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Recurrent *Aspergillus* contamination in a biomedical research facility: a case study

Christopher T. Cornelison · Bryan Stubblefield · Eric Gilbert · Sidney A. Crow Jr

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Abstract Fungal contamination of biomedical processes and facilities can result in major revenue loss and product delay. A biomedical research facility (BRF) culturing human cell lines experienced recurring fungal contamination of clean room incubators over a 3-year period. In 2010, as part of the plan to mitigate contamination, 20 fungal specimens were isolated by air and swab samples at various locations within the BRF. *Aspergillus niger* and *Aspergillus fumigatus* were isolated from several clean-room incubators. *A. niger* and *A. fumigatus* were identified using sequence comparison of the 18S rRNA gene. To determine whether the contaminant strains isolated in 2010 were the same as or different from strains isolated between 2007 and 2009, a novel forensic approach to random amplified polymorphic DNA (RAPD) PCR was used. The phylogenetic relationship among isolates showed two main genotypic clusters, and indicated the continual presence of the same *A. fumigatus* strain in the clean room since 2007. Biofilms can serve as chronic sources of contamination; visual inspection of plugs within the incubators revealed fungal biofilms. Moreover, confocal microscopy imaging of flow cell-grown biofilms demonstrated that the strains isolated from the incubators formed dense biofilms relative to other environmental isolates from the BRF. Lastly, the efficacies of various disinfectants employed at the BRF were examined for their ability to prevent spore germination. Overall, the investigation found that the use of rubber plugs around thermometers in the tissue culture incubators provided a microenvironment where *A. fumigatus* could survive regular surface disinfection. A general lesson from this case study is that the presence of microenvironments harboring contaminants can undermine decontamination procedures and serve as a source of recurrent contamination.

Keywords *Aspergillus fumigatus* · Biofilm · Industrial contamination · Microenvironment · RAPD

C. T. Cornelison (✉) · B. Stubblefield · E. Gilbert · S. A. Crow Jr
Applied and Environmental Microbiology,
Department of Biology, Georgia State University,
100 Piedmont Avenue SE, Atlanta, GA 30303, USA
e-mail: ccornelison1@student.gsu.edu

Introduction

Fungal contamination of facilities used for biomedical research and production has serious financial and regulatory consequences. In 2001, mold contamination of industrial and residential environments resulted in $1.3 billion in insurance claims, and based on current inflation rates, this would correspond to $3 billion in 2010 [4]. In a case study, a biomedical research facility (BRF) located in the metro Atlanta area experienced 3 years of recurrent fungal contamination of incubators used for culturing human cell lines. The company contacted the senior principal investigator of this article for assistance with identifying the source of the contamination problem.

Preliminary investigation revealed that *Aspergillus* spp. were the primary contaminants in the affected incubators. This was a significant finding because members of the *Aspergillus* genus have proven to be adept at colonizing human tissues; mortality rates associated with *A. fumigatus*-caused invasive aspergillosis approach 90% and pose a threat to transplant recipients [6, 11]. Additionally, *Aspergilli* are well-documented contaminants in hospitals, and significant efforts have been made to control their dissemination in these sensitive environments [5, 8]. Even with the development of novel control mechanisms,

