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Indoor Air Contamination by Yeasts in Healthcare Facilities: Risks of Invasive Fungal Infection

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Abstract: *Introduction-Aims*: Fungi are ubiquitous microorganisms that are easily dispersed through the air. In healthcare environments, indoor air can favor the spread of healthcare-associated fungal infections, compromising mainly immunocompromised hospitalized individuals. Therefore, this study aimed to evaluate indoor air contamination in healthcare environments, investigating mainly the presence of potentially pathogenic yeasts. *Method*: Indoor air samples were collected from 12 healthcare environments (hospital and medical clinics). After the growth, isolation, and purification of the yeast colonies, the isolates were identified by polymerase chain reaction using species-specific primers for yeasts of the genus *Candida* and sequencing of D1/D2 domains of the large ribosomal subunit (LSU rRNA). *Results and interpretation*: Fourteen yeast species were identified, including emerging pathogens. Species of clinical importance such as *Candida parapsilosis*, *Candida glabrata*, and *Rhodotorula mucilaginosa* were present. *C. parapsilosis* was the most prevalent species, followed by *Rhodotorula mucilaginosa*. In addition, we report the first occurrence of *Candida orthopsilosis*, *Trichosporon mucoides*, *Fereydounia khargensis*, and *Hortaea werneckii* in indoor air samples collected in healthcare environments. The present study shows that potentially fungal pathogens were present in air samples from healthcare environments, proving the role of indoor air in spreading infections. Therefore, monitoring air quality in healthcare environments is a fundamental approach to developing infection control measures, especially those related to invasive fungal infections.

Keywords: healthcare-associated infections; indoor air quality; infection control; candida

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1. Introduction

Healthcare-associated infections (HAI), defined as infections related to the patient's stay in the hospital or any healthcare setting, are a global problem and require urgent action to control the spread of pathogens within the hospital environment. More than one million hospitalized individuals are annually affected by HAIs, and the associated mortality rate is high [1]. There are many factors involved in HAIs, mainly the impaired immune condition of patients and the various invasive procedures that culminate in creating alternative infection routes [2]. Although many studies highlight concerns about the emergence of multi-drug-resistant bacteria causing HAI, attention to other emerging pathogens cannot be neglected. Considering the recent pandemic, factors related to patients and the environment

have also contributed to healthcare facilities becoming a significant niche for disseminating other emerging non-bacterial pathogens.

In this context, fungal pathogens have achieved notable prominence in recent years. This fact is mainly because of the increased susceptibility condition experienced by many hospitalized individuals, which is sometimes the result of aggressive therapeutic procedures [3]. For instance, patients who have been submitted to transplants are among the most susceptible to opportunistic fungal infections, especially those caused by *Aspergillus* sp. and *Candida* sp. [4]. One of the most frequent pathogens involved in HAI is the yeasts of the genus *Candida*. Invasive candidiasis is the most significant infection caused by these fungi, occurring mainly in Intensive Care Units (ICU) [5]. In neonatal ICUs, invasive candidiasis is the leading cause of morbidity and mortality in premature and low birth weight newborns [6]. In addition to these risk factors, the prevalence of opportunistic infections caused by *Candida* sp. can also be associated with newborns who have been subjected to invasive procedures such as the insertion of catheters [7].

In healthcare settings, infected patients are the main source of infections. However, several pieces of evidence have also demonstrated a significant role of indoor air in disseminating pathogens in these environments [8]. Door and window opening behaviors, specific hospital activities and procedures, and human occupation (professionals and visitors) can significantly compromise indoor air quality in healthcare settings. In addition, ventilation systems can also be considered relevant sources of microbial contamination for indoor air, especially when maintenance care for this equipment is neglected [9]. Recently, studies by Monteiro and collaborators [10], and Al-Bader and co-authors [11] discussed the harmful effects on human health caused by airborne contamination by bacteria and filamentous fungi in healthcare settings and highlighted the importance of this route for the occurrence of hospital-acquired infection. Along the same lines, Tang et al. [12] addressed airborne transmission as an important factor in spreading viral infections and the need for better strategies to improve patient safety, especially for immunocompromised individuals. Although many studies have investigated airborne bacteria, filamentous fungi, and, more recently, a greater emphasis on viruses contaminating indoor air in hospitals, the role of yeasts as components of bioaerosols and their impact on the occurrence of various diseases cannot be disregarded. Therefore, it is important to consider that the factors that favor the presence and dissemination of various bioaerosols in healthcare settings may also be valid for the occurrence and dissemination of infections caused by yeasts.

In recent years, countless evidence has demonstrated the role of hospital indoor air in spreading and transmitting diseases caused by fungi. This observation is crucial when considering that many invasive fungal infections outbreaks occur in this environment [13]. Belizario and coworkers [14] summarized the evidence for fungal indoor air contamination in critical hospital areas. Based on their results, it was possible to observe that a series of studies reported contamination only by *Candida* species. Also, Kayta et al. [15] evaluated the microbial load of indoor air in an Ethiopian hospital and found that yeast contamination reached 28%. Regarding species, only *Candida albicans* has been reported. In addition, in outbreaks caused by *Candida auris*, its spread within the hospital environment was related to indoor air contamination [16,17]. Based on these findings, it is reasonable to think that airborne yeasts in healthcare settings predominantly belong to *Candida* species. However, this reasoning cannot be proper. In the same way as other indoor environments, we have a set of factors that corroborate the occurrence of a greater diversity of yeasts in healthcare settings. Therefore, verifying which limitations prevent a broader knowledge about the diversity of yeasts in indoor air in healthcare settings is imperative.

