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ORIGINAL ARTICLE

EMERGENT FUNGAL DISEASE: APPLICATION OF LOW-COST PHENOTYPIC IDENTIFICATION FOR *Candida auris* LABORATORY DIAGNOSIS

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ABSTRACT

The emergent species *Candida auris* presents a significant concern to the scientific community because of its ability to cause outbreaks in hospitals and healthcare units, as well as the challenges associated with its identification via classical methodologies. This study aimed to improve the performance of an accurate and low-cost phenotypic identification method for *C. auris.* The modified salt stress resistance method was employed as a phenotypic test for diagnostic differentiation between *C. auris* and other closely related yeast species on the basis of their biochemical profiles, which can lead to diagnostic errors. The results indicated that *C. auris* isolates were thermos-halotolerant at the tested NaCl concentrations, demonstrating their ability to grow and alter the pH of the medium within 24 hours at 42 °C. This profile contrasted with that observed for other evaluated *Candida* species, including *Clavispora lusitaniae* (syn: *Candida lusitaniae*), *Pichia kudriavzevii* (syn: *C. krusei*), *Meyerozyma guilliermondii* (syn: *C. guilliermondii*), *C. albicans, C. dubliniensis*, *Nakaseomyces glabratus* (syn: *C. glabrata*), *C. parapsilosis,* and *C. haemulonii*. Complementary phenotypic methods (Vitek 2 version 9.2) and molecular techniques (MALDI-TOF MS version 3.0) successfully identified all the tested yeasts, although MALDI-TOF was unable to identify *C. auris* samples. Consequently, as automated methods are not error-free, the modified thermos-halotolerance test has proven to be a sensitive, low-cost, and effective complementary methodology for the phenotypic identification of *C. auris.*

KEY WORDS: *Candida auris*; phenotyping; diagnosis; thermos-halotolerance.

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INTRODUCTION

The inversion of the age pyramid, resulting in an aging population, contributes to an increased prevalence of metabolic comorbidities, primary and acquired immunodeficiencies, and hospitalizations (Brasil, 2022). In addition, the combination of excessive clinical medication use, climate change due to deforestation, and the overuse of antimicrobials and pesticides in agriculture has led to the emergence of unusual pathogens that act as colonizing agents responsible for opportunistic human diseases (Chakrabarti & Sood, 2021).

In this context, fungal infections caused by *Candida* spp. have shown trends in recent decades, particularly in nosocomial environments, where they rank as the fourth leading cause of morbidity and mortality (Han et al., 2020). Furthermore, the epidemiology of this genus varies significantly across geographic regions and sites of infection. For example, in the USA, *C. glabrata* is more prevalent in cases of candidemia than *C. albicans* is, whereas *C. parapsilosis* is more common in Asia (Falagas et al., 2010; Jeffery-Smith et al., 2017).

Currently, significant attention is focused on *C. auris,* a species responsible for outbreaks in hospital environments across multiple countries. The increasing incidence and prevalence of this species have increased concerns within the national and international scientific community (Wang et al., 2018; Chakrabarti & Sood, 2021; Desoubeaux et al., 2022; Melo et al., 2023).

After the emergence of *C. auris* in Japan in 2009, prospective and retrospective surveillance was undertaken to search for national and international yeast collection banks to trace the origin and emergence of this previously unknown pathogen. Three samples from different countries, dated in 1996 (South Korea), 1997 (Japan), and 2008 (Pakistan), were identified as *C. auris.* However, due to imprecise identification, underreporting occurred at that time (Lee et al., 2011; Jeffery-Smith et al., 2017; Lockhart et al., 2017; Sekizuka et al., 2019).

This pathogen (*C. auris*) has several specific characteristics, including the ability to form biofilms and robust yeast aggregates that are difficult to remove and resistance to available antifungals. Some clades exhibit pan-resistance, while others are multidrug resistant (Wang et al., 2018). Additionally, *C. auris* has a prominent layer of mannans, which is 10% greater than that found in other *Candida* species, facilitating evasion of the host immune system (Wang et al., 2018; Wang et al., 2022). Around the world, public health agencies have published technical notes to guide the screening and identification of this fungus, offering recommendations for decision-making when *C. auris* is isolated from clinical samples. The difficulty in identifying *C. auris* via classical laboratory methods reflects the need for more precise methods for the species-specific identification of pathogenic fungi. In the case of *C. auris*, accurate diagnosis enables the adoption of testing strategies for suspected

cases, characterization of the antifungal response, transmission blocking, and outbreak control (Ahmad & Alfouzan, 2021).

