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Control Strategies For Fungal Contamination In Cleanrooms

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Pharmaceutical, biotech, and medical device cleanrooms today are faced with the increasing prevalence of molds that can lead to environmental monitoring excursions. Over the years, we have noticed mold issues associated with cleanrooms, cold rooms, door kick plates, incubators, and cleanroom startups. Molds, such as Aspergillus, have come from many sources in the cleanroom, such as bags, boxes, markers, intervention equipment, and cart wheels. These occurrences seem to be more prevalent. Aspergillus, Penicillium, Trychophyton, and other molds have, in some cases, caused significant microbial excursion issues that have resulted in adverse impacts on production.

This article will provide an overview of the different types of fungi, killing mechanisms of several of the germicides commonly available, selection criteria for disinfectants, test methods, and recommendations for reducing the likelihood of environmental excursions due to molds.

FIELD OBSERVATIONS

In the past year, we have consulted for several companies where Aspergillus,Penicillium, or Trychophy-ton outbreaks have resulted in environmental monitoring data excursions exceeding alert and action limits. One company used a high impingement application device for applying germicides, which exposed the sheet rock and surfaces behind the wall, leading to endemic mold issues.Another company experienced cleanroom startup delays after a construction shutdown due to the presence of Strepto-myces spores even after triple-cleaning of the facility. The facility required additional cleaning and monitoring eventsto establish a control state for routine operation.

Two other pharmaceutical companies set limits for zero mold and zero yeast; both of these companies developed outbreaks that made their limits impossible to maintain. Both had to go back and establish limits for molds and yeast in their operations. Molds, such as Aspergillus niger, have also been problematic in cold rooms where pharmaceutical and biotech companies store raw materials for theirclean-room operations.

One company reported an instance in which Aspergillus was actually growing in the tip of a marker brought into the cleanroom.[1] In another case, a company reported an incident where Trychophyton was brought into a cleanroom by means of a severe case of athlete's foot infection. Since contamination can occur from the most unexpected sources and on any surface in a cleanroom, it is important to have a thorough solution for addressingmold issues and to have germicidal products that actually kill mold spores.

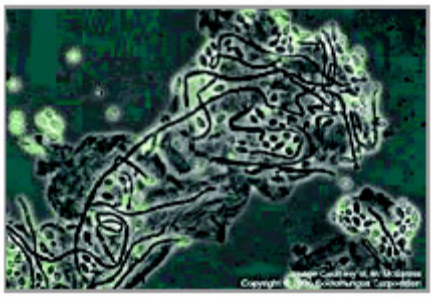

Figure 1: *Candida albicans*

FUNGAL CHARACTERISTICS

Fungi are a large and diverse group of eukaryotic microorganisms. There are approximately 100,000 species of fungi, about 200 of which are pathogens. The two groups of fungi that have practical importance in the cleanroom aremolds and yeasts.

Generally, fungi can be differentiated easily into these two types based on the macroscopic appearance of their colonies. Those that produce opaque, creamy, or pasty colonies are called yeasts and those that produce cottony, woolly, fluffy, or powdery aerial growths above the culture medium are called molds. However, this division between yeasts and molds based on growth morphology is not clear-cut, since some yeasts can produce hyphae under specific conditions (e.g., Candida albicans), while many filamentous fungi possess a yeast-like phase at some point in their life cycle. Generally, yeast cells tend to grow as single cells that reproduce asexually in a process known as budding. Molds grow as masses of overlapping and interlinking hyphal filaments and reproduceby producing masses of spores in a variety of structures.

Fungal spores range in sizes from 1–50 µm and are easily dispersed into the cleanroom environment through many routes. They can become a serious problem if the appropriate disinfectants are not used routinely in clean-rooms. Fungal contamination of pharmaceutical products can be significant risk, particularly if the products have high water activity (Aw) and stored in warm conditions. Common sources for fungal contamination include air, the interior surface of air ducts, personnel,paper, and incoming raw materials.