Studies reporting indoor air contamination by yeast in healthcare settings have described a few species. This occurs because the identification approach frequently used needs to be more precise in species definition, or special attention to the importance of invasive fungal infections caused by yeasts has not been given. Consequently, the diversity of yeasts reported as indoor air contaminants in healthcare environments remains underestimated. In a study by Alghamdi and collaborators [18], the authors described several species of

filamentous fungi and reported the yeasts identified as other fungi. Similarly, Gorzynska et al. [19] reported indoor air contamination by yeasts in selected hospital environments. However, the authors failed to identify the species, grouping them only as non-pigmented and pigmented yeasts. In common, these two studies used culture-based techniques to identify fungal bioaerosols. It is important to emphasize that these approaches help characterize fungal species in indoor air. Nevertheless, they have the limitation that some yeasts may be unable to be cultivated in the laboratory [20]. In recent years, molecular tools have emerged as an essential complementary culture-based approach for bioaerosol analysis. This has allowed us to understand more comprehensively the yeast species present in healthcare environments [21].

Given the above stated, environmental monitoring in healthcare environments should be considered an essential strategy in knowing the factors involved in HAI occurrence, especially those caused by yeasts. Therefore, considering the role that air plays in disseminating fungal pathogens, this study aimed to evaluate indoor air contamination in healthcare environments focusing on the presence of potentially pathogenic yeasts. It is essential to mention that, in the context of this study, the culture-dependent or culture-independent approach refers entirely to the techniques used to characterize bioaerosols. Therefore, we used a culture-independent approach to airborne yeast identification in healthcare settings. This survey describes a significant diversity of airborne yeasts in healthcare settings, including non-albicans *Candida* species and other potential opportunistic pathogens.

2. Materials and Methods

2.1. Study Design

The study was conducted from November 2014 (Spring season) to April 2016 (Fall season) and included a mycological examination at various locations in six medical clinics and hospitals in Maceió (Alagoas, Brazil). The city of Maceió is in the northeast region of Brazil. It consists of a coastal area with a humid tropical climate, high temperatures (average of 25 °C), and humidity throughout the year. Medical Clinics were private institutions providing ophthalmology, blood therapy, pathology, and dental services. Among the hospitals, only one was a public institution (University Hospital), while all the others were private hospitals. One of the private hospitals specialized in cardiology care, while the others performed different procedures, with one of them having an oncology center. In medical clinics, the collection sites were Doctor's office, Serology, Biochemistry laboratory, Collection room, Quality control, Reception, Operating room, Storage room, Fractionation room, Microbiology laboratory, Hemodialysis, and Relaxation room. For hospitals, the chosen locations were: Clinical screening, Occupational therapy, Sterilization room, Procedures room, Intensive Care Unit (ICU), Neonatal ICU, Storage room, Audit, Nurses station, Pharmacy, Social assistance, Macroscopy, Clinical screening, Administrative, Telephone Exchange, Coordination, Hemodynamics, Complications room, Operation room, Examination room, Post-anesthetic care unit, Urgency and Emergency, Reception, Waiting room, Ultrasound, Apartment, Operating room reception, Diagnostic center, Hemodialysis, Chemotherapy, Social assistance, Application room, Information technology and, Hospital kitchen. Selection criteria for collection sites were a high flow of people, the presence of susceptible individuals, and sites with little or no air exchange. Human occupancy and poor air circulation are known factors that can significantly influence the presence of airborne pathogens in indoor environments [22]. In addition, susceptible individuals are those at greater risk of inhaling infectious bioaerosols and the consequent development of diseases [23]. During the sampling period, the sites were operating normally, and there was no outbreak of fungal diseases. The analyses carried out were part of a routine environmental inspection plan in compliance with governmental legislation in force. Technical professionals from medical clinics and hospitals were designated for follow-up for the samplings. Figure 1 shows the experimental design of the analyses carried out in this research.

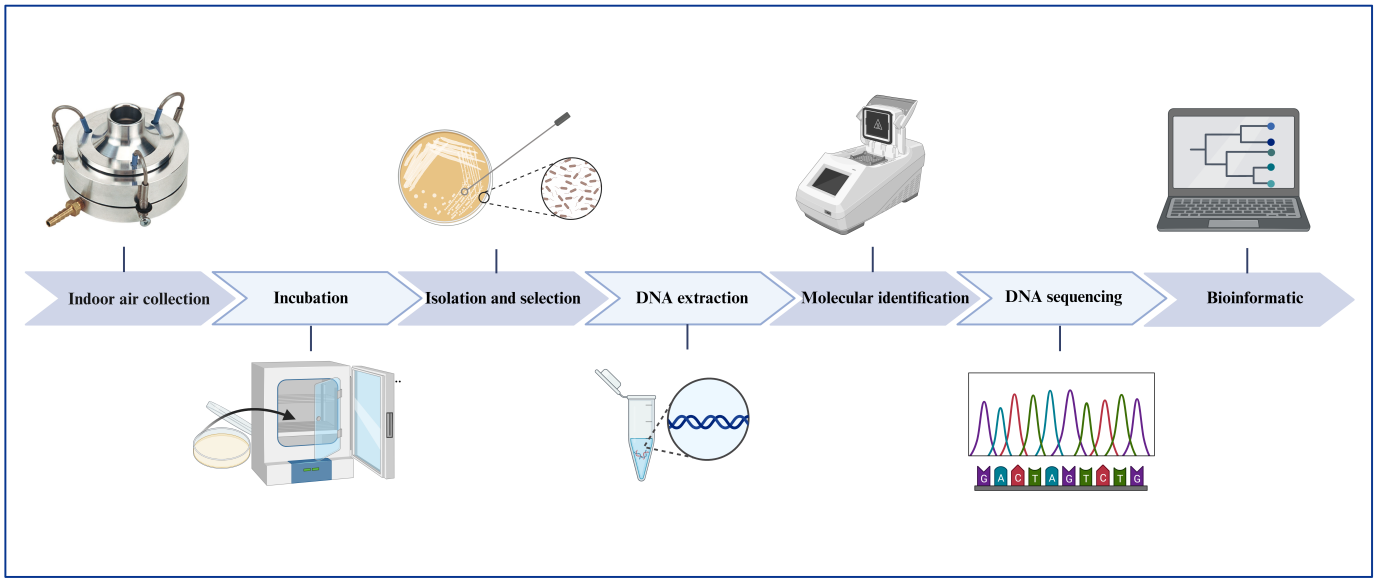

Figure 1. Experimental design of the study. Indoor air samples were obtained from healthcare settings (medical clinics and hospitals). Then, collection plates were incubated for yeast growth. After growth, colonies were isolated and selected for downstream analysis. Molecular identification of the isolates initially involved DNA extraction and polymerase chain reaction (PCR) to identify *Candida* species. Negative isolates for *Candida* species were sequenced and identified using bioinformatics tools from the obtained sequences.

