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In Vitro Activity of Disinfectants against Mold Fungi Isolated from Different Environments of the Children's Medical Center Hospital, Tehran, Iran

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ABSTRACT

***Introduction*:** Fungal aerosols cause life-threatening infections in patients hospitalized in critical wards. Antiseptics and disinfectants have broad-spectrum antimicrobial activity against the living tissue and inert surfaces microorganisms; hence, they have an essential role in controlling and preventing nosocomial infections**.** This study aimed to evaluate in vitro antifungal activity of benzalkonium chloride (BAC), chlorhexidine digluconate (CHX), and sodium hypochlorite (SH) against isolated fungal aerosols from the hospital environment.

***Materials and Methods*:** The susceptibility tests were performed on fungal aerosols isolated from various wards of Children's Medical Center, based on broth microdilution antifungal susceptibility testing of filamentous fungi approved by Clinical and Laboratory Standards Institute (CLSI) M38-A2 document. The isolates included *Aspergillus* (*Aspergillus flavus (*n = 14), *Aspergillus niger* complex (n = 12), *Penicillium* spp. (n = 14), and *Cladosporium* spp. (n = 14).

Results: The geometric means (GM) of the Minimum Inhibitory Concentrations (MICs) of the biocides across all isolates were as follows: BAC, 3.56 µg/ml, CHX, 9.45 µg/ml, and SH, 810.35 µg/ml. The highest range of MICs was found for SH (50-12800 µg/ml), while the lowest range was for BAC (1-16 µg/ml) against all fungal isolates. Generally, BAC showed the highest in vitro activity among disinfectants tested. The lowest MIC_{50} and MIC_{90} values were 4 and 8 µg/ml for BAC, followed by 16 and 32 µg/ml for CHX, and 800 and 6400 µg/ml for SH, respectively.

Conclusion: The findings showed that BAC was an effective disinfectant, which can prevent resistant species and fungal pathogens and be used as an alternative to other disinfectants and antiseptics.

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Introduction

Fungal aerosols are ubiquitous in air and can originate from soil, animals, water, and plants. They can exist on surfaces for extended periods and stay suspended in the air [1].Exposure to fungal bioaerosols was associated with a broad range of adverse health outcomes and respiratory diseases, such as allergic rhinitis, asthma, and alvelolitis [2]. Clients and patients may transfer fungal aerosols to hospital wards. Airborne

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transmission is a primary route of contamination, particularly in immunocompromised patients [3]. Aerosols of more than 80 genera of molds, including *Aspergillus*, *Penicillium*, *Cladosporium*, *and Alternaria* (which can potentially cause respiratory tract diseases [4]) are found in air conditioners [5]. The most common filamentous fungi causing nosocomial fungal infections are *Aspergillus* spp., *Fusarium* spp., *Scedosporium* spp., and *Mucorales* [6-8]. *A. niger* and *A. flavus* are among the most opportunistic molds causing lethal infections in predisposed patients [9].

Fungal airborne contaminations cause life-threatening infections for high-risk patients such as hematological malignancy, bone marrow transplantation, AIDS patients, and those who are hospitalized in intensive care units (ICUs), especially in neonatal intensive care units (NICUs) [10-12]. Antiseptics are mainly used in hospitals and health care settings to decrease microbes carried on the hands of health care staff. Disinfectants are used to disinfect clinical and industrial devices and the environment [13-15].

Considering the limited studies in the children's healthcare settings, the purpose of this study was to evaluate the antifungal activity of three biocides, a biguanide (chlorhexidine), a quaternary ammonium compound (benzalkonium chloride), and a chlorinated compound (sodium hypochlorite) against fungal aerosols isolated from indoor air of the reference children's hospital during the spring and summer of 2017 in Tehran, Iran.

Materials and Methods

Sampling wards

The studied wards included (1) pediatric intensive care unit (PICU), (2) emergency intensive care unit (EICU), (3) cardiac intensive care unit (CICU), (4) NICU, (5) pediatric intensive care unit open heart (PICU-Open Heart), (6) neonatal intensive care unit-open heart (NICU-Open Heart), (7) onco-hematology ward, (8) bone marrow transplantation ward, and (9)(10) one general and one urology operating rooms. Samples were taken between 8:00 AM and 15:00 PM from the above-mentioned wards. In total, 120 samples were collected during the sampling period with 2-week intervals for isolation of fungal bioaerosol.