FUNGAL STRUCTURE

Fungi are structurally complex, showing a bewildering variety of reproductive structures associated with sexual and asexual processes, in addition to vegetative, nonreproductive elements and hyphal structures. Their differentiation into genera, species, and varieties is made, in a large part, on a morphologicbasis — especially morphology of the reproductive structures.

Microscopic examination shows that yeasts are small, unicellular microorganisms. Yeast cells are usually spherical, oval, or cylindrical, and cell division usually takes place by budding. Yeasts usually do not form filaments or myceli-um. Figure 1 shows the yeast C. albicans and typical structures.

Molds are multicellular fila-mentous fungi that develop multicellular branching structures known as hyphae. Hyphae usually grow together across a surface and form compact tufts, collectively called a mycelium, which can be seen easily without a microscope (Figure 2A).

The mycelium arises because the individual hyphae form branches as they grow, and these branches intertwine, resulting in a compact mat. From the mycelium, other hyphal branches may reach up into the air above the surface, and on these aerial branches spores called conidia are formed. Conidia are asexual spores, often highly pigmented and resistant to drying. The spores of fungi have a large range of sizes and the range of sizes is dependent on the species, humidity, and age of the culture (Figures 2B, 3). Even within the same species they can have a narrow range of spore sizes, such as A. niger, or a wide range of sizes, such as Penicillium chrysogenum.[2] Because these spores are so numerous and spread so easily through the HEPA-filtered airflow patterns, molds can easily contaminate cleanrooms.

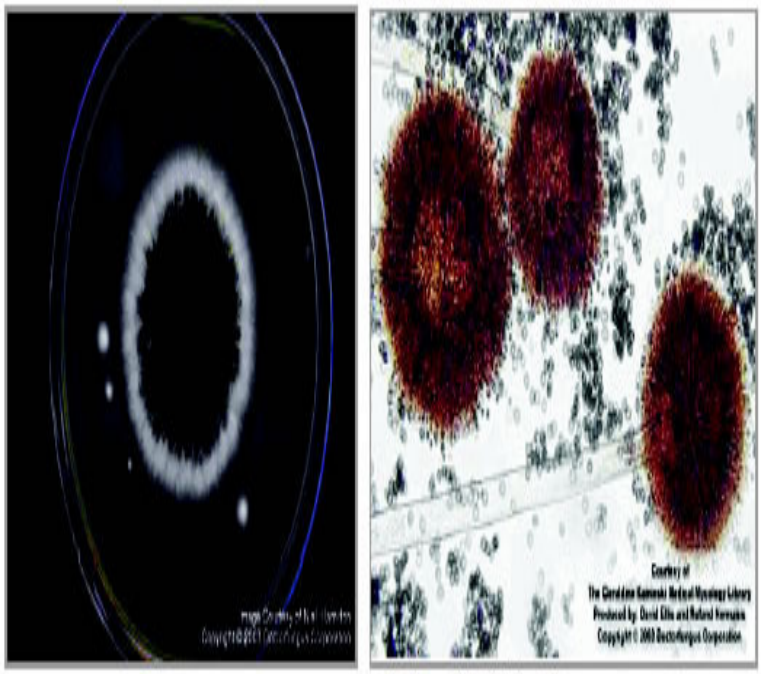

Figure 2: *Aspergillus niger*; Macroscopic Morphology (A) (left) and Microscopic Morphology (B) (right)