2.2. Indoor Air Sampling

All analyses related to indoor air quality followed the recommendations set out in Resolution No. 09/2003 of Brazil's National Health Surveillance Agency [24]. This document establishes reference standards for indoor air quality in artificially air-conditioned environments for public and collective use. The Andersen single-stage impactor sampler (FORMIS, São Paulo, Brazil) was used for the collections, with Petri dishes of 90 × 10 mm with Dichloran Rose Bengal Chloramphenicol Agar (DRBC). The impactor was placed in the center of the room and positioned 1.5 m above the floor. The flow rate was 28.3 L/min, and the length of each collection was ten minutes, totaling 283 L of air for each sampling. The equipment was disinfected with gauze soaked in 98% isopropyl alcohol during the collection interval to avoid cross-contamination. Outdoor air sampling was carried out to verify its possible influence on the bioaerosols concentration within healthcare facilities. After the collections, the plates were sealed, identified, and taken to the Laboratory of Climatized Environments of the Federal University of Alagoas for growing (incubation temperature 25 °C ± 1 °C), isolation, and identification of the microorganisms.

2.3. Isolation and Conservation of Yeasts

For isolation, selected yeast colonies were seeded on plates with YPDA medium (Yeast extract 1%, Dextrose 2%, Peptone 2%, Agar 2%, and Bromocresol dye) with 50 ppm of chloramphenicol and incubated in a microbiological incubator at 25 °C ± 1 °C for up to seven days. After growth, the colonies were purified using the same culture medium. Once the culture purity was confirmed, the colonies were kept in microtubes containing 1 mL of sterile distilled water. A total of 114 yeast isolates were obtained and identified using molecular biology tools.

2.4. Molecular Identification, Comparison of Sequences and Phylogenetic Analyses

The DNA extraction of the obtained isolates was performed according to the protocol described by [25]. The polymerase chain reaction (PCR) technique was initially used for molecular identification, using specific primers for members of the genus *Candida*. PCR reactions were performed in a total volume of 20 µL in a 0.2 mL microtube, with the

concentrations of reagents and reaction conditions according to Hsu et al. [26]. The primer sequences to identify five species of the genus *Candida* used in this study are shown in (Table 1).

Table 1. Oligonucleotides sequence used for the molecular identification of species of the genus *Candida*.

Species	Sequences (5′–3′) *	Size (pb)
Candida krusei	CKRU1:GCATCGATGAAGAACGCAGC CKRU2:AAAAGTCTAGTTCGCTCGGGCC	258
Candida albicans	CALB1:TTTATCAACTTGTCACACCAGA CALB2:ATCCCGCCTTACCACTACCG	273
Candida parapsilosis	CPA1:GCCAGAGATTAAACTCAACCAA CPA2:CCTATCCATTAGTTTATACTCCGC	300
Candida tropicalis	CTR1:CAATCCTACCGCCAGAGGTTAT CTR2:TGGCCACTAGCAAAATAAGCGT	372
Candida glabrata	CGL1:TTATCACACGACTCGACACT CGL2:CCCACATACTGATATGGCCTACAA	423

* All sequences were obtained from the study conducted by Hsu et al. [26].

We also performed the amplification of the D1/D2 domains of the large ribosomal subunit (LSU rRNA) for samples not identified with specific primers. The reactions were carried following the protocol described by Hesham et al. [27]. The primers used were NL1-F (5′-GCATATCAATAAGCGGAGGAAAAG-3′) and NL4-R (5′-GGTCCGTGTTTCAAGACGG-3′), and the expected fragment size was 600 bp. The amplification conditions were initial denaturation at 94 °C for 5 min, followed by 30 cycles of 45 s at 94 °C for denaturation, 45 s at 55 °C for annealing, extension by 45 s at 72 °C and final extension step at 72 °C for 5 min. After amplification, a 5 µL aliquot of the PCR products was applied on a 1.2% agarose gel and submitted to the electrophoresis in TBE buffer (Tris-Boric acid, 0.5 M EDTA pH 8.0) at a voltage of 85 V/cm for 1 h and 20 min. After electrophoresis, the gel was stained in ethidium bromide for two minutes and photographed using an ultraviolet light transilluminator (312 nm wavelength). The PCR products for the D1/D2 region were sent for sequencing by Macrogen Inc. (Seoul, South Korea).

The obtained sequences were evaluated to verify the degree of reliability of each of the nucleotides based on PHRED values > 30, followed by the consensus assembly through the Staden Package software (v.1.6.0, Rodger Staden's group, Cambridge, England) [28]. We initially performed local alignments using the BLAST program (Basic Local Alignment Search Tool) [29] to identify the sequenced products. For the D1/D2 region, identification at the species level occurred when a sequence similarity value was above 99%. Paired comparisons were carried out between the sequences of the isolates obtained and the yeast sequences available on GenBank. The sequences were analyzed using the Species Demarcation Tool, v. 1.0 (SDT) software [30].

The nucleotide sequences corresponding to the LSU rRNA gene were aligned using the CLUSTAL W algorithm, implemented in MEGA 6 software (Molecular Evolutionary Genetics Analysis) [31]. Phylogenetic analyses were performed through the Maximum Likelihood (ML) method using the TrN + G nucleotide replacement model. The generated tree's reliability was obtained by a statistical test of bootstrap support with a value of 1000 random pseudo-replicates. *Clavispora opuntiae* (accession number AY497691) was used as an outgroup in phylogenetic analyses.