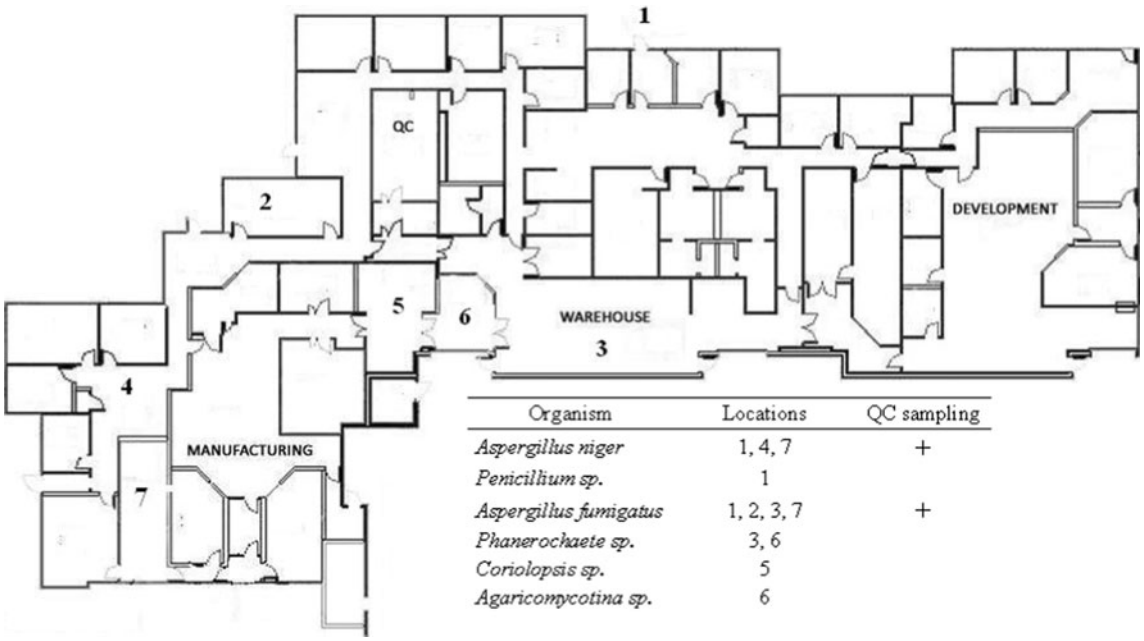
Aspergillus contamination is still a significant problem for industrial biomedical research and production facilities, with significant regulatory and financial implications. Quality-control efforts at the BRF included routine surface disinfection and UV exposure in response to contamination, but were unable to completely eradicate the fungal contaminants.

To determine the source of the contamination, air samples and swabs were taken at various locations that exhibited characteristics favorable for fungal growth throughout the manufacturing facility [1]. The resulting isolates were identified by their 18S rDNA sequences and all isolates identified as *A. fumigatus* were characterized by random amplified polymorphic DNA polymerase chain reaction (RAPD-PCR) to delineate their phylogenetic relationships [10]. This information was used to compare the isolated strains with historical samples collected in the facility over a 3-year period. We hypothesized that the persistent *A. fumigatus* contamination in the incubators could be sustained by growth as microbial biofilms within the facility rather than resulting from recurrent introduction from the environment. This hypothesis was tested by RAPD analysis and by comparing the ability of *A. fumigatus* strains obtained in the incubators to form biofilms relative to other fungal species isolated from the building. We also evaluated the efficacy of the disinfectants employed at the BRF to eradicate *Aspergillus* species. Based on the collected data, an action plan was generated to reduce the likelihood of recurrent *Aspergillus* contamination.

Materials and methods

Sampling and isolation

An Andersen single-stage impingement air sampler was used with malt extract agar (Difco, MEA) to collect air samples. Air samples were taken for 1, 2, or 5 min, depending on likely fungal particle densities as determined by visual inspection of rooms, and their proximity to facility access points. The sample durations correspond to sampled air volumes of 0.028, 0.057, and 0.12 m^3, respectively. Air samples were taken at the main employee entrance (site 1), conference room (site 2), the shipping warehouse (site 3), the corridor to the clean room (site 4), processing rooms (sites 5 and 6), and the clean room housing the incubators (site 7) (Fig. 1). Within site 7, air samples were taken in all incubators not in active use ($n = 3$), as well as near the HVAC vent. Swab samples were taken using individually packaged sterile swabs from Fisher Scientific (Pittsburgh, PA). Swab samples were collected from HVAC vents in all rooms where air samples were taken. Additional swabs were collected from the ceiling fan in site 2, storage shelves in site 3, as well as freight boxes in site 3. Within site 7, swabs were collected from the interior surfaces of all incubators not in use ($n = 3$). The interior surfaces swabbed included the baseboard and the interior water pan and around the temperature probes and stoppers. Swabs were streaked onto MEA plates and then placed in 50-ml sterile conical tubes containing 10 ml of 50 mM phosphate buffered saline (PBS). Plates were incubated at 30°C until growth was documented. Independent



