

Research Paper

## *In vitro* activity of disinfectants against *Aspergillus* spp

A.S. Mattei<sup>1</sup>, I.M. Madrid<sup>1</sup>, R. Santin<sup>1</sup>, L.F.D. Schuch<sup>2</sup>, M.C.A. Meireles<sup>1</sup>

<sup>1</sup>Laboratório de Doenças Infecciosas, Setor Micologia, Faculdade de Veterinária, Universidade Federal de Pelotas, Pelotas, RS, Brazil.

<sup>2</sup>Laboratório de Doenças Infecciosas, Setor Bacteriologia, Faculdade de Veterinária, Universidade Federal de Pelotas, Pelotas, RS, Brazil.

Submitted: March 11, 2011; Approved: July 23, 2012.

---

### Abstract

Fungi of the *Aspergillus* genus are widespread and contaminate the environment. Thousands of conidia are released from each phialide and dispersed in the air every day. These fungi are considered important mycose-causing agents in hospitals. Due to this, research to determine prevalent fungi from the *Aspergillus* genus in hospital environments, and an adequate disinfection program in these areas is needed. This study evaluated the susceptibility of *Aspergillus* spp. isolated from a veterinary environment against four disinfectants. Successive dilutions of disinfectants ( $\log_2$ ) were used according to CLSI M38-A2 microdilution technique adapted to chemical agents against 18 isolates of this genus. After 72 hours of incubation, the Minimum Inhibiting Concentration and Minimum Fungicidal Concentration capable of inhibiting 50% and 90% of the isolates were determined. Chlorhexidine-cetrimine, benzalconium chloride and a chlorophenol derivative proved to be effective against all isolates with a lower MIC than that suggested by the manufacturer, except for the *A. flavus* strain. Sodium hypochlorite was ineffective against three *A. fumigatus*, three *A. flavus* and one *A. niger* isolate. These results demonstrated that all studied disinfectants were effective against environmental isolates, with the exception of sodium hypochlorite, which showed lower effectiveness.

**Key words:** ammonium quaternary, chlorhexidine, chlorophenol derivative, filamentous fungi, sodium hypochlorite.

---

### Introduction

Fungal infections that affect immunocompromised patients are very important due to treatment difficulties, often leading to the patient's death (Morris *et al.*, 2000). A study on septicemia epidemiology reported a 207% increase in fungus cases between 1979 and 2000, some of which were related to the *Aspergillus* genus (Martin *et al.*, 2003).

Invasive aspergillosis, which is primarily caused by *Aspergillus fumigatus*, followed by *A. flavus*, *A. niger* and *A. terreus*, is one of the causes for the high mortality rate of hospitalized patients. Since this fungus is widespread, countless conidia are released from phialides and dispersed in the air every day, contaminating the environment (Latzé, 1999). Thus, an association of chemoprophylaxis with an environmental support, including positive airway pressure

in the rooms, filters for respiratory protection (HEPA) and the elimination of opportunistic fungus sources, is recommended (Falvey and Streifel, 2007).

Research to determine the prevalent fungi of the *Aspergillus* genus in hospital environments has been developed, inasmuch as these are potential infection sources (Morris *et al.*, 2000; Vonberg and Gastmeier, 2006; Falvey and Streifel, 2007). However, studies in veterinary environments are still scarce (Xavier *et al.*, 2008; Mattei *et al.*, 2011).

Basic cleaning, disinfection, sterilization and biosafety principles are similar in veterinary hospitals, but, due to some peculiarities, these processes have not been properly standardized and evaluated, resulting in cross contamination and hospital infection risks (Santos *et al.*, 2007). There are different active principles of disinfectants avail-

able, but the spectrum of desired activity, toxicity, residual power, costs and nature of the material still needs to be better defined (McDonnell and Russel, 1999). This study evaluated the susceptibility of *Aspergillus* spp. isolates from veterinary environment surfaces against four disinfectants.