All sequences of the isolates obtained in this study are publicly available on GenBank. The access numbers to the isolated sequences are: MT001237, MT001238, MT001239, MT001240, MT001241, MT001242, MT001243, MT001244, MT001245, MT001246, MT001247, MT001248, MT001249, MT001250, MT001251, MT001252, MT001253, MT001254, MT001255, MT001256, MT001257, MT001258, MT001259, MT001260, MT001261, MT001262, MT001263, MT001264, MT001265, MT001266, MT001267, MT001268, MT001269, MT001270, MT001271,

MT001272, MT001273, MT001274, MT001275, MT001276, MT001277, MT001278, MT001279, MT001280, MT001281, MT001282, MT001283, MT001284 and MN966855.

3. Results

We obtained 114 yeast isolates after the collection in healthcare environments. Out of this total, 104 (91.2%) were collected from indoor air, while 10 (8.8%) were from outdoor air samples. Two samples were discarded due to low sequence quality to be correctly identified, and then only 112 isolates were analyzed. Out of this total, 63 (56.2%) were identified by specific primers for *Candida* genus and 49 (43.8%) by sequencing the D1/D2 region. Only *Candida parapsilosis* was present in the indoor air samples identified by specific primers (Table 2).

Table 2. Molecular identification of airborne *Candida parapsilosis* identified in hospital (AYH code)/medical clinics (AYC code) environments and outdoor air (AYO code) by species-specific oligonucleotides.

Environment	Collection Site	Sample
Medical Clinic	Doctor's office	AYC02 AYC03
	Serology	AYC07 AYC08
	Biochemistry laboratory	AYC09
	Collection room	AYC10
	Quality control	AYC54 AYC55
	Reception	AYC56
Hospital	Operating room	AYH01 AYH32 AYH33 AYH49 AYH50
	Examination room	AYH04 AYH05 AYH21 AYH22 AYH23
	Post-anesthetic care unit	AYH06
	Urgency and Emergency	AYH11
	Reception	AYH12 AYH13 AYH14 AYH28
	Waiting room	AYH15 AYH16 AYH17 AYH18 AYH46
	Ultrasound	AYH19
	ICU	AYH20
	Apartment	AYH24

Table 2. *Cont.*

Environment	Collection Site	Sample
Hospital	Observation room	AYH25
	Operating room reception	AYH26 AYH27
	Diagnostic Center	AYH29 AYH30
	Hemodialysis	AYH34 AYH35 AYH36 AYH37
	Chemotherapy	AYH38
	ICU	AYH20
	Apartment	AYH24
	Observation room	AYH25
	Operating room reception	AYH26 AYH27
	Diagnostic Center	AYH29 AYH30
	Hemodialysis	AYH34 AYH35 AYH36 AYH37
	Chemotherapy	AYH38
	Doctor's office	AYH39 AYH40 AYH43 AYH44 AYH45 AYH52
	Social assistance	AYH41
	Application room	AYH42 AYH53
	Clinical screening	AYH47
	Occupational therapy	AYH48
	Sterilization room	AYH51
Outside area	Outdoor air	AYO01 AYO02 AYO03 AYO04 AYO05 AYO06 AYO07

The initial analyses performed using the BLAST algorithm and paired sequence comparisons showed considerable yeast species diversity. The identified species were *C. glabrata*, *Candida orthopsilosis*, *Fereydounia khargensis*, *Hortaea werneckii*, *Jaminaea lanaiensis*, *Papiliotrema flavescens*, *Pseudozyma hubeiensis*, *Pseudozyma siamensis*, *Rhodotorula mucilaginosa*, *Torulaspora delbrueckii*, and *Trichosporon mucoides*. (Table 3). Moreover, two samples showed 100% identity with yeasts of the genus *Moniliella*. We obtained ten isolates from outdoor air collections, and the species identified were *C. parapsilosis* and *Cystobasidium slooffiae*.

Table 3. Molecular identification of airborne yeasts identified by sequencing the D1/D2 domains of the LSU rRNA gene in hospital (AYH code)/medical clinics (AYC code) environments and outdoor air (AYO code).