Organism	Locations	QC sampling
Aspergillus niger	1, 4, 7	+
Penicillium sp.	1	
Aspergillus fumigatus	1, 2, 3, 7	+
Phanerochaete sp.	3, 6	
Coriolopsis sp.	5	
Agaricomycotina sp.	6	

Fig. 1 BRF facility layout. Locations yielding fungal isolates indicated. (+) indicates positive identification of contaminants in the BRF prior to the onset of this study

colonies were re-isolated on MEA and in Sabouraud dextrose broth (Difco, SDB). 0.1 ml of PBS from the tubes containing swabs was plated onto MEA plates and incubated at 30°C. Growth was plated for isolation, and re-isolated as needed.

DNA extraction and PCR conditions

Isolates were grown in 10 ml of SDB at 30°C until a dense mycelial mass was achieved. Cultures were then centrifuged, the media removed, and the pellet washed with 10 ml PBS a minimum of twice. After the cultures were re-centrifuged, the PBS was removed and the fungal mass was stored at −80°C for 24 h prior to lyophilization. The lyophilized samples were ground into a powder and placed in microfuge tubes. Then, 500 µl of TE buffer was added and each sample was centrifuged for 2 min at 10,000 rpm. TE buffer was then removed and 300 µl of extraction buffer (~200 mM Tri-HCl pH = 8.5, 250 mM NaCl, 25 mM EDTA, 0.5% SDS) was added. Samples were then vortexed and allowed to sit for 5 min. Then, 45 µl of proteinase K (Qiagen) and 10 µl of RNase (Roche) were added to each sample, and the samples were incubated at 37°C for 24 h. Following the incubation period, equal volumes (~400 µl) of phenol chloroform isoamyl alcohol (25:24:1) were added to each sample. The samples were then vortexed and centrifuged for 2 min at 10,000 rpm. The supernatant was then removed and saved, and the remaining phenol layer discarded. Two volumes (~600 µl) of absolute ethanol were added to each sample and incubated for 24 h at −20°C. Following incubation, the samples were centrifuged for 10 min at 20,000 rpm. The absolute ethanol was removed and the remaining DNA was washed with 70% ethanol, and centrifuged for another 10 min at 20,000 rpm. The 70% ethanol was then removed and the samples were allowed to completely dry. Once dry, the DNA was suspended in 25 µl of TE buffer. The DNA concentration of each sample was measured using a Nano-Drop (Fisher) spectrophotometer, and concentrations were optimized to 20 ng/µl. PCR was carried out using 18S whole gene primers [9] with the following thermal cycler conditions (program SAM2120): 95°C for 5 min, 30 cycles of 95°C for 30 s, 58°C for 30 s, 72°C for 1 min, and a touch-down from 58 to 54°C. Confirmation gels (1.2% agarose, 75 ml 1× TAE, 7.5 µl ethidium bromide) were run at 100 V for 1.5 h and imaged using a Bio-Rad gel imager.

Sequence sample preparation and sequence analysis

The PCR product that resulted in a single band by agarose gel electrophoresis was purified using a PCR purification kit (Qiagen). Samples for sequencing were prepared as follows: ~2 µl of PCR template, 2 µl of R or F primer, DI water to a total volume of 28 µl per sample. Samples were sent to the Georgia State University Natural Science Center Sequencing Center for processing. Sequences were analyzed using the DNAbaser program (Heracle BioSoft S.R.L.), and comparisons generated using the BLAST application provided by National Center for Biotechnology Information (NCBI).