Currently, there are chromogenic media available for the phenotypic identification of *C. auris* (Sasoni et al., 2022). However, these media require additional identification tests, as they do not effectively distinguish related species such as *C. haemulonii*, *C. pseudohaemulonii,* and *C. duobushaemulonii*, and they also do not differentiate some diverging genotypes of *C. auris* (Watkins et al., 2022; Ruiz-Gaitán et al., 2023). Phenotypic and molecular identification of *C. auris* can also be conducted via semiautomated or automated tests, including commercial identification systems such as Vitek version 7.1 (Biomerieux®), API 20C AUX (Biomerieux®), Becton Dickinson Phoenix (BD Diagnostics®), MicroScan, and MALDI-TOF Vitek-MS version 3.0 (Biomerieux®), with MALDI-TOF being one of the most recommended techniques currently for the diagnosis of *C. auris.* However, these systems often misidentify *C. auris* (Lee et al., 2011; Girard et al., 2016; Chowdhary et al., 2017; Mizusawa et al., 2017).

Several studies have been conducted to address the limitations in the identification of *C. auris*, focusing on phenotypic techniques, automation, and molecular tests. However, despite advancements, some methodologies remain unavailable in nonspecialized centers, hindering early treatment and preventing deaths and outbreaks (Ruiz-Gaitán et al., 2023). The primary concern regarding this fungus lies in the difficulty of identification via conventional phenotypic and molecular methods, as automated systems can misidentify these species, particularly those that rely on their databases for feedback (Jeffery-Smith et al., 2017). Thus, multiple methodologies for identifying this fungal pathogen are recommended (Chowdhary et al., 2017).

Regarding the phenotypic identification of *C. auris*, halotolerance is the most promising characteristic for distinguishing it from other yeasts of *Candida* genus (Alves et al., 2002; Wang et al., 2018). According to Oren (2008), organisms exhibiting this characteristic develop various metabolic adaptations and resistance mechanisms, such as high-salt-in and low-salt-in strategies. These strategies control the diffusion of NaCl into the cell, enabling the accumulation of ions in the cytoplasm and the adaptation of intracellular functions to saline stress. They also involve the accumulation of organic solutes to stabilize biological structures (Oren, 2008).

Given the limited tolerance of some *Candida* species to high concentrations of NaCl and elevated temperatures, the determination of halotolerance in various *Candida* species through growth under saline stress at 42 °C, combined with changes in pH color indicators, may serve as an additional alternative test to distinguish *C. auris* from other species (Gunde-Cimerman et al., 2009; Krauke & Sychrova, 2010; Melo et al., 2023). Therefore, the main objective of our study was to assess the thermo-halotolerance of *C. auris* to MSHB 10% as a complementary test to the identification methods used in laboratories.

MATERIAL AND METHODS

Study design

This descriptive, retrospective, qualitative study was conducted in 2023 and 2024 at the State Public Health Laboratory of Goiás (LACEN-GO) and at the Federal University of Goiás (UFG), Goiânia, Goiás State, Brazil.

Controls and tested samples

This study was conducted with 10 samples of *Candida* species selected based on research by Jeffery-Smith et al. (2017). This selection included two samples of *C. auris* and one sample of each of the following species: *Clavispora lusitaniae* (syn: *C. lusitaniae*), *Pichia kudriavzevii* (syn: *C. krusei)*, *Meyerozima guilliermondii* (syn. *C. guilliermondii*), *C. albicans*, *C. dubliniensis, Nakazeomyces glabratus* (syn. *C. glabrata*), *C. parapsilosis* and *C. haemulonii*. These eight species are considered to be confounded with *C. auris* in laboratory identification by different methodologies in the referenced study (Jeffery-Smith et al., 2017). American Type Culture Collection (ATCC) reference strains of *C. albicans* (10231) and *Nakazeomyces glabratus* MYA (2950) were also used for quality control of the tests and culture media.

Fungal cultures

All study samples were subcultured on Petri dishes containing Sabouraud dextrose agar with 1% (w/v) chloramphenicol and incubated at 30 °C ± 2 °C for 48 hours, followed by identification via different methods described below.