Sample	Environment/Collection Site	Species	Accession Number
AYH79	Hospital/Operating room	*Candida glabrata*	GenBank: MN966855
AYH57	Hospital/Reception	*Candida orthopsilosis*	GenBank: MT001254
AYC63	Medical Clinic/Doctor's office	*Candida parapsilosis*	GenBank: MT001241
AYH67	Hospital/Hemodialysis	*Candida parapsilosis*	GenBank: MT001243
AYH72	Hospital/Observation room	*Candida parapsilosis*	GenBank: MT001244
AYH74	Hospital/Observation room	*Candida parapsilosis*	GenBank: MT001245
AYH80	Hospital/Post-anesthesia care unit	*Candida parapsilosis*	GenBank: MT001246
AYH87	Hospital/Pharmacy	*Candida parapsilosis*	GenBank: MT001248
AYH88	Hospital/Examination room	*Candida parapsilosis*	GenBank: MT001250
AYH89	Hospital/Social assistance	*Candida parapsilosis*	GenBank: MT001252
AYH94	Hospital/Observation room	*Candida parapsilosis*	GenBank: MT001255
AYH95	Hospital/Doctor's office	*Candida parapsilosis*	GenBank: MT001256
AYH98	Hospital/Application room	*Candida parapsilosis*	GenBank: MT001257
AYH99	Hospital/Telephone exchange	*Candida parapsilosis*	GenBank: MT001261
AYO09	Outdoor air	*Candida parapsilosis*	GenBank: MT001265
AYO10	Outdoor air	*Candida parapsilosis*	GenBank: MT001266
AYO08	Outdoor air	*Cystobasidium slooffiae*	GenBank: MT001284
AYC58	Medical Clinic/Doctor's office	*Fereydounia khargensis*	GenBank: MT001262
AYH60	Hospital/Operating room	*Hortaea werneckii*	GenBank: MT001237
AYH61	Hospital/Operating room	*Hortaea werneckii*	GenBank: MT001242
AYC70	Medical Clinic/Fractionation room	*Hortaea werneckii*	GenBank: MT001253
AYH73	Hospital/Observation room	*Hortaea werneckii*	GenBank: MT001258
AYH100	Hospital/Coordination	*Hortaea werneckii*	GenBank: MT001259
AYH101	Hospital/Information technology	*Jaminaea lanaiensis*	GenBank: MT001264
AYC64	Medical Clinic/Doctor's office	*Moniliella* sp.	GenBank: MT001260
AYC65	Medical Clinic/Doctor's office	*Moniliella* sp.	GenBank: MT001263
AYC69	Medical Clinic/Storage room	*Papiliotrema flavescens*	GenBank: MT001249
AYH76	Hospital/Neonatal ICU	*Pseudozyma hubeiensis*	GenBank: MT001247
AYH97	Hospital/Application room	*Pseudozyma hubeiensis*	GenBank: MT001267
AYC68	Medical Clinic/Reception	*Pseudozyma hubeiensis*	GenBank: MT001240
AYH78	Hospital/Hemodialysis	*Pseudozyma siamensis*	GenBank: MT001251
AYC59	Medical Clinic/Doctor's office	*Rhodotorula mucilaginosa*	GenBank: MT001268
AYC62	Medical Clinic/Operating room	*Rhodotorula mucilaginosa*	GenBank: MT001269
AYH66	Hospital/Procedures room	*Rhodotorula mucilaginosa*	GenBank: MT001270
AYC71	Medical Clinic/Microbiology laboratory	*Rhodotorula mucilaginosa*	GenBank: MT001271
AYH75	Hospital/Urgency/Emergency	*Rhodotorula mucilaginosa*	GenBank: MT001272
AYH77	Hospital/Apartment	*Rhodotorula mucilaginosa*	GenBank: MT001273
AYH83	Hospital/Hemodialysis	*Rhodotorula mucilaginosa*	GenBank: MT001274
AYH84	Hospital/Observation room	*Rhodotorula mucilaginosa*	GenBank: MT001275
AYH85	Hospital/Waiting room	*Rhodotorula mucilaginosa*	GenBank: MT001276
AYH86	Hospital/Nurses station	*Rhodotorula mucilaginosa*	GenBank: MT001277
AYH90	Hospital/Macroscopy	*Rhodotorula mucilaginosa*	GenBank: MT001278
AYH91	Hospital/Macroscopy	*Rhodotorula mucilaginosa*	GenBank: MT001279
AYH92	Hospital/Clinical screening	*Rhodotorula mucilaginosa*	GenBank: MT001280
AYH93	Hospital/Occupational therapy	*Rhodotorula mucilaginosa*	GenBank: MT001257
AYH96	Hospital/Administrative	*Rhodotorula mucilaginosa*	GenBank: MT001282
AYH102	Hospital/Observation room	*Rhodotorula mucilaginosa*	GenBank: MT001283
AYH82	Hospital/Audit	*Torulaspora delbrueckii*	GenBank: MT001238
AYH81	Hospital/Storage room	*Trichosporon mucoides*	GenBank: MT001239

According to (Figure 2), we observed a more remarkable number of species in the hospital environment (n = 10) than in medical clinics (n = 7). The species *C. glabrata*, *T. mucoides*, *J. lanaiensis*, *P. siamensis*, and *T. delbruecki* were detected only in hospital indoor air samples. In medical clinics, we observed the strict occurrence of *P. flavenscens*, *F. khargensis*, and two isolates identified as *Moniliella* sp. Isolates of *R. mucilaginosa* and *C. parapsilosis*

were present in the hospital environment and medical clinics, with the most significant detection in hospitals. Except for *C. parapsilosis*, no other yeast species identified in the indoor air was present in the outdoor air.