RAPD PCR

Extracted fungal DNA was diluted to a concentration of 20 ng/µl. RAPD primer R108 [2] was used to produce distinct banding patterns. Reactions consisted of 12.5 µl Taq PCR Master Mix (Qiagen), 1 µl primer R108, 1 µl DNA template, and molecular grade water to a final volume of 25 µl. Amplifications were carried out in a thermalcycler (Eppendorf Mastercycler pro S) for 1 cycle of 5 min at 94°C to denature followed by 45 cycles of 30 s at 94°C, 30 s at 35°C, 1 min at 72°C, and 1 final cycle of 5 min at 72°C (program RAPD). Amplification products (10 µl) were fractionated by electrophoresis through 1.2% agarose gels with 10 µl of GelRed Nucleic Acid Stain (Biotium). Gels were imaged using FOTO/Analyst Investigator (Fotodyne) imaging system. Banding pattern analysis and comparisons were carried out using the Advanced Qualifier program (Bio Image).

Biofilm analysis

The ability to form biofilms was initially tested using a simple model consisting of a glass microscope slide submerged in 10 ml of SDB in a 200-ml Erlenmeyer flask. The flask was then shaken at 80 rpm at 30°C for 48 h. Afterwards, the slide was removed and visually inspected for evidence of biofilm formation. Biofilm formation was defined as attachment to the glass slide. Strains identified as possible biofilm formers by this simple technique were then grown in a flowcell system consisting of a plastic frame with two 4-mm channels with stainless-steel tubing at the input and output of each channel. The frame is covered on each side with traditional microscope slides and sealed with a silicone-based sealant. The flow cells were run with a constant flow of media [12]. Flowcells were analyzed after 18 h of growth. Prior to imaging, biofilms were stained with calcoflour white (10 mM) and FUN-1 (10 mM) in the absence of light for 30 min. Flowcells were visualized using confocal laser scanning microscopy. Images were produced using the LSM Image browser software (Zeiss) and attachment quantified using COMSTAT software (Hegdorn et al. 2000, *Microbiology*).

Spore harvest and disinfectant efficacy assay

Cultures for spore harvest were grown on Sabouraud dextrose agar plates (SDA, Difco) and incubated at 30°C until

spores covered the entire surface of the plate (~7 days). To harvest spores, 10 ml of conidia harvesting solution (CHS-0.05% Tween 80, 0.9% NaCl) was pipetted onto the plate. The solution was centrifuged and supernatant discarded. The resulting pellet of spores was washed with PBS. The spore suspension was filtered using glass wool to remove any mycelia. A Bright-Line hemocytometer (Reichert, USA) was used to determine conidia concentration, and the solution was diluted as required. Spores were stored at 4°C until needed. Spore suspensions of 10^4 spores/ml were used for the disinfectant efficacy assay. One milliliter of spore suspension was mixed with 5 ml of disinfectant for 1 min at room temperature. Inactivation of the active ingredients of the disinfectants was carried out with an equal volume (~6 ml) of Letheen broth for 5 min at room temperature. The suspension was then centrifuged for 2 min and the supernatant discarded. Spores were re-suspended in 3.3 ml of PBS. 0.1 ml of this suspension was spread on MEA plates and incubated at 30°C for 48–72 h. Plates were counted at the first sign of germination and monitored for additional growth for 24 h.

Results

Aspergillus fumigatus was isolated from multiple locations within the BRF including all incubators in site 7 (Table 1). Analysis of the species diversity using 18S rDNA sequences indicated the presence of at least five genera of fungi throughout the facility (Table 1). Only *A. niger* and *A. fumigatus* were isolated from clean-room incubators used for human tissue cultures. Stoppers used in conjunction with temperature probes were identified as major reservoirs for *A. fumigatus* and resulted in positive identification of *A. fumigatus* from all samples (Fig. 2a). Because *A. fumigatus* was the most prevalent organism identified from site 7, and has been shown to contaminate human tissue cultures [10], the remaining studies focus on this organism.

