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ORIGINAL ARTICLE

In vitro Efficacy of Chemical Disinfectants against Fungi isolated from Different wards of two University-Affiliated Hospitals in Tehran

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ABSTRACT
In vitro activity of benzalkonium chloride 4%, 5% and 6% solutions, Betadine® 8, 9 and 10% solutions, Chlorhexamed® 3%, 4% and 5% solutions and also Dettol® 0.5%, 1.5 % and 2.5 % solutions were assessed against fungi isolated from different wards of two Tehran university - affiliated hospitals after 15, 30 and 60 min of exposure time. 30 specimens were randomly collected from intensive care unit (ICU), cardiac care unit (CCU), examination and patient's rooms and corridors of hospitals via air and surface sampling. Fungal suspensions (0.25 ml) were supplied from each fungus and added to 3.75 ml of different disinfectant solutions and let for appropriate interaction at 15, 30 and 60 min. Dey- Engley medium (1.43 ×) was used for neutralization of disinfectants activity. Dettol® 2.5% solution was the most effective disinfectant against isolated fungi, in particular on Aspergillus niger and Cunninghamella. Betadine® was not effective disinfectant even with use of 10% solution and showed the least activity to isolated fungi. Significant correlation was seen between disinfectant and used concentration and also disinfectants and isolates (P < 0.01). More concentrated solutions than recommended concentration of disinfectants by manufacturer are needed for full fungicidal activity of disinfectants.
***Key words**: Chemical disinfectants, Saprophytic fungi, Neutralization activity, Nosocomial fungal infection*

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INTRODUCTION

Immunocompromised patients (diabetics, solid organ transplants, and patients with AIDS) are highly susceptible to nosocomial fungal infections (NFIs) [1]. NFIs are rapidly progressive, severe and hard to treat and their incidence, especially those caused by *Candida spp*. and *Aspergillus spp*., has proportionally more increased in susceptible patients [2]. National Nosocomial Infections Surveillance (NNIS) system has reported during 1981 to 1990, *Candida spp*. and *Aspergillus spp*. were responsible for 78.3% and 10.3% of NFIs, respectively [3]. Although other fungi and yeasts include *Mucor*, *Rhizopus*, *Fusarium* and *Malassezia* also, cause NFIs with lower rate in highly immunocompromised patients such as those with hematologic malignancy undergoing bone marrow transplants and severe renal failure [4]. So, with regard to importance of NFIs, more efforts are needed to delineate preventive and controlling programs of NFIs. One of the most important approaches suggested by Centers for Disease Control (CDC) for prevention of NFIs is periodic review of mycology as gravity settle plate cultures in different wards of hospitals [5] and followed by, use of appropriate methods , such as clinically useful disinfectants [6]. Chemical disinfections are widely used in busy hospitals and clinics. Classification of fungicidal proportion of disinfectants is based on the Association of Official Analytical Chemists (AOAC) suspension test, which indicates *Trichophyton mentagrophytes* var. *Interdigitale* and *Candida albicans* only can be used as the test organisms [7]. Although, there are claims of manufacturers based on fungicidal activity of some disinfectant with recommended dose and time, but it's essential for disinfectants which be tested with environmental and clinical isolated fungi to better judgment on their fungicidal activity. Unfortunately, there are very little data on fungicidal activity of disinfectants. So, in this study we tested

in vitro efficacy of four common chemical disinfectants (Benzalkonium chloride, polyvinyle pyrrolidone-iodine (Betadine®), chlorhexidine gluconate (Chlorhexamed®) and chloroxylenol (Dettol®) against isolated fungi from different wards of two university - affiliated hospitals in Tehran.

MATERIALS & METHODS

Sampling and isolation of fungi:

Fungi isolated from different wards of 2 university - affiliated hospitals including intensive care unit (ICU), cardiac care unit (CCU), examination and patient's rooms and corridors of hospitals by air and surface sampling during December 2011 to February 2012. Surface sampling was done by moistened sterile swabs over a surface approximately 25 cm^2 and air sampling was done by putting the plates (without lid) for 10 min in four areas of mentioned wards. All samples were routinely grown on sabouraud chloramphenicol agar (SC) for 7-14 days at 30°C. Isolated fungi were identified by their morphological and biochemical characteristics along with slide culture method. *Candida albicans* was used as the test micro-organism in this study.