## Material and Methods

### Isolates and inoculum

Eighteen *Aspergillus* genus fungal isolates obtained from veterinary hospital surfaces, nine of which were *A. fumigatus*, seven *A. flavus* and two *A. niger*, were tested by the plating technique.

To prepare the inoculum, the isolates were previously plated on Petri dishes containing potato dextrose agar (Neogen Acumedia®, Michigan/EUA) and incubated at 35 °C for five days so that pure young cultures could be obtained. After fungal growth, conidia and hyphae were collected from the colony surface and transferred to a sterile test-tube containing 1mL sterile salt and 1% Tween 20. After sedimentation (5 min), the supernatant was transferred to a sterile test-tube and then homogenized in a Vortex test tube shaker for 15 seconds, having its final concentration adjusted to  $5 \times 10^4$  Colony Forming Units (cfu) mL<sup>-1</sup>. This suspension was diluted to 1:50 in RPMI-1640, obtaining twice the necessary concentration to be added into each microplate well at the same volume as that of the chemical agent, according to standard protocol. To confirm the fungal inoculum concentration, the Pour-plate technique using Sabouraud dextrose agar Neogen Acumedia, Michigan/EUA and cfu count was performed.

### Chemical agents

The *in vitro* susceptibility test for *Aspergillus* spp. isolates against disinfectants and antiseptics such as chlorhexidine-cetrimide (Chemitec Agro-veterinária, São Paulo, Brazil), sodium hypochlorite (Chemitec Agro-veterinária, São Paulo, Brazil), benzalconium chloride (ammonium quaternary) (Indústria Anhembí S/A, São Paulo, Brazil) and a chlorophenol derivative (Colgate-Palmolive Indústria e Comércio LTDA, São Paulo, Brazil) was performed according to document M38-A2 of the Clinical and Laboratory Standards Institute (CLSI) adapted for chemical agents.

Six successive dilutions of the four chemical agents, namely SH (sodium hypochlorite), BC (benzalconium chloride), CC (chlorhexidine-cetrimide) and CP (chlorophenol derivative) were prepared in log<sub>2</sub> with RPMI-1640 broth (Sigma Chemical Co. Steinheim, Alemanha). The final concentrations of CB, CP and CC disinfectants corresponded respectively to 2; 1; 0.5; 0.25; 0.12 and 0.06 times the recommended concentration used by the manufacturer, and SH corresponded respectively to 4; 2; 1; 0.5; 0.25 and 0.12 times the recommended concentration.

### Microdilution test

To perform the broth microdilution technique, twelve sterile microplates (ninety-six wells) were filled with fungal inoculum added with previously diluted disinfectant/antiseptic, and adjusted to RPMI-1640 added with MOPS (Vetec Química Fina LTDA, Rio de Janeiro, Brazil). All samples were tested in duplicate against four disinfectants/antiseptics.

The first microplate column (column A) was used as growth control, and was filled with 200 µL inoculum, whereas the last column (column H) was used as sterile control and filled with 200 µL of the highest disinfectant dilution. The other columns (Column B to G) were filled with 100 µL inoculum and 100 µL chemical agent dilution sequentially from the highest to the lowest dilution of the product.

The microplates were incubated in a shaker incubator at 35 °C with constant shaking (40 cycles/min), and visual reading of the results was done after 72 hours.

The Minimum Inhibitory Concentration (MIC) was established as the highest chemical agent dilution capable of inhibiting fungal growth. The Minimum Fungicidal Concentration (MFC) was estimated by plating 10 µL from all wells and defined as the concentration which did not show apparent fungal growth on Petri dishes added with Sabouraud dextrose agar and chloramphenicol incubated at 35 °C for five days. Finally, the MIC and MFC capable of inhibiting 50% and 90% of the tested isolates were calculated.