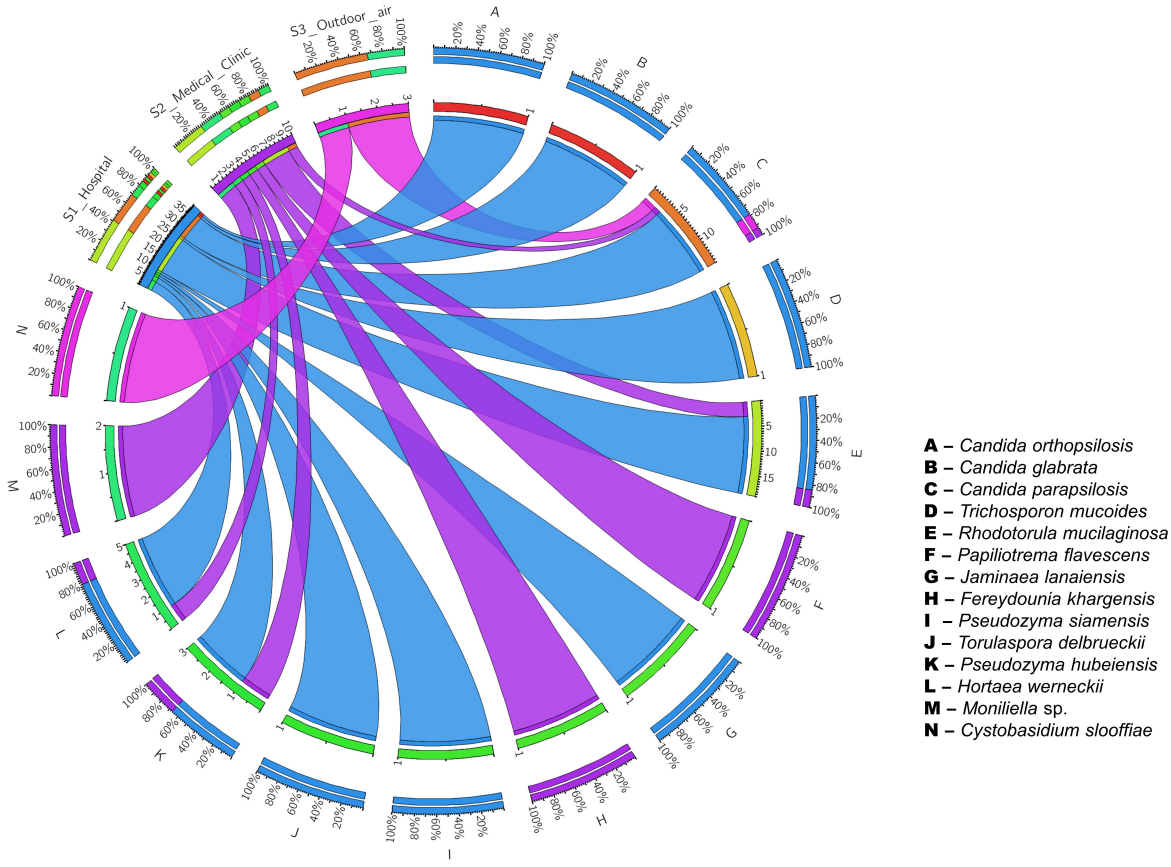

Figure 2. Prevalence of yeasts in healthcare environments and outdoor air. The circos plot displays the relative abundance of yeast species in indoor and outdoor air samples. Plots were generated from a table with the isolates obtained from D1/D2 domain sequencing from the LSU rRNA gene. The abundance of each isolate is directly proportional to the width of each ribbon connecting yeast species to their respective environments in which air samples were obtained. The outer ring represents the cumulative percent of isolates assigned to a given yeast species from each indoor environment or outdoor air sample. The inner circle represents the number of isolates assigned to a given species in a given sample (indoor or outdoor air).

Phylogenetic analyses were performed based on the sequencing data of the D1/D2 domain of the LSU rRNA gene, including sequences of isolates from the present study and sequences obtained from GenBank. Our analysis showed that the 49 isolates obtained were separated into three groups, according to (Figure 3). In group I, the most significant number of related species was present, namely: *Fereydounia khargensis* (access number KJ490644), *Jaminaea lanaiensis* (access number KY109812), *Papiliotrema flavescens* (access number EF653918), *Trichosporon mucoides* (access number KM821090), *Cystobasidium slooffiae* (access number AB566328), *Moniliella* sp. (access number JQ798180), *Pseudozyma hubeiensis* (access number AB566327), *Pseudozyma siamensis* (access number LC053502) and Rhodotorula mucilaginosa (access number KP087899). The species belonging to group II were: *Candida glabrata* (access number JN032660), *Torulaspora delbrueckii* (access number KM521824), *Candida orthopsilosis* (access number KJ817161), and *Candida parapisilosis* (access number KJ817165). The species *Hortaea werneckii* (access number AB079587) was the only member of the third group.