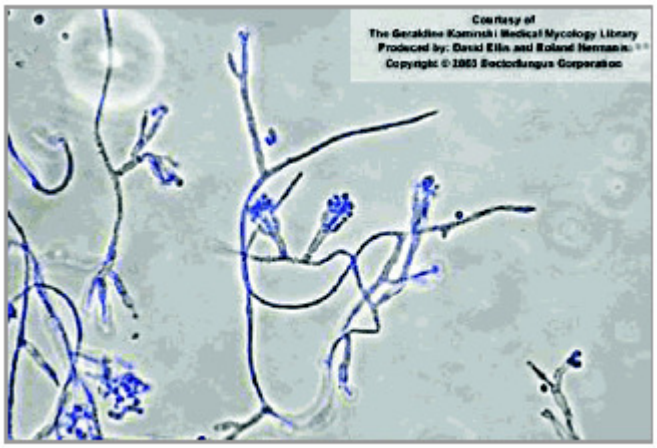

Figure 3: *Penicillium sp.* Microscopic Morphology

RESISTANCE

While a considerable amount of information is available about fungal resistance to antibiotics, data on the mechanisms of fungal resistance to chemical biocides is very limited. One commonly accepted theory about the mechanism of fungal resistance to biocides involves natural (intrinsic) resistance. A fungalcell may have an innate ability to present a permeability barrier to one or morebiocides, or to inactivate a biocide due to the presence of existing enzymes.[3]

As shown in Table 1, molds are generally more resistant than yeasts and considerably more resistant than vegetative bacteria. Fungal spores, although more resistant than non-sporulating bacteria, are less resistant than bacterial spores to disinfectants.

	Microorganism	Examples
More Resistant	Prions	Scrapie, Creutzfeld-Jacob disease, Chronic wasting disease
	Bacterial Spores	*Bacillus, Geobacillus, Clostridium*
	Protozoal Oocysts	*Cryptosporidium*
	Helminth Eggs	*Ascaris, Enterobius*
	Mycobacteria	*Mycobacterium tuberculosis, M. terrae, M. chelonae*
	Small, Non-Enveloped Viruses	Poliovirus, Parvoviruses, Papilloma viruses
	Protozoal Cysts	*Giardia, Acanthamoeba*
	Fungal Spores	*Aspergillus, Penicillium*
	Gram negative bacteria	*Pseudomonas, Providencia, Escherichia*
	Vegetative Fungi and Algae	*Aspergillus, Trichophyton, Candida, Chlamydomonas*
	Vegetative Helminths and Protozoa	*Ascaris, Cryptosporidium, Giardia*
	Large, non-enveloped viruses	Adenoviruses, Rotaviruses
	Gram positive bacteria	*Staphylococcus, Streptococcus, Enterococcus*
Less Resistant	Enveloped viruses	HIV, Hepatitis B virus, Herpes Simplex virus

Table 1. Hierarchy of Resistance[4]

BIOCIDAL ACTIVITY AGAINST FUNGI

A considerable variation exists in the response of various yeast and molds to biocides. Generally, vegetative fungi and fungal spores are more resistant than vegetative bacteria to these agents, but they are more susceptible than bacterial spores. It is often assumed that fungi are inactivated in a similar or identical fashion to vegetative bacteria. However, the different structuraland chemical properties of bacteria and fungi might challenge this assumption.

It is likely that the first interaction between biocide and fungus occurs at the cell surface, and then the biocide crosses the cell wall (or outer membrane) to reach its target sites within the cell. Some biocides are likely to have a predominate effect on the outer layers. However, few antimicrobial agents are intended to focus on the fun-gal cell wall as a major, or sole, target. The plasma membrane is another major target site for many antifungal agents.[5]

DISINFECTANT SELECTION

Chemical disinfection is a vital part of the contamination control program in aseptic processing areas. Since there are numerous ways that cleanrooms can become contaminated with fungi, the selection and use of an agent with fungicidal activity is extremely important. However, not all disinfectants typically used in the cleanroom are effective against fungal spores and itmay be necessary to use a sporicidal agent to control fungi. Sporicides by nature are more aggressive than normal disinfectants as shown in suspension testing(Figure 4) and carrier testing (Figure 5).

