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Minimum Inhibitory Concentration Determination of Chlorhexidine gluconate Against Pharmaceutical Clean Room Fungal Isolates

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ABSTRACT

The antifungal activities of most commonly used biocide chlorhexidine gluconate tested against eight pharmaceutical clean room fungal isolates like *Aspergillus niger, Aspergillus fumigatus, Aspergillus flavus, Aspergillus terreus, Penicillium sp., Curvularia sp., Cladosporium sp.,* and *Alternaria sp.* The minimal inhibitory concentration (MIC) determined by using micro broth dilution method as per clinical and laboratory standards institute (CLSI) guidelines. No data exists on the determination of MIC of chlorhexidine gluconate on pharmaceutical clean room fungal isolates. MIC of chlorhexidine gluconate against species of *Aspergillus* and *Penicillium* species were ranged between 4 and 16 µg/mL. MIC of *Curvularia, Cladosporium* and *Alternaria* species also showed less than 8 µg/mL. This is the first study report using the CLSI broth microdilution antifungal susceptibility testing to determine the MIC value of chlorhexidine gluconate.

INTRODUCTION

Moulds and yeasts are an important group of microorganisms, which are responsible for various infections, but are primarily associated with contamination of surfaces and spoilage of pharmaceuticals, cosmetic and food products. Fungal infection or contamination can be responsible for serious economic losses, for example spoilage in pharmaceutical, food or cosmetic manufacturing and increased duration in hospital [1, 2]. Antiseptics and disinfectants are used extensively in industries and hospitals to control the growth of microbes on both living tissues and inanimate objects. They are essential parts of infection control practices in hospitals and contamination control in pharmaceutical industries [3]. But a common problem is the selection of disinfectants and antiseptics because different pathogens vary in their response to different antiseptics or disinfectants [4].

In order to decide which method, or combination of methods, to be employed in disinfecting aseptic processing areas, it is important to understand the kind of microorganisms that are the prime sources of contamination [5]. The more frequently detected bacterial and fungal contaminants in the hospital and pharmaceutical production environment are species of *Staphylococcus*, *Micrococcus, Bacillus, Penicillium, Cladosporium* and *Aspergillus* [6, 7]. Biocides are used extensively to control the growth of these contaminants. Chlorhexidine gluconate of biguanides class is widely used as disinfectant and or antiseptic in pharmaceutical industries and hospitals. It is commonly used either at 0.5 to 0.75 % in aqueous solution or in some detergent preparations or at 2 to 4% in other detergent preparations [8, 9].

Antifungal susceptibility tests are performed on fungal pathogens in clinical microbiology setup, especially if they belong to a species exhibiting resistance to commonly used antifungal agents. Antifungal susceptibility testing is also important in resistance surveillance, epidemiological studies and in comparison of the *in vitro* activity of new and existing agents. Dilution methods are used to establish the minimum inhibitory concentrations (MIC) of antimicrobial agents. The MIC is defined as the lowest concentration, recorded in mg/L or µg/mL of an antifungal agent that inhibits the growth of a fungus. The MIC informs about the susceptibility or resistance of the organism to the antifungal agent and can help in treatment decisions [10, 11]. In dilution tests, fungi are tested for their ability to produce visible growth in microdilution plate wells of broth culture media containing serial dilutions of the antimicrobial agents (broth microdilution).

The development of microbial resistance to antibiotics is a well-described phenomenon but unlike microbial resistance to disinfectants. There are no published reports available for describing MIC data and resistance of pharmaceutical environmental fungal isolates against disinfectants. However, in order to test the efficacy of disinfectants the most frequently

isolated microorganisms from an environmental monitoring program may be periodically subjected to use dilution testing with the agents used in the disinfection program to confirm their susceptibility [12, 13]. When compared to bacterial clean room contaminants, the minimum inhibitory concentration of fungal isolates against common disinfectants study reports are not available.

Hence, the aim of this study is to reveal the minimum inhibitory concentration of most commonly used biocide (chlorhexidine gluconate) employed in hospitals and industrial sectors against clean room fungal isolates by microbroth dilution method.

MATERIAL AND METHODS

Isolates

Eight clean room fungal isolates were selected which included predominant hyaline and dematiaceous fungi. All the fungal isolates were collected from the environmental monitoring in microbiology division clean room facilities in Aurolab, Madurai India. The samples were collected during September 2010 and February 2011. The fungal isolates included species of *Aspergillus*, *Penicillium*, *Cladosporium, Curvularia* and *Alternaria*. Antifungal susceptibility against biocide was determined according to methods outlined in clinical and laboratory standards institute (CLSI) documents M38-A [14].