Identification via the Vitek 2 system

For the identification tests, a suspension was prepared from isolated colonies in 3.0 mL of 0.45% (w/v) saline, achieving a density of 1.8 to 2.2 on the McFarland scale, as measured by a densitometer (Densicheck Plus Biomerieux®). The YST (commercially available, Biomerieux®) card was placed on the cassette. The sample was subsequently sent to the filling chamber, transferred to the sealing chamber, and incubated for approximately 18 hours.

Mass spectrometry identification by Vitek-MS system

A thin and homogeneous layer of the colony mass was applied to a spot on a slide. After drying, 0.5 µL of 70% (v/v) formic acid was added to the colony smear. Once dried, 1 µL of the CHCA matrix (α-cyano-4-hydroxycinnamic

acid) was applied, and identification was performed via system software. For the two *C. auris* samples, two extraction protocols for Vitek-MS™ were employed: the standard protocol described above and an alternative method involving mechanical abrasion with a glass sphere, followed by homogenization through mechanical agitation, centrifugation, and the addition of 70% (v/v) formic acid and 100% (v/v) acetonitrile. Afterward, 1 µL of the protein extract was dispensed onto the spot on the slide and allowed to dry, and then another 1 µL of the CHCA matrix was added to continue the identification process.

The spectra generated via Vitek-MS™ were obtained through comparison with the IVD database (version 3.0). For IVD analysis, spectra were obtained via the device's automation control and Myla software (Biomerieux®) with the settings suggested by the manufacturer. For each acquisition group, a standard strain (*Escherichia coli* 8739) was used, which calibrates the instrument and validates the run, and quality control was carried out with *Nakazeomyces glabratus* MYA (2950); thus, the device compares the spectrum obtained with the expected spectrum of each fungus post calibration (Grenfell et al., 2016).

Complementary yeast identification by thermohalotolerance test

Previous studies have shown that *C. auris* exhibits 10% halotolerance (Falagas et al., 2010; Chakrabarti & Sood, 2021; Han et al., 2023). Therefore, Sabouraud Hypertonic Broth with 10% NaCl (w/v) supplemented with 1.6% (w/v) Bromocresol Purple was used as a pH indicator (Silva et al., 2021). Thus, all the samples were tested against the broth, with five aliquots being prepared per isolate and tests with the ATCC strains.

The yeast colonies from the subcultured medium were diluted in 3.0 mL of 0.45% saline and adjusted to a density of 0.5 on the McFarland scale, as measured by a densitometer. Subsequently, 40.0 µL of this suspension was added to 2.0 mL of modified hypertonic Sabouraud broth supplemented with 10% NaCl (w/v) and incubated for five days in a bacteriological oven at 42 °C ± 2 °C. The general workflow for the laboratory identification of *C. auris* samples (including the methodologies described above) is illustrated in Figure 1.

Ethics

The present study was approved by the research ethics committees/ CEP- Federal University of Goiás/Hospital of Tropical Diseases Dr. Anuar Auad-HDTHAA, with registration numbers 5,712,004 and 5,997,410, respectively.

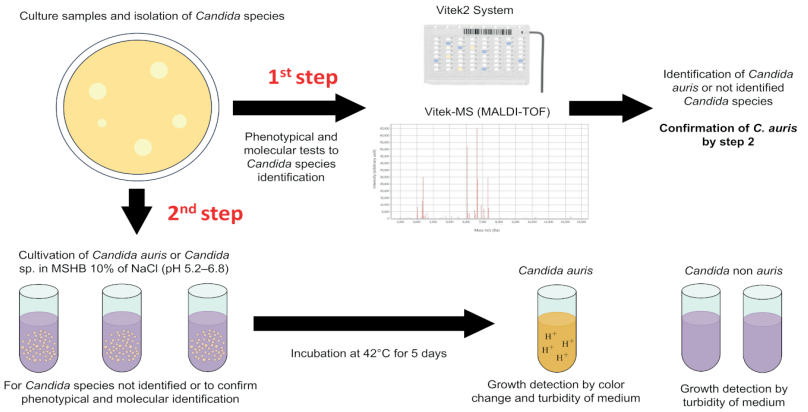

Figure 1. Workflow for laboratory identification of *Candida auris* and the control species. Step 1: Cultured samples and isolated *Candida* species were submitted for analysis via the Vitek2 System and MALDI-TOF. Step 2: Cultivation in MSHB with 10% NaCl with a pH range of 5.2–6.8. The tubes on the left contained MSH + Bromocresol Purple (MSHB) culture media, to which a suspension of *C. auris* yeast colonies were added. After incubation, cell growth produces secondary analytes that acidify the medium, turning it yellow. If *C. auris* yeast does not grow, the pH remains in equilibrium, and no color change occurs.