Fungal suspension:

Fungal suspensions were prepared from cultures grown on SC. Briefly, colonies were covered with 3 ml of sterile 85% saline and gently probed with the tip of pasteur pipette. After transfer of resulting mixture of conidia and hyphae to sterile tubes, they were vortexed for 2-3 min and then the turbid suspension were concentrated by centrifugation at 5°C and suspended with 8 ml of sterile saline in final volume. Final fungal suspensions were adjusted by counting chamber and incubated at 30°C and visible colonies were usually observed after 3 to 7 days of growth.

Disinfections:

The examined disinfectants in this study were including benzalkonium chloride, polyvinyle pyrrolidone-iodine (Betadine®), chlorhexidine gluconate (Chlorhexamed®), chloroxylenol (Dettol®). Initially, 10% concentration of mentioned disinfectants were examined for evaluation of fungicidal activity and then concentrations of disinfectants were regularly decreased until margin of effective dilution for control organisms were obtained.

Inactivation of disinfectants:

Dey-Engley (D/E) neutralizing medium was used for inactivation of disinfectants after timed exposure (15, 30, and 60 min) with 0.5×10^4 fungal cells. The compositions of this medium are presented in table 1 which was introduced by Terleckyj et al [8]. Two forms (1.43 ×) or concentrated form and (1 ×) or normal strength of D/E medium were used for neutralizing disinfectant and wash fungal cells, respectively and pH of D/E medium was adjusted in 7.6 ± 0.1. *Candida albicans* was used as test organism to determine the neutralizing capacity of D/E medium. The procedure involved adding 4.5 ml of (1.43 ×) D/E medium to 2.0 ml of different disinfectant solutions and allowing 5 min for inactivation, then 0.2 ml of *C.albicans* cell suspension was added to disinfectant – neutralizing medium solutions. After 15 min exposure, the yeast cells were centrifuged at 5°C and suspended in 6.5 ml of normal strength D/E medium. Viability counts were done to determine whether any disinfectant was still active in presence of the neutralizing components or not. Controls included exposing *C. albicans* cells to disinfectant and solution diluted with sterile distilled water (2 ml of disinfectant and 4.5 ml of H_2O) and also D/E medium (1.43 ×) diluted with sterile distilled water. (4.5 ml of concentrated medium and 2 ml of H_2O).

Components (conc. [%])	Function
Glucose(1)	Carbohydrate source (nutrient)
Tryptone(0.5)	Amino acid source (nutrient)
Yeast extract (0.25)	Vitamin and mineral source
Lecithin (0.7)	Neutralizes 1:750 quaternary compounds
Sodium thiosulfate (0.6)	Neutralizes 2% chlorine and 2% iodine
Tween 80 (0.5)	Neutralizes 2% phenolic compounds
Sodium bisulfite (0.25)	Neutralizes 2% formaldehyde and 1% glutaraldehyde
Sodium thioglycolate (0.1)	Neutralizes 1:1,000 mercurial compounds
Bromcresol purple (0.002)	Growth indicator

Table1. The components D/E neutralizing medium (normal strength, pH = 7.6) (adapted from Terleckyj et al)

Assay of fungicidal activity of disinfectants:

The quantities assay for fungicidal evaluation of different disinfectants was as follows: 0.25 ml of fungal suspension was added to 3.75ml of disinfectant solutions which resulted 4ml of use dilution concentration containing viable cells, then vortexed for 10 s and let at 25°C for appropriate interaction

time (15, 30 and 60 min). After mentioned time, 9ml of concentrated from (1.43 ×) D/E medium was added to use dilution concentration resulting final volume of 13 ml and centrifuged with 3,000 rpm for 15 min at 5˚C. Pellet cells were washed with 8ml of normal strength D/E medium and suspended with 1 ml of normal strength D/E medium. 0.15 ml of resulted suspends were inoculated into each of three sabouraud slants and incubated at 30˚C for 6 - 8 weeks. These slants were checked at least twice a week for fungal growth. Growth of fungal colonies on sabouraud slants was interpreted as survival of the fungus to disinfectants at time exposure. Controls were including 0.25 ml of the cell suspensions with 3.75 ml of sterile distilled water.