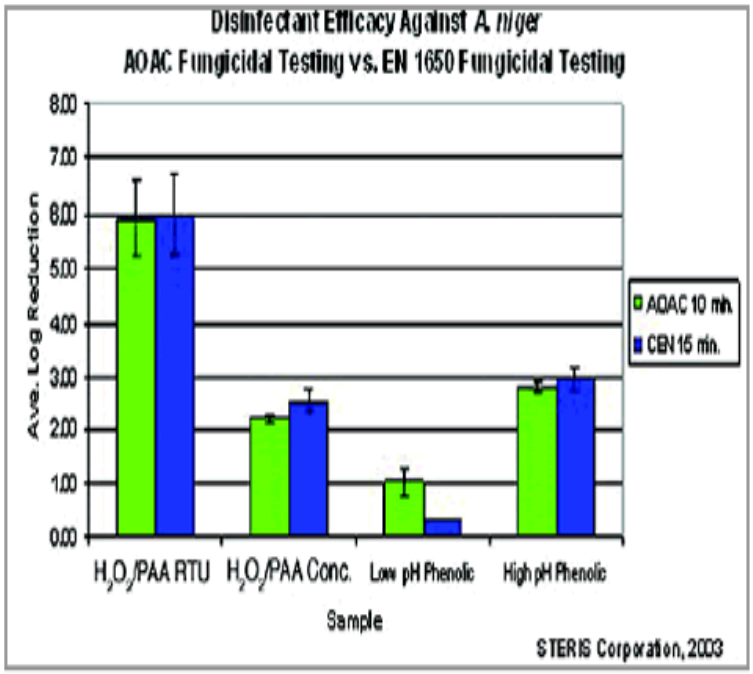

Figure 4: Comparison of H_2O_2/PAA and Phenolic Activity against *A. niger* – Suspension Test

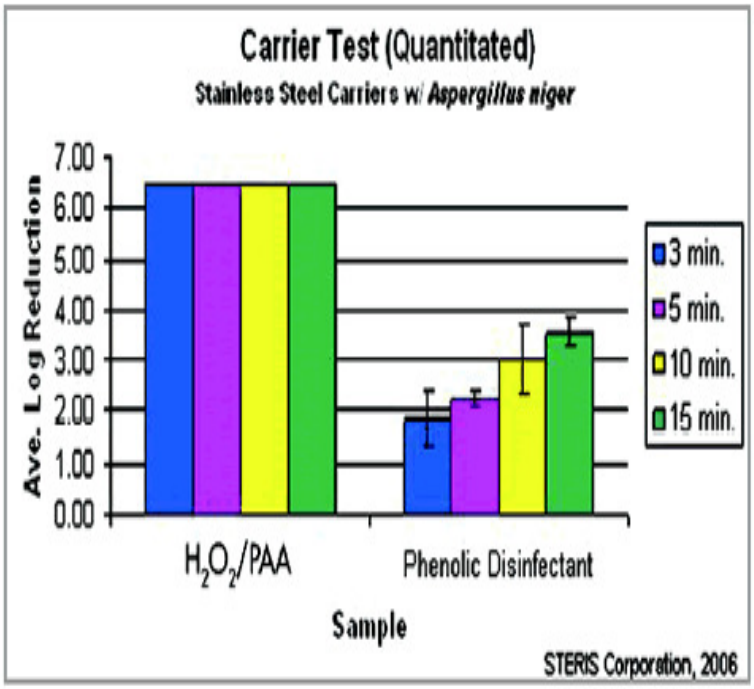

Figure 5: Comparison of H_2O_2/PAA and Phenolic Activity against *A. niger* – Carrier Test

• STEP 1: Gather all data and information

The first step in selecting a chemical disinfectant is to collect all the pertinent and available information from the supplier(s). This should include technical data sheets, material safety data sheets, and recommended directions for use. All available testing on substrate compatibility, stability, and microbial efficacy (bactericidal, fungicidal, sporicidal, and virucidal) should also be reviewed, and these tests should have been performed according to accepted standards such as AOAC, ASTM, or EN. The disinfectant should also be manufactured in accordance with local, state, and country regulations. Each lot should have a certificate of manufacture/analysis and the supplier's change control policies should be audited to ensure that customers will be notified of any significant changes in the product. Whether comparing products from the same suppler or different suppliers, it is critical to review all documentation provided and to request additional information from the suppler as needed to ensure proper selection of chemical disinfectants and sporicides.