All wards of this hospital were equipped with the central ventilation systems heating, ventilating, and air-conditioning systems (HVAC). The characteristics of hospital wards are presented in table 1. Moreover, conventional ventilation systems of ICUs, operating rooms, and bone marrow transplants wards were equipped with high-efficiency particulate air (HEPA) filters. The Children's Medical Center is located in Tehran, Iran. This study was conducted in springs and summers from March to August 2017.

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Table 1: Main characteristics of the hospital wards

Hospital ward	Year of Construction	No. of bed	Ventilation system	Manufacturer Company -Model
ICU-Open Heart	2017	7	HVAC with a HEPA filter	Saravel Company - Model: AHU-1600
Bone marrow transplant	2016	9	HVAC with a HEPA filter	Saravel Company - Model: AHU-1600
NICU-OH	2011	7	HVAC with a HEPA filter	Saravel Company - Model: AHU-1600
EICU	2009	8	HVAC with a HEPA filter	Saravel Company - Model: AHU-1600
General operating room	2009	-	HVAC with a HEPA filter	Saravel Company - Model: AHU-1600
CICU	2009	10	HVAC with a HEPA filter	Saravel Company - Model: AHU-1600
NICU	2009	12	HVAC with a HEPA filter	Saravel Company - Model: AHU-1600
Urology operating room	2000	-	HVAC with a HEPA filter	Saravel Company - Model: AHU-700
Onco-hematology	1967	20	Central operation HVAC	-
PICU	1967	10	Central operation HVAC	Saravel Company - Model: AHU-700

HEPA, high-efficiency particulate air; *HVAC*, heating, ventilating and air-conditioning systems; *PICU*, pediatric intensive care unit; *EICU*, emergency intensive care unit; *CICU*, cardiac intensive care unit; *NICU*, neonatal intensive care unit; *NICU-OH*, neonatal intensive care unit-open heart; *ICU-open heart*, intensive care unit open heart;

Air sampling procedure

Sampling was conducted using a sampler (Quick Take-30, single-stage Anderson impactor, SKC, USA) with a flow rate of 28.3 L/min for 150 s in order to sample fungal aerosols in indoor and outdoor air of the hospital. Sampling was performed according to the National Institute of Occupational Safety and Health (NIOSH) standard method [16]. Sabouraud dextrose agar (SDA, Merck Co, Germany) containing 0.5% chloramphenicol was used as a culture media for taking fungi [17]. The sampler was located at the height of 1 m above the floor at the breathing level of human [10, 18]. To record the effect of environmental parameters, relative humidity (%), and air temperature (°C), a digital humidity/temperature meter (TES-1360, Germany) was applied.

Fungal strains

After sampling, SDA plates were incubated at 24-28 ^{o} C for 3-7 days. The fungal colonies were identified using macroscopic colony morphology (color and colonial texture). The microscopic characteristics were analyzed using slide culture [19]. Moreover, yeasts were identified according to their morphological characteristics, soft and smooth to wrinkled, glossy creamy colonies [10], [19]. The concentration of airborne fungi was expressed as the number of colony-forming units per m^3 of air (CFU/m^3).

A total of 658 fungal isolates was collected from indoor air of ten critical wards and three outdoor hospital stations. As a result, 54 fungal isolates, considered as high priority in terms of frequency and pathogenicity, were used to determine the antifungal activity. The fungal isolates were: *Aspergillus* (*A. flavus* (n = 14), *A. niger* (n = 12), *Penicillium* spp. (n = 14), and *Cladosporium* spp. (n = 14).