To determine whether the *A. fumigatus* isolates collected in this study were the same or different from the isolates obtained in 2007 and 2009, a RAPD PCR banding pattern comparison was used [2]. It was hypothesized that if *A. fumigatus* was present in the clean-room incubators since 2007, then isolates from 2010 would cluster with

Table 1 Isolate list with sampling location

Isolate number	Organism	Sampling location
GSU01	*Aspergillus niger*	1
GSU02	*Penicillium* spp.	1
GSU03	*Aspergillus fumigatus*	1
GSU11	*Aspergillus fumigatus*	2
GSU21	*Aspergillus fumigatus*	3
GSU22	*Phanerochaete* spp.	3
GSU33	*Aspergillus niger*	4
GSU42	*Coriolopsis* spp.	5
GSU51	*Phanerochaete* spp.	6
GSU52	*Agaricomycotina* spp.	6
GSU61	*Aspergillus niger*	7
GSU62	*Aspergillus fumigatus*	7
GSU71	*Aspergillus niger*	7
GSU72	*Aspergillus fumigatus*	7
GSU81	*Aspergillus fumigatus*	7
GSU91	*Aspergillus fumigatus*	2009 QC isolate
GSU92	*Aspergillus fumigatus*	2010 QC isolate
GSU93	*Aspergillus fumigatus*	2010 QC isolate
GSU94	*Aspergillus fumigatus*	2007 QC isolate

See Fig. 1 for a map of sampling locations

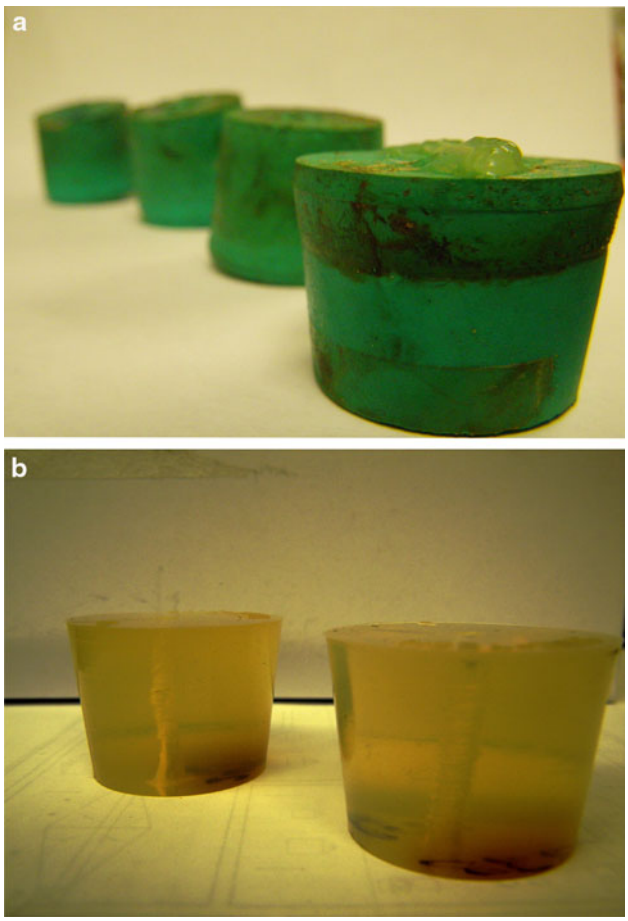


Fig. 2 Stoppers used to hold thermometers in the rear wall of the incubators with power cords leading outside of the incubators. **a** Rubber stoppers used in clean-room incubators prior to this study. Notice the biofilm accumulation characterized by textured discoloration. **b** Silicone stoppers implemented at the BRF since the completion of this study. The stoppers are routinely changed in order to prevent biofilm formation

isolates from 2007 to 2009. If on the contrary they resulted from separate contamination events, it is likely that they would produce different RAPD banding patterns. All *A. fumigatus* isolates generated distinct RAPD PCR banding patterns (Fig. 3). Two widely used matching methods and three linkage methods of clustering were used to determine the relatedness of the isolates. In all comparisons, isolates from the 2010 sampling clustered with isolates from 2007 to 2009, supporting the hypothesis of persistent fungal colonization of the BRF (Fig. 4).