## Results

The MIC and MFC obtained from four disinfectants and antiseptics against *Aspergillus* spp. isolates are shown in Table 1. The MFC from six isolates was not performed due to their growth in the most concentrated dilution of the chemical agent.

Chlorhexidine-cetrimide, benzalconium chloride and chlorophenol derivative were effective against all isolates at a lower MIC than that recommended by the manufacturer, except for an *A. flavus* strain, which was inhibited at the standard concentration for benzalconium chloride and chlorophenol derivative. Sodium hypochlorite was ineffective against three *A. fumigatus*, three *A. flavus* and one *A. niger* isolate at the growth concentration recommended by the manufacturer and the highest concentration tested.

The MIC and MFC of disinfectants capable of inhibiting 50% and 90% of the *Aspergillus* ssp. isolates from veterinary environment surfaces observed in this study are shown in Table 2.

## Discussion

Hospital environment disinfection is one of the main aspects in nosocomial disease prevention. Thus, the choice

**Table 1** - Minimum Inhibitory Concentration and Minimum Fungicidal Concentration of chlorexidine-cetrimide (CC), sodium hypochlorite (SH), chlorophenol derivative (CP) and benzalconium chloride (BC) against *Aspergillus* spp. isolates.

Isolates	CC (CI:66.7 $\mu\text{L mL}^{-1}$ )		SH (CI: 40 $\mu\text{L mL}^{-1}$ )		CP (CI: 30 $\mu\text{L mL}^{-1}$ )		BC (CI: 20 $\mu\text{L mL}^{-1}$ )	
	CIM	CFM	CIM	CFM	CIM	CFM	CIM	CFM
<i>A.flavus</i>	$\leq 4.2$	8.3	40	160	3.75	7.5	$\leq 1.25$	2.5
<i>A. flavus</i>	$\leq 4.2$	$\leq 4.2$	40	40	7.5	15	2.5	10
<i>A.flavus</i>	$\leq 4.2$	$\leq 4.2$	40	40	7.5	7.5	2.5	2.5
<i>A. flavus</i>	$\leq 4.2$	$\leq 4.2$	40	80	7.5	15	5	5
<i>A.flavus</i>	$\leq 4.2$	8.3	>160	-	15	30	2.5	2.5
<i>A. flavus</i>	$\leq 4.2$	$\leq 4.2$	80	80	7.5	7.5	2.5	2.5
<i>A.flavus</i>	$\leq 4.2$	$\leq 4.2$	>160	-	30	30	20	20
<i>A.fumigatus</i>	$\leq 4.2$	8.3	40	40	7.5	7.5	2.5	2.5
<i>A.fumigatus</i>	$\leq 4.2$	8.3	40	80	7.5	7.5	2.5	2.5
<i>A.fumigatus</i>	$\leq 4.2$	$\leq 4.2$	40	80	7.5	7.5	2.5	2.5
<i>A.fumigatus</i>	$\leq 4.2$	$\leq 4.2$	>160	-	7.5	7.5	2.5	2.5
<i>A.fumigatus</i>	$\leq 4.2$	$\leq 4.2$	40	40	3.75	7.5	2.5	5
<i>A.fumigatus</i>	$\leq 4.2$	$\leq 4.2$	>160	-	15	30	5	5
<i>A.fumigatus</i>	$\leq 4.2$	8.3	40	80	3.75	7.5	$\leq 1.25$	$\leq 1.25$
<i>A.fumigatus</i>	$\leq 4.2$	$\leq 4.2$	40	40	3.75	7.5	2.5	5
<i>A.fumigatus</i>	$\leq 4.2$	8.3	>160	-	7.5	7.5	2.5	2.5
<i>A.niger</i>	$\leq 4.2$	$\leq 4.2$	>160	-	15	30	2.5	2.5
<i>A.niger</i>	$\leq 4.2$	8.3	40	40	3.75	15	2.5	2.5

MIC: Minimum Inhibitory Concentration.

MFC: Minimum Fungicidal Concentration.