Inoculum preparation

The inoculum was prepared according to the Clinical and Laboratory Standards Institute M38-A2 document [20]. Briefly, the isolates were cultured

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on potato dextrose agar (PDA, Merck, Germany) at 25 °C for 5-7 days [9]. A seven-day-old colony was covered with 5 ml of sterile 0.85% saline and then the suspension was made. The resulting mixture of conidia and hyphal fragments was withdrawn. The massive particles were permitted to settle for 3-5 min. Later, the turbidity of the supernatants was measured using spectrophotometer at a wavelength of 530 nm and transmission was adjusted for each species as outlined in M38-A2. These supernatants were diluted in RPMI-1640 (Sigma-Aldrich, USA) medium buffered with 0.165 mol/l morpholinepropanesulfonic acid (MOPS) (34.54 g/l) at pH 7.0. The final inoculum dilutions corresponded to twice the density needed of approximately 0.45×10^4 CFU/ml [20].

Antifungal agents

In-vitro antifungal activity of benzalkonium chloride (BAC; Sigma-Aldrich, 12060, USA), chlorhexidine digluconate 20% (CHX; Sigma-Aldrich, C9394, USA), and sodium hypochlorite (SH; Sigma-Aldrich; 1.05614, USA) were studied. Each chemical agent was dissolved in sterile distilled water. The solutions were prepared at concentrations of 3200 µg/ml for BAC, 212000 µg/ml for CHX, and 61500 µg/ml for SH, which were diluted in RPMI-1640 (Sigma-Aldrich, USA) buffered to pH 7.0 with 0.165 mol/l of morpholinepropanesulfonic acid for different dilutions preparations. Serial two-fold dilutions of each antifungal agent were provided in microdilution well within concentrations ranging from 0.25-128 µg/ml for BAC, 0.125-64 µg/ml for CHX, and 25-12800 µg/ml for SH.

Antifungal susceptibility tests

The Minimum Inhibitory Concentration (MIC) describes the lowest concentration of the antifungal agent that completely inhibits any discernible growth compared to the growth of control well after 48 h. This test is widely used to evaluate the susceptibility of fungi to antifungal agent [20]. These tests were conducted as following.

A broth microdilution method was carried out following the CLSI M38-A2 guidelines. In each micro-plate, 100 µL of the final conidial suspension was dispensed to 100 µL of each of the biocide concentration [20].

Regarding the microplates, two chemical agent-free controls were kept, each with the medium RPMI-1640 alone (Sterility control) and the growth control wells were filled with 100 µL of the corresponding diluted inoculum suspension plus 100 µL of RPMI-1640 broth without antifungal agents (growth control). Later, the micro-dilution plates were incubated at 35°C for 48 h [20]. The tests were performed in duplicate in 96-well flat-bottom microplates [20].

Statistical analysis

To analyze the data, SPSS software, version 19.0 was run in this study (SPSS, Inc., Chicago, IL, USA). One-way ANOVA followed by post hoc Scheffe's test was also used to examine the difference between the average bio-aerosol concentration indexes in different wards of the hospital. The Pearson correlation coefficient was applied to determine the effect of environmental parameters on fungal contaminations. The MIC_{50}, MIC_{90}, and geometric mean (GM) MIC values were determined for four genera of fungi. They were calculated using Microsoft Office Excel 2010 and MIC distributions for g of fungi were compared using Anova-test followed by post-hoc Scheffe's tests. The level of statistical significance was set at $P \leq 0.05$.

Results

Frequency of fungal isolates

The frequency of isolated fungal species is illustrated in figure 1. *Penicillium* spp. was the most frequently identified genus (39.97%), followed by *Cladosporium* spp. (36.63%) and *Aspergillus* spp. (12.77 %) , sterilized mycelia (5.02%), *yeasts* (1.22%), *Mucor* (0.91%), *Paecilomyces* spp. (0.61%), and others (4.41%) from the hospital indoor and outdoor air samples. The detected *Aspergillus* fungal colonies included the species *A. niger* (7.14%), *A. flavus* (3.79%), *A. terreus* (0.45%); *A. hollanndius* and *A. versicolor* (0.30%), and other species (0.15%).