Rubber stoppers used in conjunction with temperature sensors in clean-room incubators exhibited growth of surface-attached communities of microorganisms (Fig. 2a); isolated fungi were determined to be *A. fumigatus* and *A. niger*. None of the other species identified at the BRF were cultured from the plugs. We hypothesized that *A. fumigatus* and *A. niger* had superior biofilm formation abilities relative to the other strains found in the BRF. To investigate this idea, biofilms of *A. fumigatus* and *A. niger* were cultivated in flowcells and imaged by confocal laser scanning microscopy (CLSM). The resulting biofilms and had an average number of attached propagules per field (field area was 0.23 mm^2) of 99 ± 22 and 47 ± 11, respectively, and were significantly greater ($p < 0.05$) than those of *Coriolopsis* sp. selected as a representative environmental isolate found in the BRF, with an average of 11 ± 3 attached propagules per field (field area was 0.23 mm^2). Image analysis demonstrated that, isolates GSU 72 (*A. fumigatus*) and GSU 61 (*A. niger*) formed dense biofilms in flow cell chambers 18 h after inoculation with spores, while GSU 42 (*Coriolopsis* sp.) did not (Fig. 5).

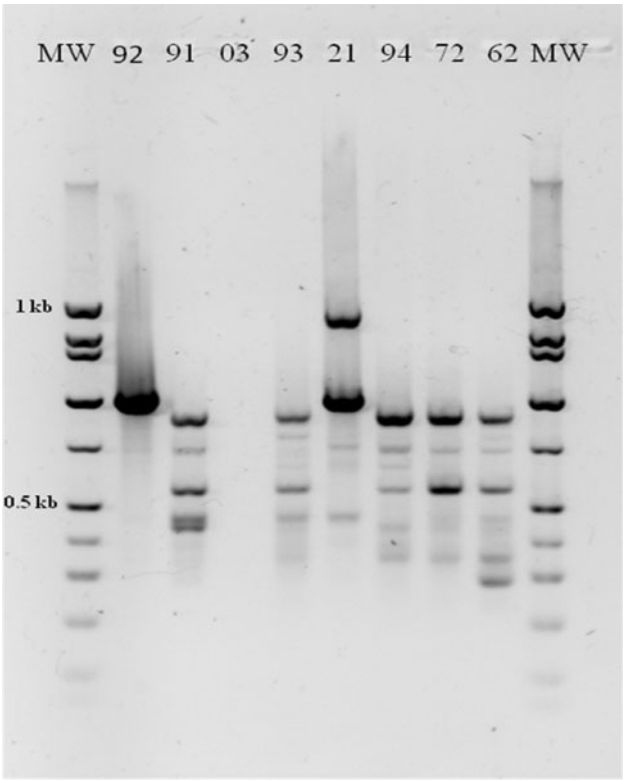


Fig. 3 Identification of *A. fumigatus* isolates by RAPD gel electrophoresis. *Numbers above lanes* indicate GSU strain number. Note the distinct banding pattern for each strain. See "Materials and methods" for details on conditions. *MW* indicates 1.5 kb ladder

To determine the efficacy of the cleaning regime employed at the BRF on *A. fumigatus* eradication we tested three disinfectants employed in routine cleaning