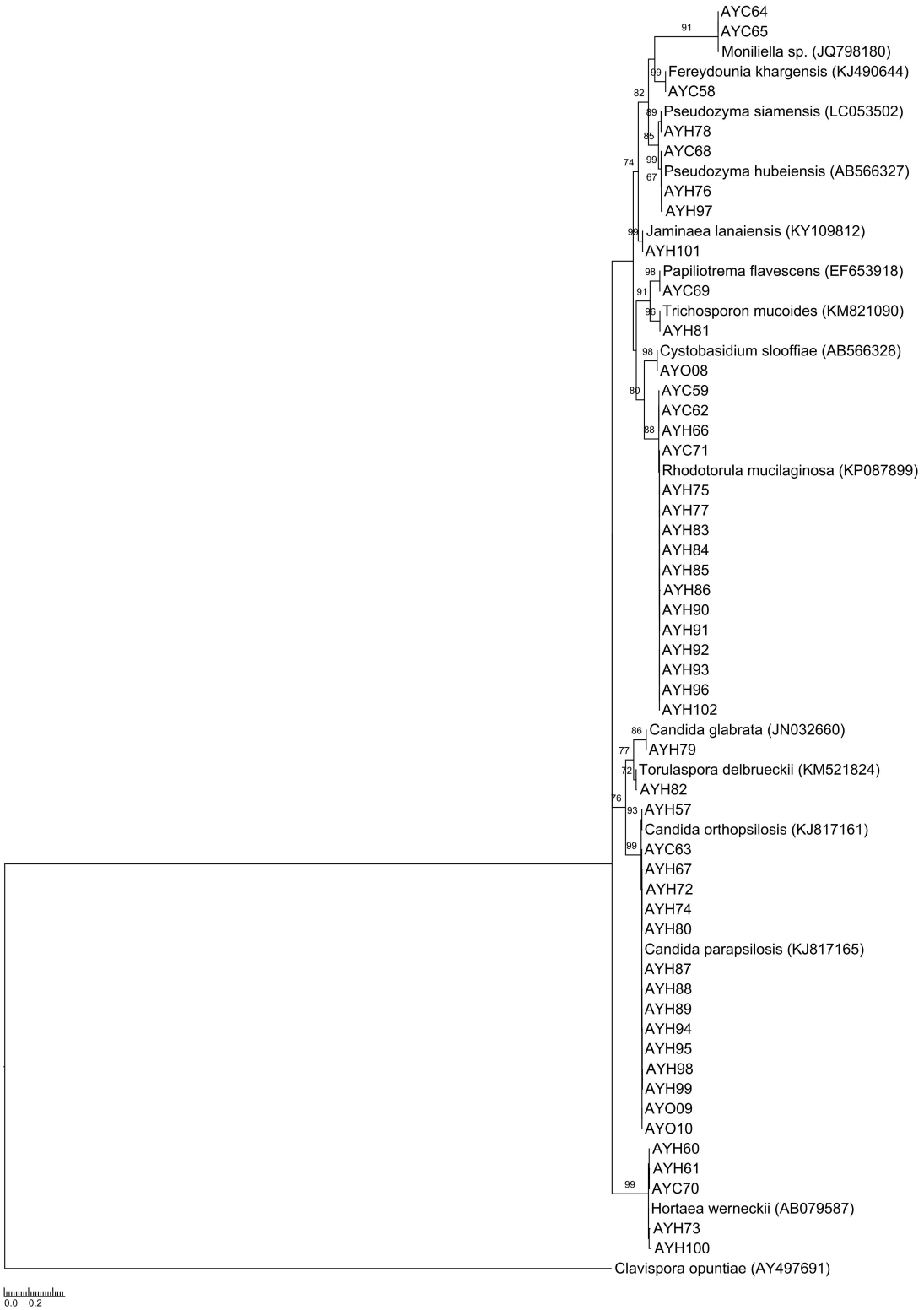

Figure 3. Phylogenetic tree of maximum likelihood generated by the PhyML software (v3.0, Montpellier Bioinformatics, Montpellier, France) built based on the multiple alignments of partial sequences of the yeast LSU rRNA gene. The nucleotide replacement model used was TrN + G, provided by the jModeltest (v2.1.10, Computer Architecture Group, Coruna, Spain) software. Node numbers indicate percentage bootstrap values (1000 bootstraps). *Clavispora opuntiae* was used as an outgroup.

4. Discussion

In hospitals and clinic environments, indoor air quality can aggravate infection outbreaks, favored mainly by the presence of immunocompromised or susceptible individuals in these places. In this survey, we reported indoor air contamination in healthcare environments by various yeast species. Also, our results provide evidence about exposure to airborne pathogens in hospital areas, which can determine HAI occurrence. Despite indoor air contamination by filamentous fungi, other airborne fungi, such as yeasts, can also severely threaten human health, especially in health settings.

In this study, we observed more significant indoor air contamination and a greater diversity of airborne fungi in hospital environments than in medical clinics. This observation may be due to the higher number of sick people in hospitals and the performance of specific procedures in these environments, contributing to more aerosolization and dissemination of microorganisms. Compared to other environments, hospitals constitute important reservoirs for yeasts, which can be aerosolized and spread throughout the building's spaces. Like us, Veysi and co-authors [32] also found a higher average concentration of bioaerosols and other indoor air contaminants in hospitals than in other indoor environments. On the other hand, Guo and colleagues [33] assessed the concentration of airborne fungi in one hospital and other buildings and reported divergent results. They found that the indoor air fungal concentration in the hospital environment was lower than in homes. This discrepancy can be explained by the fact that homes are subjected to a more significant influence of factors that contribute to the increase of indoor air fungal concentration compared to hospitals. Therefore, concerning healthcare environments, our finding confirms the importance of hospital settings as reservoirs for a wide range of pathogenic fungi species and their possible involvement in HAIs.

Of all identified yeasts, detecting *Candida* isolates is noteworthy in the hospitals and medical clinics analyzed. We have two possible explanations for this result. First, species of this genus constitute the microbiota of the human body and may have patients and workers as sources of contamination for indoor air. This assumption has already been observed in the literature, primarily reports of aerosolizing commensal bacteria from the skin and oral mucosa [34,35], which can also occur for yeasts. Second, invasive procedures in critically ill patients may also be responsible for aerosolizing *Candida* into the air. Mirhoseini et al. [36] also found that dental procedures significantly influence the abundance of airborne opportunistic pathogens in dental clinics. In many of the sites where airborne *Candida* were reported in our monitoring, there is a low occurrence of invasive procedures and a massive circulation of people (e.g., Doctor's office and Reception). However, other unusual sources can also serve as yeast reservoirs with the potential for aerosolization into indoor air. Bottled mineral water from dispensers and tap water has been reported as an essential source of pathogenic microorganisms, including *Candida* species detected in our study [37]. Also, Tischner et al. [38] carried out a mycological investigation of bottled water dispensers in healthcare facilities and verified contamination by filamentous fungi and yeasts with pathogenic potential. Therefore, we speculate that yeasts reported in our study may have been aerosolized mainly from patients' or healthcare professionals' skin and oral cavities. Nonetheless, other potential sources should also be considered.

Investigations of the composition of bioaerosols in indoor air have primarily used a culture-dependent identification approach. However, these choices have significant limitations, sometimes making it challenging to identify microorganisms at the species level and restricting the understanding of the microbial signature of a given environment. Therefore, culture-independent techniques such as PCR assays and sequencing have been widely used in airborne microbiology today. Our study used culture-independent tools, which allowed us to observe a considerable diversity of yeasts in healthcare settings. In Brazil, Venceslau and co-authors [39] evaluated indoor air contamination in critical and non-critical hospital areas and reported contamination by *Candida* sp. However, due to a culture-dependent approach, the authors could not identify the isolates obtained at the

species level, limiting the knowledge of *Candida* diversity in the analyzed environments. Like us, Pedrosa and colleagues [40] used a culture-based approach and the PCR assay to identify yeast isolates in indoor air at a University Hospital. They found contamination in critical areas by *Candida tropicalis*, *Candida krusei*, and *C. parapsilosis*. Therefore, combining culture-dependent and independent approaches can be an exciting strategy to understand better the diversity of yeasts composing bioaerosols in healthcare settings.

Candida parapsilosis was the most prevalent species in the analyzed environments. Its detection occurred in hemodialysis and chemotherapy rooms and critical areas such as operating rooms and ICUs. This finding deserves attention since individuals in these places represent the leading risk group for infections by this emergent species. Nosocomial candidemia is a growing hospital problem in several countries, including Brazil. For example, Yamin et al. [41] reported this species as the leading cause of candidemia in patients in ICUs and oncology wards. Also, an epidemiological surveillance study in 16 hospitals in five Brazilian regions shows that *Candida* sp. was the 7th most prevalent agent in cases of nosocomial bloodstream infection and that *C. parapsilosis* was the second most prevalent [42]. In agreement with our results, Souza and co-authors [43] also found this species the most frequent among yeasts isolated in air samples from neonatal intensive care units (NICU). In contrast, Sudharsanam and coworkers [44] found these indoor bioaerosols concentrations from a hospital ward and reported *Candida krusei* as the only yeast species found. This result difference may be due to the limited number of hospital sites analyzed. For example, while we evaluated several sites, the authors performed air sampling only in an orthopedic ward. Therefore, hospital air quality assessment can be essential in providing important epidemiological data about the occurrence and spread of *Candida* sp. in the hospital environment. In addition, it provides data to assist in developing infection control measures.