Inoculum preparation

The inoculum was prepared by overlaying mature slants with sterile distilled water and gently scraping the surface with a wooden applicator stick. The suspension was permitted to sit for five minutes to allow large particles to settle down and then adjusted spectrophotometrically to the correct optical density for each species as outlined in M38-A, providing an inoculum concentration of 0.4 – 5 X 10^4 conidia /mL, which was verified by colony count. The inoculum suspensions were diluted (1:50) in RPMI-1640 (Roswell Park Memorial Institute) media buffered with 0.165M morpholino propanesulfonic acid (MOPS) (34.54 g per liter) at pH 7.0

Antifungal agents

Chlorhexidine gluconate (20 %, Unilab chemicals, Mumbai, India) of biguanides group was selected as antifungal agents. The stock solution was prepared by dissolving disinfectant at a concentration of 1000 µg/ mL in sterile distilled water following the protocol of CLSI. From the stock, further dilutions were made using RPMI test medium.

Preparation of microdilution plates

Using sterile plastic, disposable, 96 well microdilution plates with flat-bottom wells with a nominal capacity of approximately 300 µL, 100 µL from each of the tubes containing the corresponding concentration (2 x final concentration) of target disinfectants was dispensed into the wells in each column (from 1 to 10). For example, column 1 the medium containing 128 µg/ ml (128 mg/L) was dispensed, to column 2 the medium containing 64 µg/ ml was dispensed, and so on to column 10 where the medium containing 0.25 µg/ ml was dispensed. To each well of column 11 and 12, 100 µL of RPMI 1640 medium was dispensed. Thus, each well in columns 1 to 10 contained 100 µL of twice the final antifungal drug concentrations in RPMI medium. Columns 11 and 12 contained double-strength RPMI 1640 medium. The final well concentrations reached were 0.125 µg/ ml to 64 µg/ ml after addition of inoculum (100 µL). Microdilution plates were stored at - 70ºC prior to use.

Antifungal susceptibility testing

The MICs were determined following the microdilution method recommended by CLSI, approved standard M38-A (14). This involved testing each fungal isolate in duplicate. Each microdilution well containing 100 µl of the two fold drug (biocide) concentration was inoculated with 100 µl of diluted inoculum suspension. For each test plate, two drug free controls were included, one with the medium alone (sterility control) and the other with 100 µl of medium plus 100 µl inoculum suspension (growth control). The biocide concentrations assayed ranged from 0.125 to 64 µg/ml.

Incubation time and Temperature

The microdilution plates were incubated at 35^0 C for 48 hours or until growth was visible in the drug (biocide) free control well

Table No.1 : Minimum inhibitory concentration of chlorhexidine gluconate against clean room fungal isolates

Fungal Group	Name of the fungal isolates	MIC value (µg/mL)
Hyaline fungi	*Aspergillus flavus*	4
	Aspergillus fumigatus	4
	Aspergillus niger	8
	Aspergillus terreus	16
	Penicillium sp.	8
	MIC Range	4 – 16
Dematiaceous fungi	*Cladosporium sp.*	4
	Curvularia sp.	2
	Alternaria sp.	8
	MIC Range	2 – 8

Reading and interpretation

Endpoint determination values were read visually with the aid of an inverted reading mirror. The minimum inhibitory concentration (MIC) was noted as the lowest concentration that exhibited a 100% visual reduction in turbidity when compared with the control well at 48 hours. The test for each fungal strain was repeated twice and the geometric mean of the MIC only was considered as final result.

RESULTS

All fungal isolates tested produced detectable growth after 48 – 72 hours of incubation. Eight fungal isolates were grouped as two categories named hyaline and dematiaceous fungal groups. The MIC's for all species of *Aspergillus* and *Penicillium sp*. were observed in the range between 4 and 16 µg/mL. In dematiaceous fungal group, the lowest MIC was found against *Curvularia sp*. (2 µg/mL) *Cladosporium sp*. and *Alternaria sp*. were recorded as are 4 and 8 µg/mL respectively. Table 1 summarizes MIC data for hyaline clean room fungi and dematiaceous fungi.

DISCUSSION

Chlorhexidine gluconate is probably the most widely used biocide in antiseptic products, in particular in hand washing and oral products but also as disinfectant and preservative. It is low to intermediate level of disinfectant of biguanides group. It interacts with the cell surface and promotes membrane damage, which in turn causes an irreversible loss of cytoplasmic components [15, 16]. Chlorhexidine antifungal activities have been studied by various authors in the context of dental products and the oral environment [17 – 19]. However, limited data are available on the susceptibility and mechanisms of resistance of reference strains of molds to biocides. The aim of this study was to evaluate the efficacy by determining the MIC of chlorhexidine gluconate against various fungal isolates of pharmaceutical environment origin by using micro broth dilution technique.