RESULTS

Identification via the Vitek 2 system

In the Vitek 2 automated test, 100% of *Candida* samples met the system's reproducibility criteria (≥95%), with identification rates between 95% and 99%. For the MALDI-TOF method, 80% (8 samples) achieved 99.9% similarity with the IVD–Vitek-MS reference database, whereas 20% (two samples) returned unidentified samples. In the halotolerance test using MSHB with 10% NaCl, only the two *C. auris* samples demonstrated thermos-halotolerance (Table 1).

Table 1. Identification of *Candida* sp. by Vitek 2, Vitek-MS, and MSHB in 10% NaCl.

Yeasts samples	Vitek 2[a]	Vitek-MS[b]	MSHB10%[c]
Candida auris MYA 5000	97%	Not identified	Yellow
Candida auris Control LACEN 2018	99%	Not identified	Yellow
Clavispora lusitaniae (syn. *Candida lusitaniae*)	97%	99.9%	Unchanged
Pichia kudriavzevii (syn. *Candida krusei*)	98%	99.9%	Unchanged
Meyerozima guilliermondii (syn. *Candida guilliermondi*i)	97%	99.9%	Unchanged
Nakazeomyces glabratus (syn. *Candida glabrata*) MYA 2950	98%	99.9%	Unchanged
*Candida albicans*10231	99%	99.9%	Unchanged
Candida haemulonii	95%	99.9%	Unchanged
Candida dubliniensis 646	99%	99.9%	Unchanged
Candida parapsilosis 22019	98%	99.9%	Unchanged

[a] Version 9.02, % indicates the similarity rate of the identified agent; [b] Version 3.0 and [c] MSHB 10% - Yellow (halotolerant); Unchanged (non-halotolerant).

Mass spectrometry identification by Vitek-MS

Eight of the ten study samples were identified by MALDI-TOF using only the first protocol (formic acid). For *C. auris*, two protocols were used: one for yeasts and the other for filamentous fungi, and no identification was obtained, as shown in Figure 2.

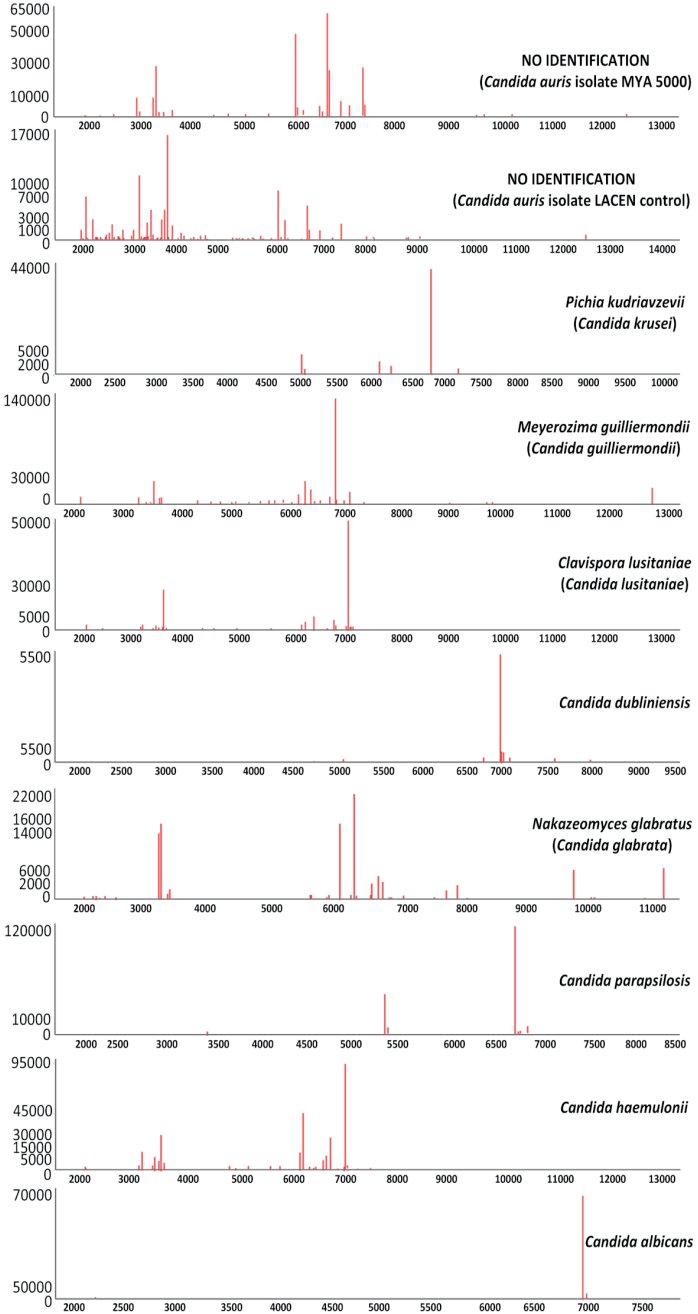
NO IDENTIFICATION
(Candida auris isolate MYA 5000)
NO IDENTIFICATION
(Candida auris isolate LACEN control)
Pichia kudriavzevii
(Candida krusei)
Meyerozima guilliermondii
(Candida guilliermondii)
Clavispora lusitaniae
(Candida lusitaniae)
Candida dubliniensis
Nakazeomyces glabratus
(Candida glabrata)
Candida parapsilosis
Candida haemulonii
Candida albicans