Surprisingly, we have not detected *Candida albicans* in the air samples analyzed, which had already been observed in other studies [42,45]. Although *C. albicans* is considered one of the most important pathogens of the genus *Candida*, other Candida-non-albicans species have also emerged in the HAI context. We also described the detection of *C. orthopsilosis* and *C. glabrata* in the hospital reception and operating room, respectively. In line with us, studies in Brazilian hospitals and other countries have also reported detecting *C. glabrata* in indoor air samples [45,46]. *C. orthopsilosis* occurrence in hospital air samples has not yet been demonstrated in the literature until the completion of this study. This species and other *Candida* may be inferred as significant indoor air contaminants in hospitals. However, due to the use of culture-dependent approaches for airborne yeast studies, their detection has not yet been reported. Therefore, we highlight the first report on this species as a contaminant of indoor air in healthcare environments. Furthermore, we emphasize the need for continuous monitoring of air quality in these environments to characterize other *Candida* species. Given the relevance of *Candida auris* in recent outbreaks in Brazil [47], future surveys are also necessary to inform about the occurrence of this emerging pathogen and its airborne spread in healthcare settings.

Here, we also reported *Rhodotorula* in our indoor air quality microbiological monitoring. Its detection has substantial clinical relevance because it has emerged as a significant opportunistic pathogen in recent years, especially in immunocompromised patients. In our finding, *R. mucilaginosa* was the second most frequent species encountered in hospitals and medical clinics, mainly in non-critical areas. Although we have not observed its occurrence in critical areas, indoor air can be essential in spreading to these places. Similarly, other studies have reported *Rhodotorula* sp. as an indoor air contaminant in critical and semi-critical hospital areas [48,49]. Equally, Souza et al. [43] evaluated fungal contamination of indoor air in two neonatal intensive care units and reported *R. mucilaginosa*. However, unlike us, the authors of this study additionally reported the presence of *Rhodotorula minuta*. Since *Rhodotorula* sp. has been recognized as a relevant opportunistic pathogen in various types of infections, studies to evaluate indoor air quality may be helpful to clarify better the role of these yeasts in the occurrence of HAIs.

Studies on fungal bioaerosols as contaminants in hospital indoor air mainly focus on filamentous fungi. Therefore, there are few yeast species reported in the literature. In addition to the clinically relevant species already discussed, other yeasts with the potential to cause opportunistic infections were identified in our study. We report the first occurrence of the *Trichosporon mucoides*, *Fereydounia khargensis*, and *Hortaea werneckii* species in indoor air samples collected in healthcare environments. Except for *H. werneckii*, which is strictly associated with benign superficial infections [50], *F. khargensis* and *T. mucoides* have stood out as relevant opportunistic emerging pathogens, especially in transplanted patients, cancer patients, and critical areas [51,52]. Although *Candida* sp. is the most reported yeast in hospital indoor air quality analysis and the most significant in causing HAI, our results indicate that hospital air can also be an essential factor in the occurrence of invasive fungal infections by emerging or even unknown pathogens.

To infer a possible influence of outdoor air on the diversity of yeast species in indoor air, collections were made in open areas close to the evaluated hospitals and clinics. Overall, based on the few isolates obtained in our study, we believe that outdoor air did not influence the concentration of airborne yeast in the analyzed environments. We verified that *C. parapsilosis* was the most prevalent indoor air sample species detected in outdoor air. This fact may indicate a slight influence in some environments, at least for this species. Some studies have reported the contribution of outdoor air to high indoor concentrations of filamentous fungi, which has been mainly attributed to opening doors and windows [53]. Although *Rhodotorula* ssp. seems to have a common occurrence in outdoor air; we have not verified its occurrence in our outdoor air samples. Similarly, Bezerra et al. [54] did not find this fungus analyzing air in outdoor areas in São Luís, State of Maranhão, Brazil. Nonetheless, our results differ from those found by Gonçalves and collaborators [55] who reported *Rhodotorula* sp. in the outdoor air of areas located in the metropolitan region of São Paulo, Brazil. A possible explanation for this difference may be related to the characteristics and climate variations observed in different regions of Brazil. For example, the city of São Paulo is in a region where temperatures are generally lower and the climate is more humid, while Maceió and São Luís are in a region with annual higher temperatures and a drier climate. Based on this, we can assume that the concentration of this fungus in the outdoor air seems to fluctuate seasonally. Therefore, the diversity of fungal contaminants observed in our study is likely to have been influenced by internal factors that must be better understood. Further studies are essential to identify the key factors influencing the concentration of airborne yeast in the healthcare setting and confirm the role of outdoor air on yeast diversity.

5. Conclusions

In this study, we report a considerable diversity of yeasts in indoor air samples in healthcare facilities, with hospitals exhibiting a greater diversity of yeasts than medical clinics. Several sites were positive for airborne yeast contamination, including those with a higher probability of infections for exposed patients, such as operating rooms, ICUs, and hemodialysis. Clinically significant species such as *C. parapsilosis* and *R. mucilaginosa* were the most prevalent in the evaluated environments, in addition to opportunistic or emerging pathogens.

Our results further reinforce the evidence about the role of indoor air as an essential factor to be considered in the spread of HAI, with a greater risk for immunocompromised individuals. In this way, indoor air quality monitoring consists of a fundamental approach to target the elements involved in the aerial spread of fungal infections and guiding actions to implement effective infection control programs.

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