Statistics:

ANOVA along with regression test were used for evaluation of effects of concentration and type of disinfectants used on antifungal activity and ($P < 0.01$) was considered significant.

RESULTS

Fungus	Samples (% percent)
Penicillium spp.	11(36.67)
Aspergillus niger	7 (23.34)
Cuninghamella	3 (10)
Rhizopus spp.	2 (6.67)
Alternaria spp.	2 (6.67)
Mucor spp.	1 (3.33)
Cladosporium spp	1 (3.33)
Fusarium spp.	1 (3.33)
Acremonium	1 (3.33)
Zopfia rosatii	1 (3.33)
Total	30(100)

Table 2- Type and rate of isolated fungi from different wards of university- affiliated hospitals.

Disinfectants (Concentration %)	Fungal growth after 15,30 and 60 min interactions with disinfectants		
	15 Min No. of growth (%)	30 Min No. of growth (%)	60 Min No. of growth (%)
Benzalkonium chloride (4)	30 (100)	27 (90)	21 (700)
Benzalkonium chloride (5)	18 (60)	16(53.3)	7 (22.3)
Benzalkonium chloride (6)	3 (10)	0 (0)	0 (0)
Betadine® (8)	30 (100)	30 (100)	30 (100)
Betadine® (9)	30 (100)	30 (100)	28 (93.3)
Betadine® (10)	30 (100)	29 (96.6)	27 (90)
Chlorhexamed® (3)	30 (100)	26 (86.6)	23 (76.6)
Chlorhexamed® (4)	29 (96.6)	24 (80)	19 (63.3)
Chlorhexamed® (5)	21 (70)	15 (50)	0 (0)
Dettol® (0.5)	30 (100)	24 (80)	22 (73.3)
Dettol® (1.5)	11 (36.7)	8 (26.6)	2 (6)
Dettol® (2.5)	4 (13)	0 (0)	0 (0)

Table 3- Fungal growth rate (survival of isolated fungi) after 15, 30 and 60 min interactions with various disinfectants.

30 samples were randomly selected via cluster sampling from different wards of 2 university- affiliated hospitals. Isolated fungi are shown in Table 2. In presence of benzalkonium chloride 4% solution within 30 min of contact time, 3 colonies of *Penicillium spp* were sensitive but after 60 min, 9 colonies including 4 colonies of *Penicillium spp*, 3 colonies of *Cunninghamella*, 1 colony of *Aspergillus spp* and 1 colony of *Alternaria* were sensitive. Ungrown colonies within 15 min were including 4 colonies of *Penicillium*, 2 colonies of *Aspergillus niger* and 1 colony from each of *Cunninghamella*, *Rhizopus*, *Alternaria*, *Cladosporium*, *Fusarium* and *Acremonium* in presence of benzalkonium chloride 5% solution. After 60 min, both isolates of *Alternaria* were resistant to benzalkonium chloride 5% solution and other resistant colonies were 3 colonies of *Aspergillus niger*, 1 colony of *Penicillium* and 1 colony of *Alternaria*. Three resistant colonies against benzalkonium chloride 6% solution were *Aspergillus niger*, *Rhizopus* and *Zopfia rosatti* after 15 min of contact time. Chlorhexidine 3% solution had no effect in 15 minutes. It prevented the growth of 4 and 7 fungal colonies and didn't show a good antifungal effect in times of 30 and 60 minutes, respectively but chlorhexidine 5% solution showed the complete fungicidal effect in 60 minutes. It destroyed 50% of the colonies in 30 minutes (50% of fungicidal effect). Dettol® 5% solution showed a