Figure 2. The spectrum generated by the Vitek-MS system for the proteomic profiles of *Candida* isolates evaluated in this study were obtained following the yeast extraction protocol. The spectral patterns of the 10 yeast species analyzed via Vitek-MS were processed via Myla software. The molecular mass and peak intensity values are represented on the X and Y axes, respectively. The X-axis spans a mass range from 2,000 to 20,000 Da, whereas the Y-axis intensity indicates that higher peaks correspond to the most abundant ions, with no fixed units. Yeasts 1 and 2 (*C. auris* controls) did not match any spectra in the database with Vitek-MS version 3.0 and were not identified. Yeasts 3 to 10 were successfully identified, each showing a 99% similarity to its respective reference species.

Complementary yeast identification by thermo-halotolerance test

All the samples studied were subjected to a thermotolerance test via modified Sabouraud hyaluronic broth (MSHB) with 10% NaCl at 42 °C. After the incubation period, eight isolates, *C. lusitaniae, P. kudriavzevii, M. guilliermondii*, *C. albicans, C. dubliniensis, N. glabratus, C. parapsilosis*, and *C. haemulonii*, showed no growth (no color change) and were considered negative (Figure 3).

In contrast, the two isolates of *C. auris* tested were positive, exhibiting growth and causing a pH and color change (to yellow) in the medium due to acidification (Figure 3). It is important to ensure that the quality control of the medium is rigorously adequate so that interfering factors do not compromise the assay. Two key points are highlighted: coloration after the sterility test and checking the pH, which should be between 5.2 and 6.8 before the medium is released for use.

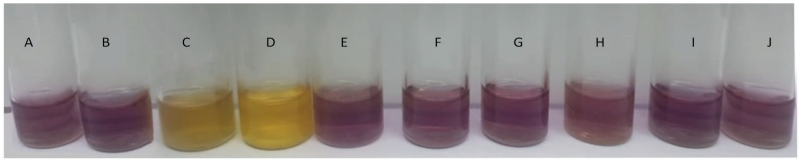

Figure 3. Yeasts were cultured on Modified Sabouraud Hypertonic Broth with 10% NaCl at 42 °C. The color of *C. auris* is yellow in the MSHB test. Test tubes: A. *N. glabratus*; B. *C. albicans*; C. *C. auris* (MYA 500); D. *C. auris* (Control LACEN 2018); E. *C. lusitaniae;* F. *Pichia kudriavzevii;* G. *Meyerozyma guilliermondii;* H. *C. dubliniensis*; I. *C. parapsilosis*; and J. *C. haemulonii.*

DISCUSSION

This study proposes a low-cost complementary phenotypic test for diagnosing and screening *C. auris,* which can be particularly useful when there are discrepancies between current automated methodologies or when resources are limited. While automation for yeast identification in routine mycology is a reality, challenges remain, including ensuring inoculum purity and the need for continuous software and equipment updates (Graf et al., 2000; Girard et al., 2016; Mizusawa et al., 2017). Our study compared the effectiveness of identifying *C. auris* via the globally recognized methods Vitek 2 and Vitek-MS and modified thermos-halotolerance tests.