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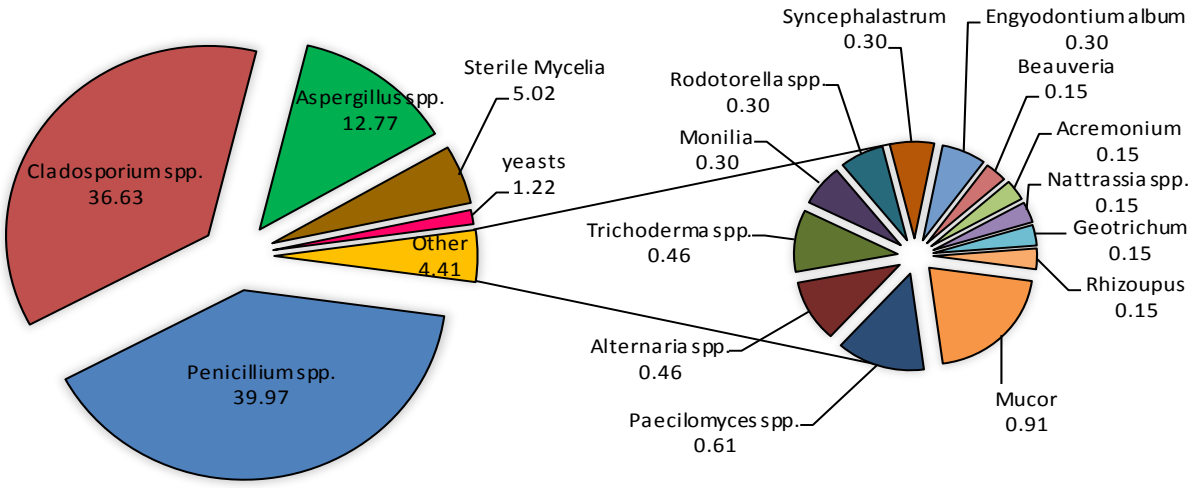


Figure 1: The percentage of fungal species isolated from indoor (10 wards) and outdoor (3 stations) area during the sampling period in the Children's Medical Center.

Antifungal susceptibility testing

Table 2 and figures 2, 3, and 4 summarize the MIC range, MIC mode, GM, and MIC_{50}, and MIC_{90} distribution of the three biocides against 54 fungal isolates in the hospital air.

The GMs for MICs of antifungal agents among isolates were as the following (in increasing order): BAC, 3.56 µg/ml; CHX, 9.45 µg/ml; and SH, 810.35 µg/ml; respectively. Based on the findings, BAC appeared to be the most effective antifungal agent against all fungal isolates (MIC_{90}, 8.0 µg/ml). The highest MIC_{90} value was 6400 µg/ml for SH against all isolates, which was significantly different from the other antifungal agents ($p< 0.05$). However, this difference was not significant in the MIC_{90} value of BAC with CHX ($p > 0.05$).

The ranges of MICs were SH (50 to 12800 µg/ml), CHX (0.5 to 64 µg/ml), and BAC (1-16 µg/ml) against all isolates of fungi. Our findings showed that the most active biocide against *Cladosporium* spp. was BAC (GM MIC, 2.69 µg/ml) followed by CHX (GM MIC, 10.24 µg/ml) and SH (GM for MIC, 116.01 µg/ml). Furthermore, for *Penicillium* spp., CHX (GM for MIC, 2.0 µg/ml) had the highest activity, followed by BAC (GM for MIC, 3.44 µg/ml) and SH (GM for MIC, 464.05 µg/ml). In the same regard, BAC (GM for MIC; 4.64 µg/ml and GM for MIC, 3.77 µg/ml, respectively) was found as the most potent biocide against both *A. flavus* and *A. niger* compared with CHX (GM for MIC, 22.62 µg/ml and GM for MIC, 19.02 µg/ml, respectively) and SH (GM for MIC, 6400 µg/ml and GM for MIC, 1345.43 µg/ml, respectively).

