Published in final edited form as:

Appl Biosaf. 2015 ; 20(3): 154–158.

Effective Disinfectants for *Coccidioides immitis* and *C. posadasii*

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Abstract

The lack of published data on effective disinfectants and contact times for use on the fungal pathogens *Coccidioides immitis* and *C. posadasii* prompted the authors to investigate the fungicidal activity of three commonly used laboratory disinfectants on arthroconidia harvested from *C. immitis* strain 2009. They tested the ability of 10% bleach, 70% ethanol, and Vesphene® IIse to inactivate 10^7 arthroconidia in an aqueous suspension within 1, 2, 5, 10, or 20 minutes of contact time. Both 10% bleach and 70% ethanol provided a 7-log¹⁰ reduction in arthroconidia in less than 1 minute, with no growth observed at any of the tested time points. Vesphene® IIse was less effective, providing a 6-log¹⁰ reduction in arthroconidia after 5 minutes, but was unable to completely inactivate all of the arthroconidia, even after 20 minutes of contact time.

Keywords

Coccidioides immitis; *Coccidioides posadasii*; Disinfectant; Fungicide; Coccidioidomycosis; Valley Fever

Introduction

Most published research on effective fungicides has focused on the food industry due to the needs of that industry to increase shelf life and decrease loss due to spoilage caused by various contaminating fungi. Other fungicide research has centered on the use of disinfectants in clinical settings and the need for general-use disinfectants with broad-spectrum activity, including fungicidal activity, for use on various surfaces. Thus, tested fungal agents have tended to be various food industry important fungi (Bundgaard-Nielsen & Nielsen, 1996) or a few representative fungi that may be of concern in clinical settings (e.g., opportunistic fungal pathogens) (Gupta et al., 2001, 2002). Unfortunately, fungicides identified for use in either of these venues do not necessarily directly translate to the disinfectant needs of a research laboratory conducting fungal research. For example, simply delaying or slowing fungal growth may be sufficient for food industry fungicide needs, and most clinical setting fungicide use is going to be targeted at a general all-purpose type disinfectant that may or may not be effective against all of the potentially contaminating fungi. Indeed, fungi vary widely in their susceptibility to various disinfectants not only by species but also by life-cycle stage within a species. Arthroconidia, for example, are very resistant to adverse environmental conditions and, by extension, to disinfectants. Thus, a

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research laboratory conducting fungal research is going to prefer to use a disinfectant with known fungicidal activity against the fungal pathogens and life-cycle stages with which it is working. This becomes even more important when working with higher-risk fungal pathogens in high-containment. Among the fungal pathogens, few are recognized as requiring BSL-3 containment (CDC & NIH, 2007).

Among these are the etiological agents of coccidioidomycosis (also known as valley fever), *Coccidioides immitis*, and *C. posadasii*. These closely related species are endemic to certain arid and semi-arid regions of the Western Hemisphere, including the southwestern United States extending into Mexico and other isolated pockets in Central and South America, with *C. immitis* being restricted to central and southern California and *C. posadasii* occupying the rest of the geographic range (Nguyen et al., 2013). They are the only fungal agents that were ever included among the U.S. Department of Health and Human Services (HHS) and Overlap Select Agents considered important to human health (U.S. HHS, 2005), although they were later removed from the Select Agent list in 2012 (U.S. HHS, 2012). The risk group 3 status of these agents and the related recommendation for BSL-3 containment are due to the aerosol transmission risk of the arthroconidia produced by these dimorphic saprophytic fungi. These arthroconidia are resistant to adverse environmental conditions, remaining viable for years in laboratory storage, dust, and soil. Other growth stages of *Coccidioides* spp. include mycelia, which grow saprotrophically in the environment and produce fresh arthroconidia, and spherules, which are specialized structures unique to *Coccidioides* spp. These structures develop from arthroconidia inhaled by susceptible mammalian hosts in the parasitic phase of the life cycle. These spherules develop over a 4- to 5-day period, expanding in size up to 100 µm in diameter and then rupturing, releasing 100 to 300 endospores that can disperse in the host and produce new spherules (Lewis et al., 2015; Nguyen et al., 2013).