USP <1072>, Aseptic Processing Guide, and The Orange Guide recommend a rotation of a sporicidal agent in addition to a broad spectrum disinfectant. Rotation is commonly viewed as a rotation of two disinfectants and a sporicidal agent or a rotation of a single disinfectant and a sporicidal agent. Refer to USP <1072> Disinfectants and Antiseptics, Aseptic Guidance Document[6], and The Orange Guide[7]for additional information.

• STEP 2: Test the disinfectants for effectiveness in your cleanroom

Several factors are known to influence the effectiveness of active ingredients in biocides including but not limited to: concentration, type of surface, contact time, pH, the presence of organic soil, bioburden, water hardness, and temperature. For this reason, it is difficult to identify specific actives for use against fungal spores, as many of them are fungistatic or fungicidal depending upon the factors mentioned above, particularly concentration and

contact time. Futhermore, general disinfectants (pheno-lics, quaternary ammonium compounds, and chlorine) may not be effective in controlling mold outbreaks. Because of these factors, it is critical to perform in-house disinfectant effectiveness testing on agents to be used in cleanrooms.

Sound cleaning and disinfection procedures are critical to controlling fungal contamination in the cleanroom. Disinfectant application should be considered a critical processing step that requires validation. USP <1072> Disinfectants and Antiseptics provides some guidance as to how to structure disinfectant validation studies. A series of tests to demonstrate the efficacy of a disinfectant/sporicide against fungal spores within the pharmaceutical manufacturing cleanroom environment is necessary as many agents that are effective against vegetative fungi are not effective against fungal spores. Specifically, the following testing methods and comparisons should be considered:

1. Suspension tests. While varying to some degree in their methodology, most of the proposed procedures tend to employ a standard suspension of the microorganism in appropriate dilutions of the test disinfectant. Tests are carried at room temperature and, at selected time intervals, samples are removed and viable counts are performed following neutralization of any disinfectant remaining in the sample. Residual disinfectant can be neutralized by dilution or by adding specific agents, such as lecithin or Tween 80. Suspension tests are valuable for determining the time necessary to achieve an acceptable log reduction of organisms, but they do not address the variations in efficacy that occur when a disinfectant is applied to different surface types.

2. Carrier tests. These tests are useful in evaluating the antifungal properties of potential hard-surface disinfection agents under conditions that simulate a specific facility's application. Most of the methods use stainless steel carriers, but in reality, all surfaces within a cleanroom will be exposed to the disinfectant/sporicide. The choice of test surfaces is matched to the types of surfaces present in cleanroom where the test disinfectant will be used. USP <1072> provides a list of typical clean-room surfaces including stainless steel, epoxy, lexan®*, terrazzo flooring, vinyl, glass, and other hard surfaces.

3. Statistical comparisons: Suspension tests and surface tests give a fairly reliable evaluation of disinfectant efficacy under laboratory conditions, but cannot guarantee efficacy in the actual cleanroom environment. Therefore, it is necessary to perform a statistical comparison of the frequency of isolation and the numbers of organisms isolated before and after the implementation of a new cleaning and disinfection program. It is important to trend environmental data over the course of a year, specifically in regard to fungal growth, to identify possible seasonal variations that may indicate facility and/or maintenance issues. Refer to USP <1116> Microbiological Evaluation of Clean Rooms and Other Controlled Environments8, Aseptic Guidance Document, and The Orange Guide, Annex 1 for additional information regarding environmental testing practices, limits, and controls.