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Table 2: Geometric means of MICs (GM), MIC ranges, as well as MIC_{90} and MIC_{50} values obtained by testing susceptibilities results of 54 environment fungal isolates to three conventional biocides

Origin of isolation (no. of isolates)	Antifungal agents	MIC parameter (µg/ml) Range	MIC_{50}	MIC_{90}	GM	Mode
All isolates (n = 54)	SH	50-12800	800	6400	810.35	100
	BAC	1-16	4	8	3.56	4
	CHX	0.50-64	16	32	9.45	16
Cladosporium spp. (n = 14)	SH	50-400	100	200	116.01	100
	BAC	1-4	4	4	2.69	4
	CHX	4-16	8	16	10.24	16
Penicillium spp. (n=14)	SH	100-1600	400	800	464.05	400
	BAC	2-4	4	4	3.44	4
	CHX	0.50-8	2	8	2	1
Aspergillus flavus (n = 14)	SH	400-12800	6400	12800	6400	6400
	BAC	2-16	4	8	4.64	4
	CHX	8-64	16	32	22.62	16
Aspergillus niger (n = 12)	SH	400-3200	1600	1600	1345.43	1600
	BAC	2-8	4	8	3.77	2
	CHX	16-32	16	32	19.02	16

SH: Sodium hypochlorite; BAC: Benzalkonium chloride; CHX: Chlorhexidine digluconate; GM: geometric mean; MIC_{50}: minimal effective concentration that inhibited the growth of 50% of isolates; MIC_{90}: minimal effective concentration that inhibited the growth of 90% of isolates.

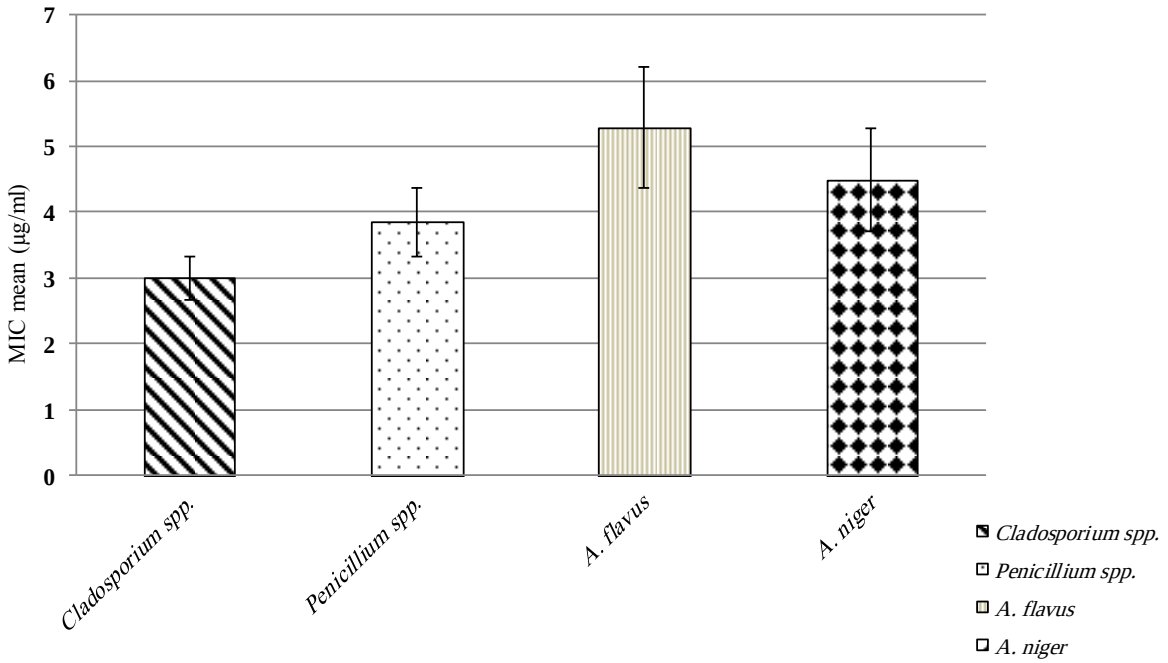


Figure 2: Minimal inhibitory concentration (MIC) distribution of fungi for BAC.