Despite the importance of these pathogens, almost no information exists on effective disinfectants for use with these species. The Public Health Agency of Canada has published a series of pathogen safety data sheets (PSDS), including one for *Coccidioides* spp., that are widely regarded as sources of general biosafety knowledge on various pathogenic agents (PHAC, 2010). The *Coccidioides* spp. PSDS indicates that *Coccidioides* spp. are susceptible to a 1:10 dilution of bleach, 6% hydrogen peroxide, 8% formaldehyde, and 3% phenolics with a contact time of 20 minutes or more (PHAC, 2010). However, this report is based on a 2009 expert opinion article entitled, “What to Do When There Is *Coccidioides* Exposure in a Laboratory” (Stevens et al., 2009) that presents these disinfectants as “cleaning agents [that] have been used” for cleaning laboratory surfaces. However, the effectiveness of these cleaning agents specifically to inactivate *C. immitis* or *C. posadasii* does not appear to have been documented by either Stevens et al. or by any references reported by them (Stevens et al., 2009). Furthermore, the 20 minute contact time suggested in this article appears to be a generalized contact time with additional generalized advice to extend exposure to the cleaning agent to 6–10 hours to achieve “sterilization and the killing of spores,” again without documented evidence that this treatment would be effective against *Coccidioides* spp. (Stevens et al., 2009). Two older publications (Kruse et al., 1963, 1964) investigated the disinfection of laboratory surfaces exposed to aerosolized “tissue” (i.e., spherule) (Kruse et

al., 1963) and “culture” (i.e., arthroconidia) (Kruse et al., 1964) phases of four and three, respectively, different pathogenic fungi, including *C. immitis*. These publications suggest that a number of disinfectants are effective against *Coccidioides* spp., given sufficient concentration and contact time, including ethanol, sodium hypochlorite, formaldehyde, phenolics, peracetic acid, an iodophor, and a quaternary ammonium compound. However, the unreported fungal concentration and nature of the fungal suspensions deposited on the test surfaces, along with the data presentation style, make these publications difficult to interpret, beyond clearly illustrating the greater susceptibility of the tissue phase as compared to the culture phase to all of the tested disinfectants (Kruse et al., 1963, 1964).

In this article the authors investigate the fungicidal activity of three commonly used laboratory disinfectants against arthroconidia derived from *Coccidioides immitis*.

Materials and Methods

Arthroconidia were harvested from *C. immitis* strain 2009 for use in the disinfectant efficacy tests using a modified arthroconidia harvest procedure. Specifically, *C. immitis* strain 2009 was grown for 6 weeks at 28°C on 2X glucose yeast extract (GYE) agar (pH 6.5) in four 75 cm² tissue culture flasks with 0.22 µm hydrophobic, vented, filtered caps (Genesee Scientific, San Diego, CA). After 6 weeks, 15 ml of phosphate buffered saline (PBS) was added to each tissue culture flask and the surface of the agar was gently scraped using a cell scraper to suspend the culture material in the PBS. The suspended culture material was then poured through a sterile funnel lined with a sterile Miracloth filter (EMD Millipore, Billerica, MA) into a 50 ml conical centrifuge tube to separate the arthroconidia from the mycelia culture material. A 100 µl aliquot of the resulting arthroconidia suspension was removed and fixed by adding 900 µl of 10% formalin and incubating overnight at 28°C. The fixed suspension was then pelleted by centrifugation, the fixative removed, the pellet washed using 1 ml PBS and repelleted by centrifugation, the wash solution removed, and the pellet resuspended in 100 µl fresh PBS. The resuspended fixed arthroconidia were then quantified by microscopy and the resultant total counts confirmed by viable counts. Specifically, 100 µl aliquots of appropriate dilutions from a 10-fold serial dilution of the unfixed arthroconidia suspension were plated on 2X GYE agar (pH 6.5) plates in triplicate, incubated at 28°C for 3 days, and the CFUs counted and used to confirm the total counts obtained via microscopy.

The purified arthroconidia from *C. immitis* strain 2009 were used to test the effectiveness of 10% bleach, 70% ethanol, and Vesphene® IIse (STERIS® Corporation, Mentor, OH) as disinfectants for *Coccidioides* spp. A starting stock of 10⁸ arthroconidia was diluted using PBS to give a total of 10⁷ arthroconidia for each disinfectant test and then dispensed into 2 ml screw-cap tubes. Volumes were adjusted such that, when each test suspension was mixed with the tested disinfectant, the final volume would be equal to 1 ml (Table 1). Disinfectant was added to each test suspension, briefly vortexed to mix, and then spun down using a microcentrifuge (USA Scientific item number 2631-0006, Ocala, FL). Spin times were appropriately delayed and/or adjusted to ensure that each disinfectant was in contact with the test suspensions for only the specified tested contact time (i.e., contact times include spin steps). The supernatant was then removed and each pellet resuspended in 1 ml PBS to wash

