ABSA INTERNATIONAL

Applied Biosafety:
Journal of ABSA International
2019, Vol. 24(1) 30-33

DOI: 10.1177/1535676018818560
journals.sagepub.com/home/apb
SAGE

Evaluation of Virex® II 256 and Virex® Tb as Disinfectants of the Dimorphic Fungi *Coccidioides immitis* and *Coccidioides posadasii*

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Abstract

Introduction: To date, limited published data exists regarding the efficacy of commonly used disinfectants in inactivating the Risk Group 3 dimorphic fungal pathogens, *Coccidioides immitis* and *Coccidioides posadasii*. Newer generation quaternary ammonium compounds, like Virex® II 256 and Virex® Tb, have not been previously evaluated.

Methods: Herein, these disinfectants are evaluated against 10% bleach and 70% ethanol, for their ability to inactivate 5×10^7 arthroconidial spores of *C immitis* RS or *C posadasii* strain Silveira within 2, 5, 10 or 20 minutes contact time in aqueous solution.

Results: Evidence is provided that both Virex® II 256 and Virex® Tb are highly effective alternatives to 10% bleach or 70% ethanol for the disinfection of 5×10^7 arthroconidia of *Coccidioides spp*. within 2 minutes of contact time.

Discussion: Both 70% ethanol and 10% bleach were seen as less effective than the other disinfectants, requiring a longer contact time to completely inactivate the same number of arthroconidia.

Conclusion: A contact time of > 2minutes is adequate for the disinfection of 5×10^7 *Coccidioides spp*. arthroconidia when using either the Virex® II 256 and Virex® Tb formulations.

Keywords

Coccidioides spp., Virex II 256, Virex Tb, dimorphic fungal pathogens, disinfectant, BSL-3

Institutions that perform biosafety level 3 (BSL-3) procedures or animal BSL-3 (ABSL-3) procedures frequently share communal areas and equipment that are used by multiple investigators. It is not uncommon for one BSL-3 or ABSL-3 facility to house viral, fungal, and bacterial organisms concurrently. The optimal disinfectants used in such facilities need to be carefully evaluated by institutional biosafety committees (IBCs). Ideal disinfectants would (a) be effective for all organisms used in the facility, (b) have a favorable safety profile, (c) be easy to use, (d) be economical, and (e) be chemically compatible with the composition of surfaces and other chemicals used in the facility.

Among the most commonly used disinfectant compounds for high-containment organisms, including select agents, are sodium hypochlorite (bleach), phenolics, quaternary ammonium compounds (quats), and alcohols. However, efficacy for killing/inactivation is known to vary by organism and must be assessed on a case-by-case basis. With regard to the dimorphic fungal pathogen *Coccidioides posadasii*, no study evaluating the efficacy of common disinfectants has been published in over 50 years, and only 1 study evaluating *Coccidioides immitis* has been published within the same time period.[1] The published study evaluated 10% bleach, 70% ethanol, and a phenolic compound, Vesphene IIse, against the highly infectious spore form of the fungus. These authors found that 10% bleach and 70% ethanol were effective in providing a 7-log^{10} reduction in fungal arthroconidia of *C immitis* strain 2009 in less than 1 minute, whereas Vesphene IIse was less effective, providing only a 6-log^{10} reduction of arthroconidia within a longer contact time of 5 minutes. The authors recommended the use of 10% bleach or 70% ethanol with a contact time of 1 to 2 minutes for normal laboratory procedures or longer in the event of a biological spill.

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Although a 10% bleach solution is generally accepted as the gold standard for inactivation of infectious agents, it carries a number of safety warnings and health concerns. Stock bleach is considered a category 1 hazardous chemical by the 2012 Occupational Safety and Health Administration (OSHA) Hazard Communication Standard (29 CFR 1910.1200).[2] Even when diluted to 10%, bleach is highly corrosive, not only to metals and some plastics but also to human tissues such as eyes, skin, and mucus membranes. In addition, repeated inhalation of vapors is associated with an increased risk of chronic lung diseases such as adult-onset nonallergic asthma and chronic obstructive pulmonary disease.[3,4] Finally, when mixed with acids or other commonly used cleaners, it can release chloroform or chloramine gas. Since the purpose of biosafety regulations is the minimization of risk and to provide a safe environment for working with biological pathogens, alternatives to bleach should be evaluated. Although 70% ethanol was also found to be effective in the previously published study, the utility of ethanol as a primary disinfectant is not practical. It is an effective secondary disinfectant, useful to wipe down residues left by other disinfectants. As ethanol evaporates quickly, a continuous contact time of >2 minutes required to inactivate fungal arthroconidia would require an impractically voluminous amount of ethanol if it were to be used as a primary disinfectant.