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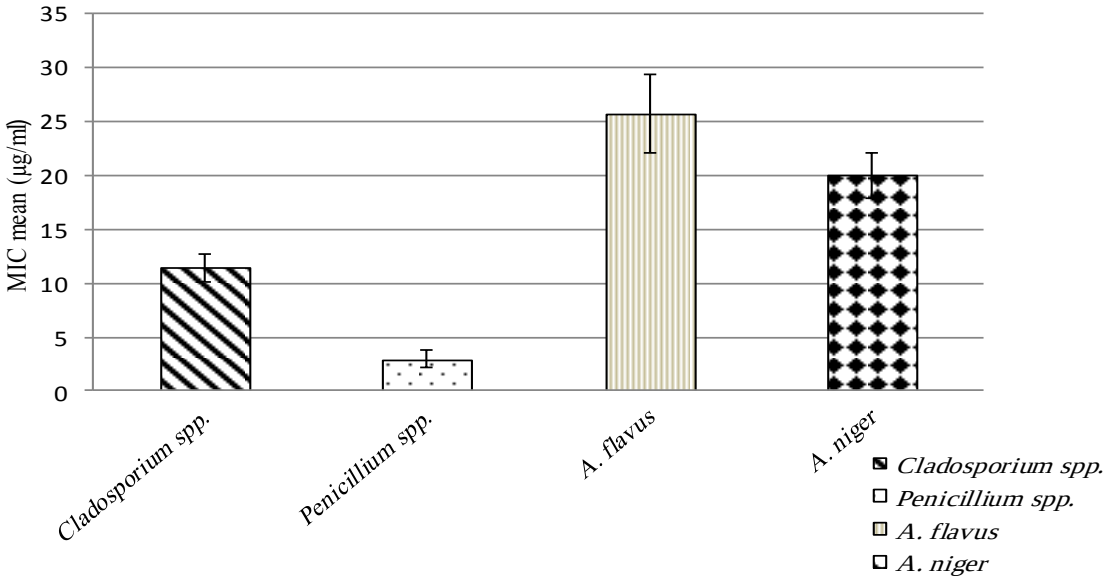


Figure 3: Minimal inhibitory concentration (MIC) distribution of fungi for CHX

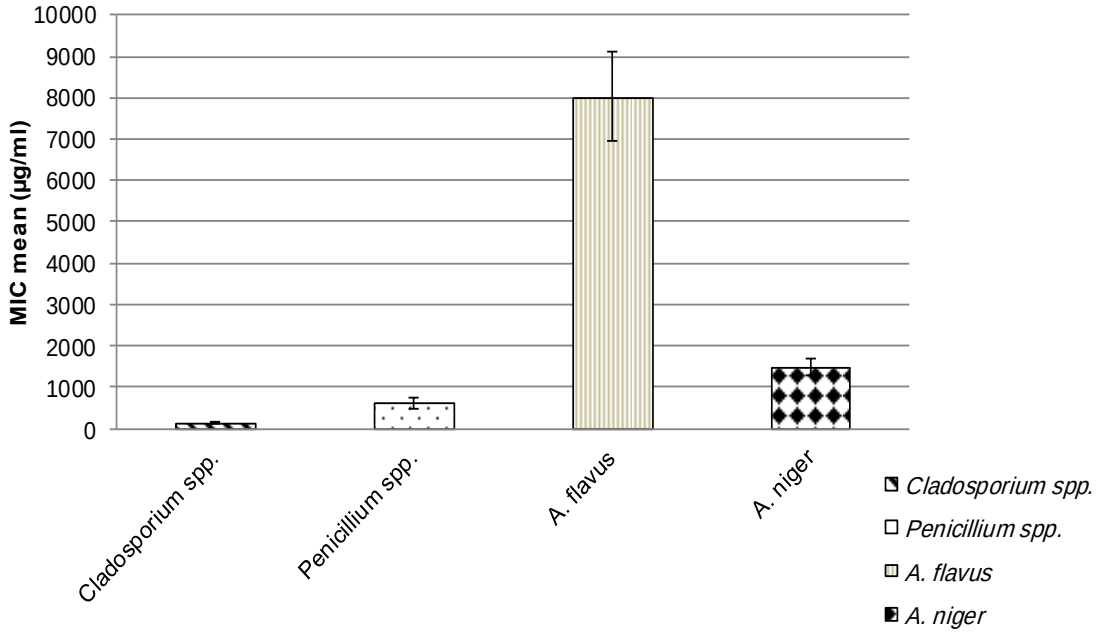


Figure 4: Minimal inhibitory concentration (MIC) distribution of fungi for SH.
No. of isolates susceptible by MIC (µg/ml)