the pellet. Each test suspension was then spun again using the microcentrifuge, the supernatant removed, and the washed pellet resuspended in 100 μ l PBS. The entire suspension was then plated onto a 2X GYE agar (pH 6.5) plate. Contact times of 1, 2, 5, and 10 minutes were tested in triplicate for each disinfectant, with an additional 20-minute time point tested for Vesphene® IIse. The agar plates were incubated at 28°C for 15 days and checked for growth every 3 days. The highest colony count observed over this 15-day period was recorded as CFUs for each agar plate. A positive control consisting of a 1 ml suspension of 10^7 arthroconidia was similarly processed in triplicate, as above, except that no disinfectant was added. A 10-fold serial dilution of each positive control was made and 100 μ l of appropriate dilutions plated on 2X GYE agar (pH 6.5) plates in triplicate. The viable count plates were incubated at 28°C for 3 days and the CFUs counted on the third day. These viable counts verified that 80% to 95% of the starting arthroconidia concentration could be successfully recovered following the centrifugation and wash steps, validating the use of this procedure for the disinfectant efficacy tests.

Results

Both 10% bleach and 70% ethanol were highly effective at inactivating the test suspensions of arthroconidia, with no growth observed at any of the tested time points, suggesting that a 7- $\log_{10}$ reduction in arthroconidia occurred in less than 1 minute. The Vesphene® IIse solution was less effective, with overgrown plates observed after 1 and 2 minutes of contact time. The Vesphene® IIse solution was able to achieve a 6- $\log_{10}$ reduction in arthroconidia after 5 minutes of contact time. However, based upon the observed CFUs on some of the 10- and 20-minute contact time plates, >20 minutes of contact time would be required to achieve inactivation of all arthroconidia (Table 2).

Discussion

These data strongly suggest that both 10% bleach and 70% ethanol are effective disinfectants for inactivation of *Coccidioides* spp. arthroconidia. The Vesphene® IIse solution was also effective, but required a longer contact time (Table 2). These findings are consistent with previous findings by Kruse et al. in that they also found sodium hypochlorite, ethanol, and various phenolic compounds to be effective at inactivating the tissue and culture phases of *C. immitis*, given sufficient concentration and contact time (Kruse et al., 1963, 1964). However, the contact times required for inactivation that the authors observed differed significantly from those observed by Kruse et al. Specifically, the inactivation curves presented by Kruse et al. suggest that contact times of at least 17.5 minutes and 22.5 minutes were required for inactivation of the culture phase (i.e., arthroconidia) of *C. immitis* strain Silveira using 10% bleach and 70% ethanol, respectively. Similarly, Kruse et al. found that contact times of 8 minutes, 1.5 minutes, 5.5 minutes, and 17.5 minutes were required to inactivate the culture phase of *C. immitis* strain Silveira using 10% concentrations of phenol, a cresylic product (*o*-phenylphenol) containing soap and alcohol, phenolic “A” (containing *o*-benzyl *p*-chlorophenol, *p*-tertiary amyl phenol, and *o*-phenylphenol), and phenolic “B” (containing *o*-benzyl *p*-chlorophenolate and potassium ricenoleate), respectively (Kruse et al., 1964). Undiluted Vesphene® IIse contains the active ingredients *o*-phenylphenol (9.09% w/v) and *p*-tertiary amylphenol (7.66% w/v) (STERIS®

Corporation, 2015), making the concentration of active phenolic compounds in the Vesphene® IIse solution tested here approximately 0.13%, which is far lower than the 10% phenolic concentrations reported above. The lowest phenolic disinfectant concentration tested by Kruse et al. was 1%, at which none of the tested phenolic compounds were able to inactivate the culture phase of *C. immitis* within 30 minutes of contact time (Kruse et al., 1964). The Vesphene® IIse solution tested here, although almost 10-fold more dilute than this, was able to achieve a 6-log₁₀ reduction in CFUs by 5–10 minutes of contact time, although the amount of time necessary for complete inactivation was undetermined (Table 2). These results are consistent with product information produced by STERIS® Corporation, which indicates that a 1:128 dilution (i.e., ~0.78%) of Vesphene® IIse passes the A.O.A.C. Use-Dilution Test against the fungal species *Trichophyton mentagrophytes*, *Candida albicans*, and *C. parapsilosis* in the presence of 5% organic soil (serum) in 10 minutes at 20°C (STERIS® Corporation, 2015).