Use recognized methods

There are several currently published methods to determine fungicidal activity in suspension tests or carrier tests. The BS EN methods (1650 and 13697) established by the European Committee for Standardization and the AOAC (Association of Official Analytical Chemists) methods to test fungicidal activity provide general test procedures to

use when performing fungicidal activity testing. However, since these methods are used specifically by disinfectant manufacturers to register a product for sale in the U.S. or Europe, certain requirements in these methods are not necessarily applicable to cleanroom disinfectants for the pharmaceutical manufacturing, compounding pharmacy, or medical device industries. Reasonable modifications can be made so long as the method remainsscientifically sound.

Use reference cultures and environmental isolates

A common question related to disinfectant effectiveness testing is which fungus (e.g., yeast or mold) should be used as a test microbe. It is generally recommended to use fungal spore suspensions of both reference cultures and environmental isolates. For example, the AOAC fun-gicidal test uses Trichophyton mentagrophytes as a reference organism. This is somewhat misleading since it does not present as great of a challenge to disinfectants as A. niger (Figure 6) and is not as common in pharmaceutical, biotech, and medical device cleanrooms. Therefore, the suggested procedure is to include A. niger and C. albicans as reference cultures. Regulatory authorities also expect to see the specific environmental isolates most frequently found in the facility included in the disinfectant effectiveness testing.

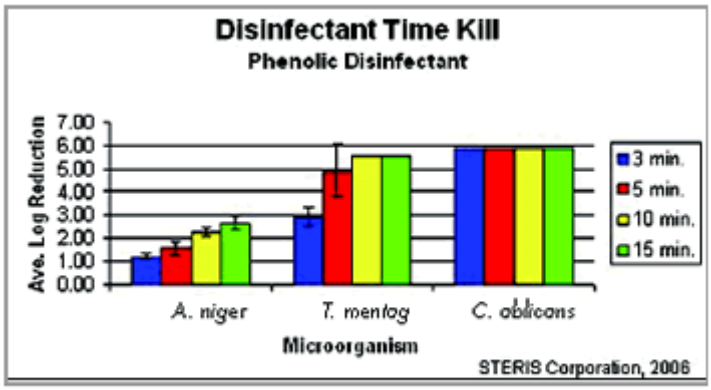

Figure 6: *A. niger* Resistance

Prepare suspensions for optimal disinfection challenge

Yeast suspensions should be prepared from yeast that has been propagated on media that results in rapid growth and smooth colonies. The technique used for propagation of yeast can be important because different morphological structures show differences in susceptibility to fungicides.

The morphologic structures present in mature mold cultures are quite different from those encountered in yeast cultures, and they are often more resistant to the effects of fungicides. Molds used in disinfectant validation testing should be prepared as conidia suspensions with hyphal fragments removed since the conidia present the greatest disinfection challenge.

CONCLUSION

A thorough comtamination control program includes a process for determining the most effective disinfectant formulations to address a myriad of microbial challenges, including specific mold species that may be present in the environment. In addition, a well-managed cleanroom microbial control program evaluates and makes improvements to the condition

of the facility, and provides constant training and reinforcement of personnel practices in order to prevent problematicbehaviors.

In order to choose the most effective disinfectant, cleanroom professionals must take a multitude of factors into consideration. For example, it is important to determine the actual mold species because some molds are more resistant to disinfectants than others. In addition, in-house testing must be completed that validates the efficacy of a particular disinfectant for a specific cleanroom application. This testing should address the many variables that can influence the effectiveness of active ingredients in biocides, such as concentration, type of surface, contact time, pH, the presence of organic soil, the amount of bioburden, water hardness, and temperature. It should include carrier tests, suspension tests, and year-long statistical comparisons account for seasonal variations.

Once the optimal disinfecting agents have been selected, a complete contam ination control program can be devel oped to prevent mold growth, reduce risk of excursions in a facility, and ulti mately to safeguard product quality.

**Lexan is a registered trademark of General Electric Corporation.*

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