Quaternary ammonium compounds have broad-spectrum efficacy against a number of organisms and are considered to have a better user health safety profile than bleach. They are widely used in laboratories and hospitals as disinfectants and floor and surface cleaners. Two of these "quats," a 1-step Virex II 256 formulation and a ready-to-use formulation, Virex Tb, are Environmental Protection Agency (EPA) registered as bactericidal, virucidal, and fungicidal. Importantly, they are not classified as hazardous when diluted to the specified working dilution. They have a relatively neutral pH of 8.8, are not corrosive to metals, and have an odor that is well tolerated. However, quats have been implicated in the triggering of asthma symptoms and contact dermatitis in some people.[5] In addition, some quats can cause degradation of certain plastics and rubber.

The only previous publication evaluating quaternary ammonium compounds against *Coccidioides* spp. was published in 1964.[6] This study evaluated a first-generation quat, benzalkonium chloride (n-alkyl [50% C12, 30% C14, 17% C16, 3% C18] dimethyl benzyl ammonium chloride), in parallel to phenolics, hypochlorite, formaldehyde, and peracetic acid, against an undisclosed number of arthroconidia. This quat required a contact time of at least 7 minutes against *C immitis* arthroconidia, and it was determined that *C immitis* arthroconidia were more resistant than arthroconidia from 2 other pneumonia-causing dimorphic fungi, *Histoplasma capsulatum* and *Blastomyces dermatitidis*. A modern evaluation of currently used third- and fourth-generation quat formulations against a precise number of *Coccidioides* spp. arthroconidia is thus warranted.

The purpose of this study was to investigate whether the quaternary ammonium compounds Virex II 256 and Virex Tb are efficacious alternatives to the previously published 10% bleach or 70% ethanol for the inactivation of 5×10^7 *Coccidioides* sp. arthroconidia within 2, 5, 10, or 20 minutes of contact time in an aqueous solution of disinfectant.

Methods

Arthroconidia were harvested from *C immitis* strain RS and *C posadasii* strain Silveira using a modified spin bar harvest procedure.[7] Briefly, the strain was cross-streaked onto 2× glucose yeast extract (GYE) agar plates and wrapped in 3 M venting tape 394 (3 M, Maplewood, Minnesota) for 4 days at 30°C, prior to incubating for a subsequent 5 weeks at room temperature. Arthroconidia were then aseptically harvested by pouring 10 mL of 0.9% sterile saline into the center of each plate and slowly disrupting the mycelial lawn with a sterile 12.7 × 3-mm micro stir bar (VWR, Radnor, Pennsylvania) spinning on top of a magnetic stir plate. This arthroconidial suspension was extracted from the plate using a 23-gauge blunt needle and syringe (VWR), passaged twice through a Falcon 40-µm cell strainer (Fisher, Hampton, New Hampshire) to remove mycelial fragments, and washed twice in sterile saline with centrifugation for 5 minutes in gasket-sealed rotors at 3000 rpm in a Thermo Forma Multi RF tabletop centrifuge (Thermo Fisher Scientific, Waltham, Massachusetts). An aliquot of arthroconidia was fixed in 10% formalin for 30 minutes prior to microscopic cell counting in duplicate. Tenfold dilutions of the arthroconidial harvest were then plated onto fresh 2× GYE agar and incubated for 3 days at 30°C to confirm spore counts determined by microscopy and assess the number of viable colony-forming units (CFU).

Harvested arthroconidia were adjusted to 5×10^7/mL viable *C posadasii* strain Silveira arthroconidia in 0.9% sterile saline, and this batch was used for all subsequent experiments. For each testing condition, 5×10^7 arthroconidia were put into 1.5-mL Eppendorf tubes (VWR) and centrifuged at 4000 rpm for 1 minute. Supernatants were carefully removed by pipetting prior to adding 1 mL of the respective disinfectants or mock disinfectant (0.9% sterile saline) for the noted contact times. The disinfectants used in this study were 10% bleach diluted to 0.8% sodium hypochlorite (Germicidal Bleach; The Clorox Company, Oakland, California), 70% ethanol (SANIHOL70; Decon Labs, King of Prussia, Pennsylvania), Virex II 256 (Diversey, Sturtevant, Wisconsin), and Virex Tb (Diversey). The 70% ethanol and Virex Tb disinfectants were purchased as ready-to-use formulations, whereas fresh volumetric dilutions of 10% bleach (1 part bleach:9 parts water) were made within 1 hour of use within sterile 50-mL conical tubes with tap water. Similarly, fresh preparations of Virex II 256 were made in accordance with the manufacturer's instructions.