Statistics

The MIC distributions of SH showed a significant difference in the fungal isolates (Anova test, *P = 0.00*). *A. flavus* differed significantly from *Cladosporium* spp., from *A. niger*, and *Penicillium* spp. (*p = 0.00*, Post Hoc Tests, scheffe).

The MIC distributions of CHX indicated a significant difference between the fungal isolates (Anova test, *P = 0.00*). *Penicillium* spp. differed significantly from *A. niger* and *A. flavus* (*p = 0.00*, Post Hoc Tests, scheffe). However, this difference in the MIC value BAC showed no significant

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difference between the fungi isolates (Anova test, *p = 0.11*).

Discussion

BAC and CHX

In the present study, we investigated the in vitro susceptibility of 54 fungal isolates obtained from the air to BAC, CHX, and SH. Moreover, BAC was found a potent inhibitor of *A. niger*, *A. flavus*, and *Cladosporium* spp. isolates; whereas, less activity was observed in SH.

Based on the MIC_{50} and MIC_{90} values, BAC had lower activity than CHX (4-fold) and SH (200- to 800-fold) against all isolates. Similarly, based on the geometric mean MIC values, BAC was more effective than CHX and SH against *A. flavus*, *A. niger*, and *Cladosporium* spp. isolates.

Nonetheless, the BAC MIC, MIC_{50}, and MIC_{90} ranges obtained in our study were comparatively lower than those for *Penicillium* spp. and *Cladosporium* spp. isolates (2 to 4 µg/ml, 2 µg/ml, and 8 µg/ml, respectively, for *Penicillium* spp; 4 to 16 µg/ml, 4.0 µg/ml, and 8 µg/ml, respectively, for *Cladosporium* spp.) reported by Sandle and Vijayakumar et al. [21, 22].

Xu et al. [23] reported that the MIC_{90} values of BAC were 32, 32, and 16 µg/ml for *Fusarium* spp., *Aspergillus* spp., and *A. alternata*, respectively. In our study, the MIC_{90} values of BAC for *A. flavus* and *A. niger* (8 µg/ml) were much lower than those reported by Xu et al. [23].

In line with our findings, Sandle et al. [21] evaluated the MIC ranges of CHX, BAC, and cetrimide against hyaline fungi (*A. flavus*, *A. niger*, *A. fumigatus*). Moreover, the MIC ranges of the three mentioned biocides against *Alternaria* spp were observed to be < 32 µg/ml and 8 to 16 µg/ml against other dematiaceous fungi, which were lower than the MIC ranges found in the present study.

The MIC ranges of BAC for *A. niger* and *A. flavus* (2-8 µg/ml and 2-16 µg/ml, respectively) in our study were higher than those reported by Stupar et al. [24]; 0.1-4 µg/ml. These significant differences can depend upon variations in methodology or may be due to the application of other media like MEA and the introduction of solvents such as water or tween 80 and the used quaternary ammonium compounds (QACs). The activation of QACs varied with the temperature and pH by agar that reduced activity [25, 26].

In a study conducted in Brazil by Bernardi et al. [27], the effective performance was seen only at the highest concentration of BAC (5%), even though *C. cladosporioides* and *P. polonicum* with sensitivity at the intermediate concentrations (2.5%) were exceptions. Moreover, it was mentioned that BAC and quaternary ammonium should not be applied as a sanitizer used for cheese industries due to their limited efficacy of action against *P. commune* and *P. roqueforti*, which are in contrast with the obtained findings in the present study [27]. As a result of a similar survey carried out in Brazil, all fungal isolates were found susceptible to an antimicrobial product containing active sodium hypochlorite, except for *A. terreus* [5].