CI: Concentration indicated by the disinfectant manufacturer.

**Table 2** - Minimum Inhibitory Concentration and Minimum Fungicidal Concentration of the four disinfectants against *Aspergillus* spp. isolated from an isolates environment.

Disinfectants	MIC ( $\mu\text{L mL}^{-1}$ )		MFC( $\mu\text{L mL}^{-1}$ )	
	50%	90%	50%	90%
Benzalconium chloride	1.90	4.2	2.2	4.9
Chlorophenol derivative	5.3	11.3	7.5	18.2
Sodium hypochlorite	55	>160	72.9	>160
Chlorexidine-cetrimide	<4.2	<4.2	<4.2	7

MIC: Minimum Inhibitory Concentration.

MFC: Minimum Fungicidal Concentration.

of the most adequate product for this purpose is crucial to reduce the occurrence of opportunistic pathogens in these places (Medeiros *et al.*, 2009; Sherlock *et al.*, 2009).

Pedrini and Margatho (2003) tested different sodium hypochlorite dilutions against standard strains of clinical bacteria causing mastitis and found that the 0.5% dilution was not effective against these isolates. 1% benzalconium chloride was active only against gram positive bacteria, whereas 0.5% chlorexidine obtained the best results against the tested strains. Chlorexidine effectiveness against patho-

genic microorganisms such as *Staphylococcus* spp. and *Aspergillus* spp. has been described by other authors (Xavier *et al.*, 2007; Xavier *et al.*, 2008; Medeiros *et al.*, 2009). In agreement with these results, this study observed that sodium hypochlorite did not have a satisfactory action against the isolates tested, while chlorexidine-cetrimide showed efficacy against the same *Aspergillus* spp. isolates.

The need for regular assessment of the disinfectants used on dairy farms has been suggested due to the variations in the sensitivity and resistance profile of *Staphylococcus* genus bacteria to disinfectants, which can jeopardize disease control programs (Medeiros *et al.*, 2009).

*In vitro* sensitivity studies of fungal pathogens to disinfectants are scarce. Xavier *et al.* (2007) described chlorexidine and the benzalconium chloride efficacy against *Aspergillus* spp. isolated from a veterinary environment, as well as clinical cases of aspergillosis in penguins. Furthermore, they found that all isolates were iodine-resistant in the tested dilutions. Similarly, *Aspergillus* spp. isolates were sensitive to disinfectants in this study; however, six species were resistant to sodium hypochlorite and four were sensitive at the standard concentration, showing that this product should not be recommended for disinfection in the research site.

According to literature, *A. niger* is sensitive at a 200 µg mL<sup>-1</sup> chlorexidine and 100-200 µg mL<sup>-1</sup> benzalconium chloride concentration (McDonell and Russel, 1999); however, Xavier (2003) and Xavier *et al.* (2007) demonstrated that a 40 µg mL<sup>-1</sup> chlorexidine-cetrimide and a 2.5 µL mL<sup>-1</sup> ammonium quaternary derivative concentration sufficed to inhibit the growth of this species. Therefore, our results are in agreement with those in literature in relation to these two disinfectants. At lower concentrations, however, such as 4.2 µg mL<sup>-1</sup>, chlorexidine-cetrimide and the benzalconium chloride were found to inhibit the growth of this microorganism.

The chlorexidine-cetrimide MIC capable of inhibiting 90% of isolates was lower as compared to that of other disinfectants, whereas the SH MIC was higher, though the lowest MFC was obtained from BC. Thus, CC has an action against environment isolates and BC could be used as an alternative disinfectant by reducing costs and avoiding the emergence of resistant strains.

In some isolates, MFC differed from MIC for the tested disinfectants, demonstrating the importance of using the correct disinfectant dilution when applying this technique so as to eliminate pathogens in hospital environments more efficiently.