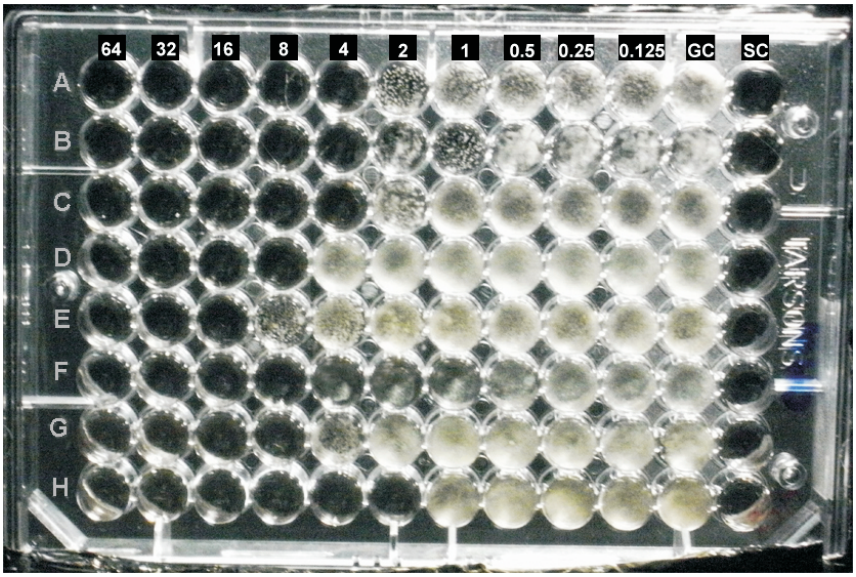
In the present study, it was found that the MIC for chlorhexidine gluconate against hyaline fungi like *Penicillium sp.* and species of *Aspergillus* ranged between 4 and 16 µg/mL. The MIC range of *Cladosporium sp.*, *Curvularia sp.* and *Alternaria sp.* was found to be 4, 2 and 8 µg/mL respectively (Fig.1). The killing action of **c**hlorhexidine at relatively low concentrations (e.g., 2–2.5 µg/mL) is similar to the action of some antibiotics. At higher concentrations (> 20 µg/mL), **c**hlorhexidine causes coagulation of cytoplasm and precipitation of proteins and nucleic acids of bacteria and yeasts [20].

Species of *Cladosporium*, *Curvularia* and *Alternaria* contain melanin pigment in their cell wall and called as dematiaceous fungi. This melanin pigment in their cell wall might be involved with cellular resistance to physical and chemical agents [21]. But in our study there was no significant MIC value difference found between hyaline and dematiaceous fungal group. However, this could be confirmed with other dematiaceous fungi in further studies.

There have been no reports of the MIC of chlorhexidine gluconate against environmental fungi. Therefore comparison of MIC results with other fungal reports might be difficult. However, Rahman et al., reported that chlorhexidine might have potential to control the growth of fungal pathogens and might used as primary treatment for fungal corneal ulcers in circumstances where specific antifungal agents are not available [22]. Other studies have described the chlorhexidine to be potent anticandida biocide[23].

Several authors have investigated invitro effect of chlorhexidine gluconate against bacterial pathogens and reported MIC values of *E. coli* 0.5 – 1 µg/mL, *Streptococcus pneumoniae* 1 µg/mL, *Staphylococcus aureus* 1 µg/mL, *Salmonella sp.* 3.3 µg/mL, *Proteus vulgaris* 5 µg/mL and *Pseudomonas aeruginosa* 5 – 12.5 µg/mL [24, 25]**.** Odore et al., reported that the MIC values of chlorhexidine gluconate against dermatophytes such as *Microsporum gypseum*, *Microsporum canis* and *Trichophyton mentagrophytes* were 12.5, 50 and 6.25 µg/mL respectively [26]. This is comparable with our findings where MIC values of environmental fungal isolates ranged between 2 and 16 µg/mL which seems chlorhexidine appears to be less active against fungal cells than against non sporulating bacteria. This conforms that molds are more resistant than yeasts and considerably more resistant than non sporulating bacteria to antiseptics and disinfectants [21]. However, it has to be noted that testing protocols varied greatly and therefore comparison of results might be difficult.

Fig.1 : Micro broth dilution plate showed MIC (µg/mL) of chlorhexidine gluconate against fungal isolates



A – *Aspergillus flavus*, B – *Aspergillus fumigatus*,
C – *Cladosporium sp*., D – *Penicillium sp*.,
E – *Aspergillus terreus*, F – *Alternaria sp*.,
G – *Aspergillus niger*, H – *Curvularia sp.*
GC – Growth Control, SC – Sterility control

CONCLUSION

Over all, our present study reveals that minimum inhibitory concentrations of chlorhexidine gluconate against environmental fungal isolates were 2 to 16 µg/mL. The lowest MIC was observed against *Curvularia* species. This is the first report using the CLSI broth microdilution antifungal susceptibility testing to determine the MIC values of chlorhexidine gluconate versus clean room fungal isolates. Further work is needed in this field to increase our understanding of biocides against different fungal isolates and to enable to design more efficient disinfection and contamination control programs in pharmaceutical industrial sectors and hospitals.

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