentified by Vitek-2 system | Low discrimination by Vitek-2 system | Correctly identified by conventional methods | Misidentified by conventional methods |
|----------------------------------|----------------------------------------|-------------------------------|-------------------------------------|----------------------------------------------|-------------------------------------|
| C. albicans | 139 | 126 | 11 | 2 | 138 | 1 |
| C. tropicalis | 14 | 12 | 1 | 1 | 14 | 0 |
| C. krusei | 10 | 10 | 0 | 0 | 10 | 0 |
| C. parapsilosis | 5 | 3 | 1 | 1 | 5 | 0 |
| C. glabrata | 4 | 4 | 0 | 0 | 4 | 0 |
| Total | 172 | 155 | 13 | 4 | 171 | 1 |

C. albicans: Candida albicans, C. tropicalis: Candida tropicalis, C. krusei: Candida krusei, C. parapsilosis: Candida parapsilosis, C. glabrata: Candida glabrata, C. Famata: Candida Famata, K. ohmeri: Kodamaea ohmeri, G. capitatum: Geotrichum capitatum
agreement between the Vitek-2 AST system and CLSI broth microdilution method by Kappa was 99%.

The correlation coefficient index (CCI) between Vitek-2 ID system and conventional methods of identification was 0.938, and it was statistically significant ($P<0.05$). The CCIs values between the Vitek-2 AST system and the CLSI broth microdilution method for the antifungal agents (fluconazole and amphotericin B) were also highly significant [Table 3]. Correlation coefficient indices were expressed to a maximum value of 1. All the correlation coefficient indices were statistically significant ($P<0.05$).

**DISCUSSION**

Candida species is an important cause of systemic mycosis in hospitalized patients, and morbidity and mortality worldwide, especially in critically ill patients. Among Candida species, C. albicans, C. glabrata, C. tropicalis, C. parapsilosis, and C. krusei were the most common species encountered in routine clinical laboratory samples.[23] In our study, we have found C. albicans (80.8%) as the predominant species followed by C. tropicalis (8.13%), C. krusei (5.8%), C. parapsilosis (2.9%), and C. glabrata (2.3%), which is consistent with a previous study of Jha et al.,[24] in which the majority of Candida species were C. albicans (70%) followed by C. tropicalis (13.33%), C. krusei (10%), C. parapsilosis (3.33%), and C. stellatoidea (3.33%).[24] A study from South India by Kumari et al. in 2014 reported with overall predominance of NAC spp. and the predominant species identified was C. albicans.[25]

In this study, we compared the fully automated Vitek-2 ID system with conventional methods for identification of Candida species. Of 172 Candida isolates, Vitek-2 identified 155 (90.12%) Candida isolates correctly, 13 (7.56%) were misidentified, and 4 (2.32%) were identified with low discrimination. Massonnet et al.[8] in their prospective study reported that Vitek-2 identified 41 (67.21%) Candida isolates correctly, 10 (16.39%) were not identified, 3 (4.91%) were misidentified, and 7 (11.47%) isolates were identified with

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**Table 2: Antifungal susceptibility testing pattern of the Candida spp. isolates by the Vitek-2 system and Clinical Laboratory Standards Institute broth microdilution method (n=172)**

| Species name     | Identification method | FLU, n (%) | AMB, n (%) |
|------------------|-----------------------|------------|------------|
|                  |                       | S* (≤8 μg/ml) | R* (≥8 μg/ml) | S* (≤3 μg/ml) | R* (≥9 μg/ml) |
| C. albicans (139) | Vitek-2               | 139 (100)  | 0          | 139 (100)    | 0           |
|                  | CLSI                  | 125 (97.1) | 4 (2.9)    | 139 (100)    | 0           |
| C. tropicalis (14) | Vitek-2               | 24 (100)   | 0          | 14 (100)     | 0           |
|                  | CLSI                  | 11 (98.6)  | 3 (1.4)    | 14 (100)     | 0           |
| C. krusei (10)   | Vitek-2               | 0          | 10 (100)   | 7 (70)       | 3 (30)      |
|                  | CLSI                  | 0          | 3 (100)    | 6 (60)       | 4 (40)      |
| C. parapsilosis (5) | Vitek-2             | 5 (100)    | 0          | 5 (100)      | 0           |
|                  | CLSI                  | 5 (100)    | 0          | 5 (100)      | 0           |
| C. glabrata (4)  | Vitek-2               | 2 (25)     | 3 (75)     | 4 (100)      | 0           |
|                  | CLSI                  | 2 (50)     | 2 (50)     | 4 (100)      | 0           |

*S*: Susceptible range, *R*: Resistant range. FLU: Fluconazole drug, CLSI: Clinical and Laboratory Standards Institute, AMB: Amphotericin B drug, C. albicans: Candida albicans, C. tropicalis: Candida tropicalis, C. krusei: Candida krusei, C. parapsilosis: Candida parapsilosis, C. glabrata: Candida glabrata
A majority of Candida isolates were susceptible to both antifungal drugs tested by AST-YS06 Vitek-2 cards and the CLSI M27-A3 method. All the isolates of C. kruzei (100%) were resistant to fluconazole drug by both the methods also seen by other workers, emphasizing its intrinsic resistance toward azoles and poor susceptibility to other antifungals, including amphotericin B. In current clinical management practices, fluconazole is not recommended as a treatment option for C. kruzei infection or susceptibility testing.

The measurement of percentage agreement between the Vitek-2 AST system and CLSI broth microdilution method by Kappa was 94% for fluconazole, quite in concordance with a study by Bourgeois et al., where the agreements were 94.6%. Earlier in a study, the overall essential agreement between the Vitek-2 AST system and the broth microdilution MICs has been found to range from 97.9% with 24 h broth microdilution result used as reference. However, we found that for amphotericin B drug susceptibility testing, the measurement of percentage agreement between the Vitek-2 AST system and CLSI broth microdilution method by Kappa was 99%. While the overall essential agreement between the Vitek-2 AST system and the broth microdilution, MICs were found to range from 96.7% (voriconazole) to 99.1% (amphotericin B and flucytosine) with the 24 h broth microdilution result used as the reference in another study.

The CCIs values between all the methods were statistically significant (P < 0.05). The CCIs values for the Vitek-2 AST system and the CLSI broth microdilution method were also statistically significant. However, the CCIs values for the Vitek-2 AST system were lower than those observed for the CLSI broth microdilution method; this may be because the ranges of antifungal agents in the Vitek-2 AST system were lower than those observed for other methods also seen by other workers.

CONCLUSION

The present study revealed that Vitek-2 system reduces the period required for identification and improves the
rate of identification of *Candida* species isolates. However, conventional identification methods are still considered reference standard methods, in spite of time-consuming along with ambiguities results, and hence, suited better for research purposes. Therefore, Vitek-2 system appeared to be an alternative method for identification and AST for the *Candida* species to prescribe appropriate antifungal agents for the better management of opportunistic infection among immunosuppressed patients.

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**Conflicts of interest**

There are no conflicts of interest.

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