remarkable fungicidal effect in 30 and 60 minutes. Dettol® 1.5 % solution prevented the growth of 36.7% fungi in 15 minutes but its effect on *Aspergillus niger*, *Cunninghamella* and *Alternaria* was 100% in 60 minutes. Dettol® 2.5% solution prevented the growth of the whole fungal isolates in 30 and 60 minutes, and just 13 percent of fungi grew in 15 minutes of contact time. In presence of Betadine® 8% solution, all colonies grew and only 3 colonies didn't grow in presence of Betadine® 10% solution. Betadine® was not effective disinfectant even with use of 10% solution and showed the least activity to isolated fungi (Table 3).Totally, significant correlation was seen between disinfectant and used concentration (benzalkonium chloride , Betadine®, Chlorhexamed® and Dettol® , $P < 0.01$, $\beta = 0.35$,0.49 and 0.21 , respectively) and also disinfectants and isolates ($P < 0.01$, $df = 21$).

DISCUSSION

Prevalence of NFIs is increasing in all types of hospitals specially, in university – affiliated hospitals [9, 10]. Hospital environment has a pivotal role in the epidemiology of NFIs. So, with understanding of epidemiology of fungi causing NFIs and use of preventive measures (such as use of effective disinfectant), could decrease the incidence of NFIs. Penicillium spp., and Aspergillus spp., were the most isolated fungi from different wards of hospitals. Kordbacheh et al. has reported Penicillium spp., and cladosporium spp., were the most isolated fungi from ICU and transplant wards in a teaching hospital [4]. In this study, four chemical disinfectants were used for evaluation of their neutralization activity against isolated fungi from different wards of two university- affiliated hospitals. Benzalkonium chloride 4% solution (current form of used in hospitals) wasn't full effective disinfectant even with 60 min of time exposure but its 6 % solution was shown full efficacy after 30 and 60 min of time exposure to isolated fungi. In a study carried by Marchetti et al. [11], benzalkonium chloride 2% solution has been shown 97 % efficacy against mild and moderate contamination of *Microsporum canis*. Chlorhexamed® 3 % and 4 % solutions had no good efficacy even after 30 and 60 min of time exposure and only in presence of Chlorhexamed® 5 % solution after 60 min, growth of fungi were not seen. Theraud et al. [12] has reported 0.5 % chlorhexidine was effective on clinical and environmental isolates of *Candida albicans* and *Cryptococcus neoformans*, which didn't agreed with our results. In our study even with use of eight- fold concentration of 0.5 % chlorhexidine, good results didn't obtained. Dettol® 2.5 % solution was the most effective disinfectant against isolated fungi, in particular *Aspergillus niger* and *Cunninghamella*. Benzalkonium chloride 6% solution was in second level among four disinfectants from aspect of antifungal activity.

Betadine® was not effective disinfectant even with use of 10 % solution (maximum concentration suggested by manufacturer) and showed the least activity to isolated fungi (in spite of manufacturer's claim). Theraud et al. [12] also has reported Betadine® showing the least activity against clinical and environmental filamentous fungi and yeasts which was in accordance with our study. Among the isolated fungi, *Cunninghamella* showed the most susceptibility to used disinfectants and *Mucor spp.* and *Cladosporium spp.* were in following levels. In the lowest dilution of Dettol®, Benzalkonium chloride, Chlorhexamed® and three dilutions of Betadine® at 15 min contact time, all isolates were resistant but with increase of contact time, more fungal isolates were destroyed. So, time and concentration were two important factors in increasing of antifungal activity of disinfectants which time showed more effective role than concentration. In this study, sampling was done after sterilization program and isolation of these fungi in different wards specially, in CCU showed the inappropriate sterilization in these important wards which is alertness in immunocompromised patients. With regard to isolation of fungi after sterilization program and low efficacy of most used disinfectants, in spite of manufacturer's claim, more concentrated solutions for full fungicidal activity of disinfectants and also further studies about efficacy of disinfectants are recommended.

CONCLUSION

Our study showed the importance of fungicidal activity evaluation of disinfectants on isolated fungi. A fungicidal agent should be able to kill most resistant fungi otherwise, by use of questionable disinfectants, sterilization targets will be not accessible.

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