Identification by automated and semiautomated methods may be a problem for some fungal strains. Studies by Hanzen & Howell (2003) and Hata et al. (2007) reported issues in the automated identification of yeast species, specifically *P. kudriavzevii, N. glabratus, C. parapsilosis,* and *C. tropicalis*, via the Vitek 2 system. Melhem et al. (2014) noted a similar challenge with *M. guilliermondii* isolates. In these studies, the authors reported low discrimination in fungal identification, which occurs when the generated biopattern cannot discriminate between two taxa, necessitating additional tests to define the species accurately.

While automated phenotypic identification systems are valuable, using classical methods such as recently reformulated chromogenic media, including CHROMagar CandidaPlus®, to identify some yeast species requires caution (Tamura et al., 2022). Sasoni et al. (2022) reported that some samples of *C. parapsilosis* exhibit color profiles similar to those of *C. auris* on chromogenic media. In addition, Jeffery-Smith et al. (2017) analyzed more than 28 studies and reported that *C. auris* can be misidentified as other species by automated and semiautomated methods. The authors emphasized the importance of molecular methods, particularly the sequencing of the internal transcribed spacer (ITS) region of rDNA, as a "gold standard" for fungal identification.

Although some studies have addressed discrepancies in the identification of *C. auris* via Vitek 2, recent software updates have reportedly increased the identification rate of *C. auris* to 99%. Nevertheless, additional tests remain necessary when automated systems yield inconclusive results (Sidrim & Rocha, 2004; Melhem et al., 2014; Biomerieux, 2017; Parra-Giraldo et al., 2018). The identification guidelines for *C. auris* provided by ANVISA in Brazil emphasize the importance of monitoring software versions of automated methodologies. Confirmation through an alternative method, whether phenotypic or molecular, is essential, as illustrated by the identification workflow for *C. auris* isolates (ANVISA, 2022).

Our data obtained through mass spectrometry (MALDI-TOF, Vitek-MS) demonstrated high sensitivity for identifying most *Candida* species, yet it failed to identify *C. auris.* This outcome was unexpected, given that matrix-assisted

laser desorption/ ionization mass spectrometry is considered a high-throughput identification tool (Kolecka et al., 2016). In terms of nonidentification, two hypotheses are considered potential challenges: (i) the greater rigidity of the cell wall or (ii) the greater quantity of mannans expressed by *C. auris,* which is 10% greater than that of other yeasts in the genus, as demonstrated by Wang et al. (2022). This increase could hinder the precipitation of peptides necessary for species identification by this methodology. We also highlighted that other researchers have reported the misidentification of *C. auris* via the Vitek-MS system (Ghosh et al., 2015; Grenfell et al., 2016; Wattal et al., 2017). After confirming that there were no errors, the most likely explanation is that the Vitek-MS database may not be updated (Ghosh et al., 2015; Wattal et al., 2017). In addition, unlike molecular methods, mass spectrometry techniques, such as Vitek-MS, do not differentiate between the five (I to V) genotypes of *C. auris* (Jamalian et al., 2023).

The thermos-halotolerance test using a colorimetric indicator in saline medium proved efficient in identifying *C. auris*, serving as a reliable and low-cost additional test to confirm the identification of this pathogen. This study underscores the importance of accurate pathogen identification for tracking infections or colonization by *C. auris.* For the first time, a colorimetric thermo-halotolerance test was implemented for the rapid diagnosis of *C. auris,* which was proposed as a complementary laboratory test to distinguish this species. The use of MSHB to confirm or map *Candida* strains can be an effective strategy for monitoring these yeasts in healthcare facilities, contributing to the prevention and control of this emerging pathogen.

The MSHB has demonstrated efficiency and is free from false negatives, as turbidity was replaced with a pH indicator that provides an ideal turning point. Through colorimetry, a clear assessment of halotolerance is achievable, thereby improving the test's accuracy and establishing it as a promising and low-cost supplementary method for *C. auris* identification, mainly in epidemic outbreaks and/ or their use in underdeveloped and developing countries.

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CONFLICT OF INTEREST

We declare for all due purposes that there are no conflicts of interest.

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