Quaternary Ammonium Salts (QAS) show antimicrobial activities against yeasts, bacteria, viruses, and spores at a low concentration between 10 and 50 mg/l depending on the target microorganism and type of used QAS [28, 29].

The CHX ranges of MIC, MIC_{50}, and MIC_{90} obtained for all isolates in the current investigation were 1 to 4-fold, 4-fold, and 4-fold higher, respectively compared with BAC. Consistent with our findings, Xu et al. [30] found that MIC_{90} values obtained for CHX were 32 µg/ml against both *F. solani* and *A. falvus*, respectively. In our study, CHX showed relatively high MICs (MIC of range: 8-64 µg/ml; 16-32 µg/ml) against *A. flavus* and *A. niger*, respectively (Table 2), which is not in agreement with a previous study reporting that the MIC range of CHX against *Aspergillus* strains were within the range of 4-16 µg/ml [22].

Sodium hypochlorite

Sodium hypochlorite disinfectant solutions play an essential role in preventing the growth and propagation of fungal aerosols in indoor air environments. They are also recommended to be used at concentrations of less than 0.04% to prevent fungal growth on vegetables, seeds, and

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nuts [4]. In the same regard, SH solution is a common disinfectant suitable for the hard-surfaces. Hypochlorous acid inactivates bacterial DNA and damages the oxidative phosphorylation and membrane activity [29, 31].

Some studies [32, 33], similar to our findings, stated that SH had the broadest range of MICs and did not performe appropriately against *A. fumigatus*, *A. flavus*, and *A. niger*, while chlorhexidine-cetrimide showed high activity against these fungi. A study reported that SH was the most efficient agent against the fungal species at the concentrations evaluated as 0.1%, 0.5%, and 1.5% even in the presence of organic matter [27], which is in contrast with our findings. Reynold et al. [34] mentioned that SH concentration reduced by 2.4% with regard to the total mold counts (*Cladosporium*, *Mucor*, *Rhizopus*, *Alternaria*, and *Aspergillus*) more than five logs after five minutes of exposure in home showers. Recent studies indicated that most disinfectants investigated in healthcare settings (10 of 13, 77%) are active (>3 log reduction) against *Candida auris*, except for a 1:50 dilution of sodium hypochlorite 5.25% and a quaternary ammonium compound (water-based) [35].

Some factors such as formulation of biocide [21], complex interactions between antifungals and fungal isolates, different ecological factors [36], as well as unusual behaviors of the strains within a species against biocides [4] use different protocols in the methodology [36]. Moreover, different distributions of fungal isolates are possible reasons for the differences among the results in other regions of the world. The success of antimicrobial agents depends not only on the characteristics of disinfectants, but also on the type of microorganisms present in the environment and their sensitivity to the antimicrobial employed [27].

Abundant melanin that exists in the spores of *A. niger* has a protective role and is likely responsible for higher resistance against physical and chemical agents. Furthermore, changes in the structure and physiology of fungal elements, including swelling and rupture of the mycelium, are among the functions of disinfectants [36].

Finally, no clear criteria exist for the concentrations and breakpoints of antiseptics and disinfectants. Concerning the limited number of in vitro studies on antifungal activity of disinfectants and antiseptics in healthcare settings, a comparison of MIC results with other reports would be problematic. The different outcomes may suggest that further studies are necessary in this field [21].

The limitation of this study included investigating antifungal activity of BAC, CHX, and SH only in one children's hospital and only in two seasons. Future researchers are suggested to investigate a higher number of hospitals over more extended periods and other sampling sources such as hospital surfaces. This study was a general description over the fungal bio-aerosol disinfection of the hospital. Therefore, as BAC becomes more commonly used in surface disinfection, further studies are needed on how BAC-treated products affect fungal contamination in the hospital environment.

Conclusion

In conclusion, BAC has potent in vitro activity against *A. flavus*, *A. niger*, and *Cladosporium* isolates, as effective disinfectants, along with commercial disinfectants. This may lead to the prevention of resistant fungal pathogens and be a suitable alternative to other biocides.

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Conflict of interests

The authors declare no conflict of interest.

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