Disinfectants were added to the arthroconidial pellets and briefly resuspended by vortexing prior to centrifugation at 4000 rpm for 1 minute and careful removal of the supernatant by pipetting. Pellets were washed once with 1 mL of sterile saline by inversion and centrifuged again. Disinfectant contact times were defined as the moment that the disinfectant was added to the initial arthroconidial pellet until the moment 1 mL of wash saline was added. Contact times of 2, 5, 10, and 20 minutes were tested in quintuplicate experiments performed on separate

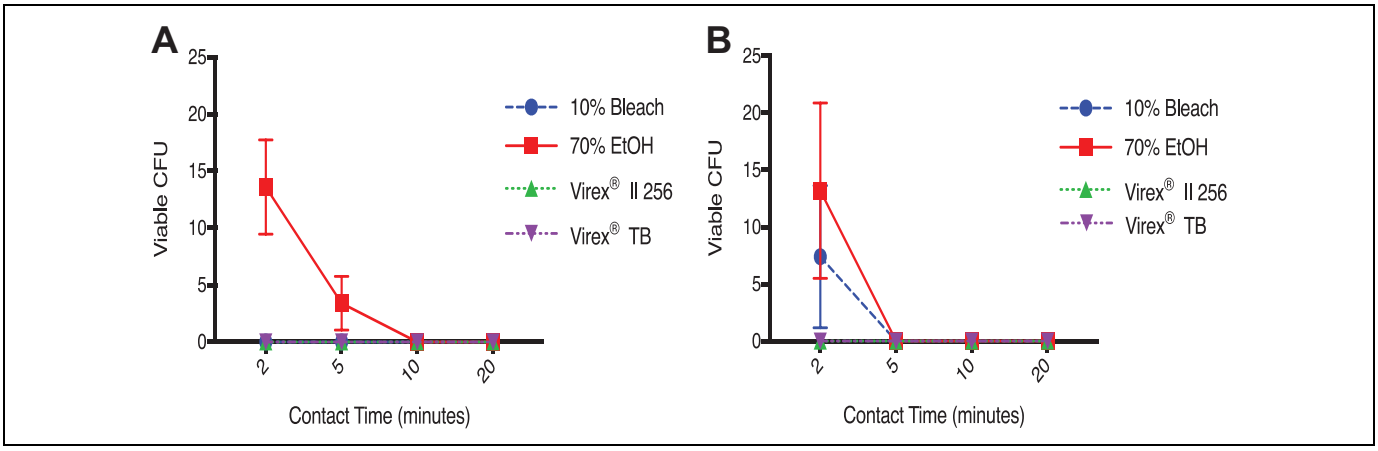

Figure 1. Efficacy of disinfectants in inactivating 5×10^7 coccidioidal arthroconidia. Viable colony-forming units (CFU) of *Coccidioides immitis* strain RS (A) or *Coccidioides posadasii* strain Silveira (B) after a contact time of either 2, 5, 10, or 20 minutes with the various disinfectants are shown. Values are presented as the mean ± standard error of the mean.

days to ensure repeatability. The washed pellet was brought up to 100 µL in fresh 0.9% sterile saline and plated in its entirety onto fresh 2× GYE agar plates. The mock disinfected samples were treated in the same manner as the other disinfectant samples but were 10-fold diluted prior to plating. All culture plates were incubated at room temperature for 7 days prior to initial colony counting and left for a further 3 weeks prior to disposal to ensure no late breakthrough growth.

Results

As seen in Figure 1A, Virex II 256, Virex Tb, and 10% bleach were effective in complete inactivation of *C immitis* RS arthroconidia within 2 minutes of contact time. However, 70% ethanol disinfection was only able to achieve a 6-$\log_{10}$ reduction in viable arthroconidia within contact times of either 2 or 5 minutes. A mean of 13.6 viable CFU (range, 0-23) after 2 minutes and a mean of 3.4 viable CFU (range, 0-12) after 5 minutes of contact time with 70% ethanol were seen.

As seen in Figure 1B, Virex II 256 and Virex Tb were effective in complete inactivation of *C posadasii* strain Silveira arthroconidia within 2 minutes of contact time. However, both 70% ethanol and 10% bleach were only able to achieve a 6-$\log_{10}$ reduction in *C posadasii* arthroconidia within 2 minutes of contact time. For 70% ethanol, there was a mean of 13.2 viable CFU (range, 0-42) after 2 minutes of contact time, and for 10% bleach, there was a mean of 7.4 viable CFU (range, 0-32). All disinfectants were able to inactivate all *C posadasii* strain Silveira arthroconidia within 5 minutes.

Mock disinfected samples treated with 0.9% saline for 20 minutes recovered 92% to 88% of the original inoculum, recovering an average of 4.5×10^7 viable arthroconidia.