Several factors can affect the ability of a disinfectant to inactivate microorganisms, including, number and location of the microorganisms, innate resistance of the microorganisms, concentration and potency of the disinfectant, physical and chemical factors, and organic and inorganic matter (Rutala et al., 2008). Experimental procedure differences leading to potential differences in several of these factors may have influenced the contact time differences observed between the experiment presented here and the experiments reported by Kruse et al. (Kruse et al., 1963, 1964). In the Kruse et al. disinfectant tests, the test material was aerosolized onto 1 in² test surfaces made of a variety of materials, including painted wood, glass, stainless steel, neoprene, and asphalt floor tile. They then immersed these test surfaces into the tested disinfectants for the tested contact times, removed them from the disinfectant, immersed them in a neutralizing buffer, swabbed them, and dissolved the swabs in an appropriate buffer. They then tested for surviving fungi by incubating plated samples from the buffer used to dissolve the swabs, plated filters through which they had filtered the neutralizing buffer (to recover any viable fungi that had been washed off during the neutralization step), and the swabbed test surfaces immersed in appropriate broth (to recover any viable fungi that remained on the test surfaces after they were swabbed). Graphs plotting the inactivation time in minutes against concentration of the tested disinfectant were then produced for each fungus × disinfectant combination (Kruse et al., 1963, 1964). No information on the concentration of fungi impregnated on the test surfaces was provided, and, if much higher than the concentrations tested here, could have extended the required inactivation times. Also, in the case of the culture phase tests conducted by Kruse et al. (Kruse et al., 1964), dried arthroconidia were aerosolized onto the test surfaces by bursting a gelatin capsule into which the arthroconidia had been placed. The resulting presence of gelatin with the arthroconidia, as well as the use of dried material on a test surface instead of the aqueous arthroconidia suspension tested here, also could have extended the required inactivation times. The different test surfaces could also have been a factor and, in fact, were reported by Kruse et al. to affect the inactivation times required for inactivating the tissue phase (Kruse et al., 1963), although not the culture phase of the tested fungi (Kruse et al., 1964). As for disinfectant concentration and potency, the authors have attempted to compare results for the same concentrations of disinfectants. However, this was not possible for the Vesphene® IIse solution, which did not have a direct equivalent in the

tests conducted by Kruse et al. Similarly, the 10% bleach test results may not be fully comparable either, since the 10% bleach used here and by Kruse et al. was prepared volumetrically and not according to active ingredient concentration, which may vary by manufacturer. Related to this, the pH of the 10% bleach solutions tested could also have differed and may have affected the required inactivation times since an increase in pH is known to decrease the antimicrobial activity of hypochlorites (Rutala et al., 2008).

In conclusion, these data strongly suggest that both 10% bleach and 70% ethanol are effective disinfectants for use against *Coccidioides* spp. In addition, these results suggest that, for normal laboratory procedures, a contact time of 1–2 minutes is probably acceptable when using these disinfectants. Longer contact times should be considered in the event of a biological spill or when disinfecting more resistant materials (e.g., porous surfaces, etc.), and should especially be considered in the case of biological spills involving dry arthroconidia that would be associated with months-old solid agar cultures.

Acknowledgments

Disclosure

This project was partially funded by an NIH/NIAID K22 AI104801 grant, an Arizona Biomedical Research Corporation young investigator grant, and TGen startup funds to Bridget Barker.

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Table 1

Arthroconidia test suspension and added disinfectant volumes.

Tested Disinfectant	Suspension Volume ^b	Volume Disinfectant Added	Final Disinfectant Concentration
Bleach ^a	900 µl	100 µl undiluted household bleach	10%
Ethanol	260 µl	740 µl 95% ethanol	70%
Vesphene® Ilse	921.9 µl	78.1 µl 10% Vesphene® Ilse	1:128 ^c

^a Obtained from WAXIE Sanitary Supply® with manufacturer's indicated concentration of 6% sodium hypochlorite.

^b Each suspension contained 1×10^7 arthroconidia total.

^c Final dilution of 1:128 (equivalent to 0.781%) prepared according to manufacturer's directions.

Table 2

Fungicidal activity against *Coccidioides* spp.

Tested Disinfectant	1 min ^b	2 min ^b	5 min	10 min	20 min ^c
10% Bleach	0	0	0	0	-
	0	0	0	0	-
	0	0	0	0	-
70% Ethanol	0	0	0	0	-
	0	0	0	0	-
	0	0	0	0	-
Vesphene® IIs ^e ^a	TNTC	TNTC	1	0	0
	TNTC	TNTC	0	3	1
	TNTC	TNTC	0	0	3

^a Prepared according to manufacturer's directions at a 1:128 (i.e., 0.781%) dilution.

^b TNTC: too numerous to count.

^c -: Time point not tested.