## Conclusion

By the the results obtained, it could be concluded that chlorexidine-cetrimide, benzalconium chloride and the chlorophenol derivative were efficient against *Aspergillus* spp. isolated from veterinary environments, whereas sodium hypochlorite showed lower efficiency.

## Acknowledgments

The authors would like to thank Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Coordenação de Aperfeiçoamento de Nível Superior (CAPES) and Fundação de Amparo a Pesquisa do Estado do Rio Grande do Sul (FAPERGS) for financing this research.

## References

CLSI - M38-A2 (2008) Reference Method for Dilution Antifungal Susceptibility Testing of filamentous fungi. Approved Standard - Second Edition. pp 281-35.

- Falvey D, Streifel A (2007) Ten-year air sample analysis of *Aspergillus* prevalence in a university hospital. *J Hosp Infect* 67:35-41.
- Latgé JP (1999) *Aspergillus fumigatus* and Aspergillosis. *Clin Microbiol Rev* 12:310-350.
- Martin GS, Mannino DM, Eaton S, Moss M (2003) The epidemiology of sepsis in the United States from 1979 through 2000. *N Engl J Med* 348:1546-1554.
- Mattei AS, Madrid I, Santin R, Coimbra H, Nobre M, Meireles M (2011) Isolamento de fungos filamentosos e gênero *Staphylococcus* em locais de atendimento veterinário. *A Hora Veterinária*. 180:19-22.
- McDonell G, Russel D (1999) Antiseptics and Disinfectants: Activity, Action, and Resistance. *Clin Microbiol Rev* 12:147-179.
- Medeiros ES, Santos MV, Pinheiro Júnior JW, Faria EB, Wanderley GG, Teles JÁ, Mota RA (2009) Avaliação *in vitro* da eficácia de desinfetantes comerciais utilizados no pré e pós-dipping frente amostras de *Staphylococcus* spp. isoladas de mastite bovina. *Pesqui Vet Bras* 29:71-75.
- Morris M, Kokki MH, Anderson K, Richardson MD (2000) Sampling of *Aspergillus* spores in air. *J Hosp Infect* 44:81-92.
- Pedrini SCB, Margatho LFF (2003) Sensibilidade de microrganismos patogênicos isolados de casos de mastite clínica em bovinos frente a diferentes tipos de desinfetantes. *Arq Inst Biol* 70:391-395.
- Santos L, Scalco Neto J, Rizzo N, Bastiani P, Rodrigues L, Ferreira D, Schwants N, Barcellos H, Brun M (2007) Avaliação dos procedimentos de limpeza, desinfecção e biossegurança no Hospital Veterinário da Universidade de Passo Fundo (HV-UPF). *Acta Sci Vet* 35:357-362.
- Sherlock O, O'Connell N, Creamer E, Humphreys H (2009) Is it really clean? An evaluation of the efficacy of four methods for determining hospital cleanliness. *J Hosp Infect* 72:140-146.
- Vonberg R, Gastmeier P (2006) Nosocomial aspergillosis in outbreak settings. *J Hosp Infect* 63:246-254.
- Xavier PCN (2003) Levantamento de contaminação fúngica em ambiente hospitalar e avaliação de eficiência do desinfetante à base de derivado de amônia quaternária. *O Mundo da Saúde* 27:579-588.
- Xavier MO, Madrid IM, Meinerz ARM, Cleff MB, Schuch LFD, Nobre MO, Meireles MCA (2007) Atividade "in vitro" de três agentes químicos frente a diferentes espécies de *Aspergillus*. *Arq Inst Biol* 74:49-53.
- Xavier MO, Meinerz ARM, Cleff MB, Osório LG, Schuch LFD, Nobre MO, Silva Filho RP, Meireles MCA (2008) Eficácia da clorexidina-cetrimida na desinfecção ambiental contra *Aspergillus* spp. *Arq Bras Med Vet Zootec* 60:873-877.

All the content of the journal, except where otherwise noted, is licensed under a Creative Commons License CC BY-NC.