Discussion

Our data suggest that Virex II 256 and Virex Tb are effective disinfectants for the inactivation of 4.5×10^7 viable *Coccidioides* spp. arthroconidia within 2 minutes of contact time. A fresh solution of 10% bleach did not completely inactivate all coccidioidal arthroconidia within 2 minutes, as it required 5 minutes to inactivate all *C posadasii* strain Silveira arthroconidia. Likewise, 70% ethanol did not inactivate all coccidioidal arthroconidia, requiring a contact time of 10 minutes to inactivate all *C immitis* RS and 5 minutes to inactivate all *C posadasii* strain Silveira arthroconidia. These data suggest that 70% ethanol is less effective in disinfecting *C immitis* RS, and both 10% bleach and 70% ethanol are less effective in disinfecting *C posadasii* strain Silveira arthroconidia than the Virex formulations tested herein.

Several attempts were made to perform 1-minute disinfectant evaluations in this study, but we were unable to safely and accurately perform the procedures within precisely 1 minute due to technical constraints of our centrifuge. These results differ from the Vogler et al[1] study, which found that 10% bleach and 70% ethanol were equally as effective in inactivating 1×10^7 arthroconidia within a 1-minute contact time. It is unlikely that the different bleach formulations used between the 2 studies brought about these differences, as the formulation used by Vogler et al[1] includes 6% sodium hypochlorite, whereas our study used an 8.3% sodium hypochlorite formulation. The most likely explanation for the differing results of 10% bleach efficacy is that our study evaluated the disinfectants against a 5-fold higher concentration of arthroconidia. It is also possible that the differences in results are due to the difference in *Coccidioides* sp. strains between the 2 studies. Our study used the *C immitis* strain RS and *C posadasii* strain Silveira, whereas Vogler et al[1] used *C immitis* strain 2009. The potential for the same disinfectants to have different inactivation efficacies dependent on strain, harvesting condition, or storage condition is interesting and warrants further investigation.

When working with microbial pathogens, effective disinfectants are crucial components of workplace safety. *Coccidioides* spp. require BSL-3 laboratory conditions, where personal protective equipment (PPE) like Tyvek suits (DuPont, Wilmington,

Delaware) and powered air-purifying respirators (PAPRs) or disposable N-95 respirators are often worn. The filtering capacity of PAPR and N-95 respirators is excellent at filtering infectious agents and particulates. However, gas molecules like chlorine, hydrogen sulfide, chloramine, chloroform, and ammonia can all pass freely in the spaces between the fibers in N-95 or HEPA-only PAPR respirators. Therefore, vapors from chemicals sprayed onto PPE to disinfect them prior to removal are continually inhaled by the user. The infectious dose of coccidioidal arthroconidia is thought to be as low as a single arthroconidium,[8] and aerosolization of stray arthroconidia could occur during removal of PPE. Thus, prior to doffing PPE, it must be sprayed with an effective disinfectant for the appropriate contact time.

Bleach is a cheap and effective disinfectant when used at the sufficient contact time but has been associated with chemical irritation and chronic lung disease. Frequent use of bleach is associated with nonallergic adult-onset asthma, elevated neutrophil counts, and lower airway symptoms in women.[4] In addition, in a study using job-task-exposure matrix estimates (JTEM) to quantify effects from cumulative exposure to commonly used disinfectants, formaldehyde, glutaraldehyde, bleach, hydrogen peroxide, and enzymatic cleaners were associated with poor asthma control (all $P_{\text{trend}} < .05$), whereas exposure to quats and alcohol was not.[9] Data from our study indicate that decontamination using 10% bleach would require $\geq$5 minutes of continual contact time to inactivate 5×10^7 arthroconidia. This extended time is not optimal for direct application onto metals, as it will accelerate the degradation of these materials. In addition, if fumes are continually inhaled by the user during the contact time period, this can be particularly problematic for persons with prior lung sensitivity or asthma.

Based on the findings reported here, we recommend a minimum of 2 minutes of contact time with Virex II 256 or Virex TB as a preferred disinfection strategy for any otherwise clean surface potentially contaminated with of coccidioidal arthroconidia, such as decontamination of PPE prior to doffing.

Conclusion

To avoid the inhalational hazards and corrosion of laboratory materials by bleach, alternative disinfectants are needed. Two such options are Virex II 256 and Virex Tb, as a contact time of $\geq$2 minutes is adequate for the disinfection of 4.5×10^7 viable *Coccidioides* spp. arthroconidia when using both the Virex II 256 and Virex Tb formulations.

Acknowledgments

We thank the guidance, diligence, and experience of the Biosafety Committee and the Environmental Health and Safety Department at Arizona State University. We also thank Bridget Barker and Heather Mead from the Northern Arizona University for the *Coccidioides* strains used in this study.

Ethical Statement

Not applicable.

Statement of Human rights

Not applicable.

Statements of Informed Consent

Not applicable.

Declaration of Conflicting Interests

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Funding

The authors disclosed receipt of the following financial support for the research, authorship, and/or publication of this article: Arizona Biomedical Research Council (ABRC) grant 16-162513 to TEG and DFL; American Achievement Rewards for College Scientists (ARCS) Foundation Award to NMM.

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