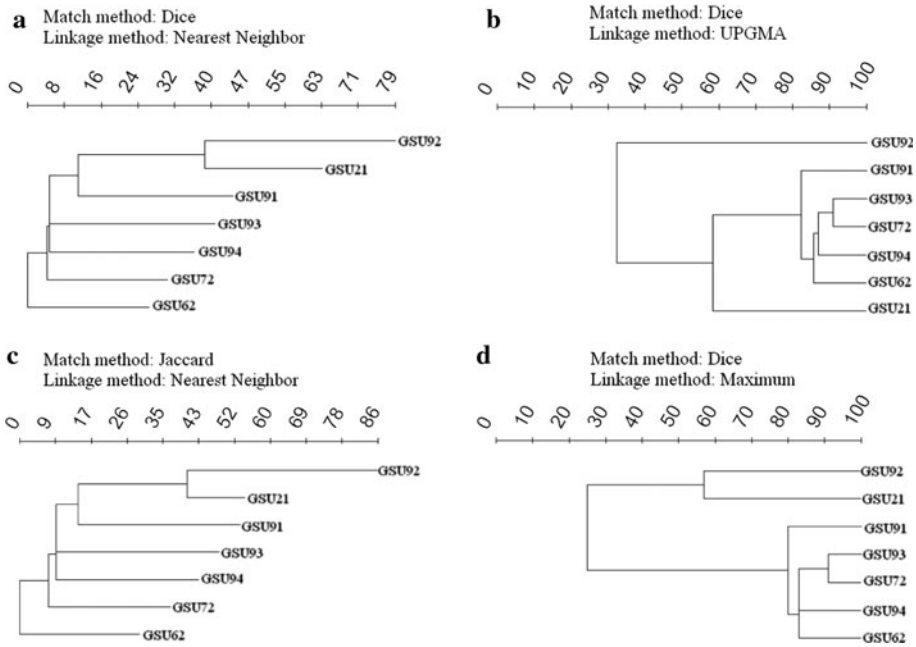


Fig. 4 RAPD banding pattern cluster analysis of *A. fumigatus* isolates: **a** match method Dice and linkage method nearest neighbor, **b** match method Dice and linkage method UPGMA, **c** match method Jaccard and linkage method Nearest Neighbor, **d** match method Dice and linkage method Maximum similarity. All combinations of matching and linkage methods resulted in the same primary cluster consisting of GSU91, GSU93, GSU72, GSU94, and GSU62

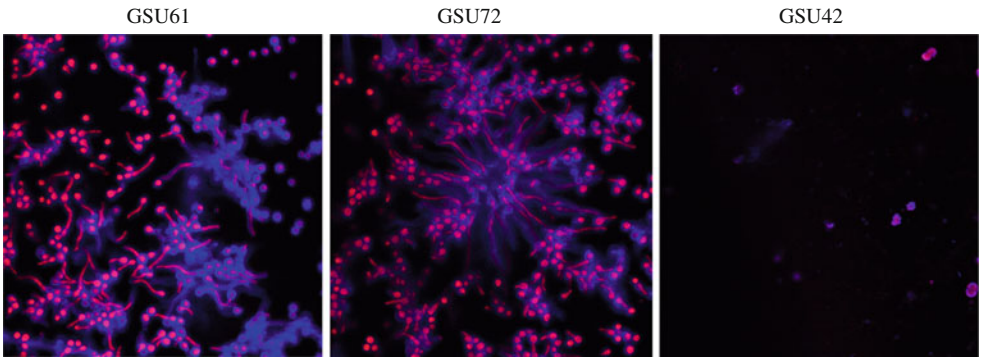


Fig. 5 Confocal laser scanning microscopy images of biofilm formation in flow cell models. *Red* indicates cell cytosol; *blue* indicates extra polymeric substance (EPS). Magnification ×400. All cultures were grown for 18 h before staining. Note the extensive attachment of GSU61 (*A. niger*) and GSU72 (*A. fumigatus*) isolated from the rubber stoppers compared to GSU42 (*Coriolopsis* sp.) isolated from elsewhere in the facility. See "Materials and methods" for details on flow cell and CLSM conditions

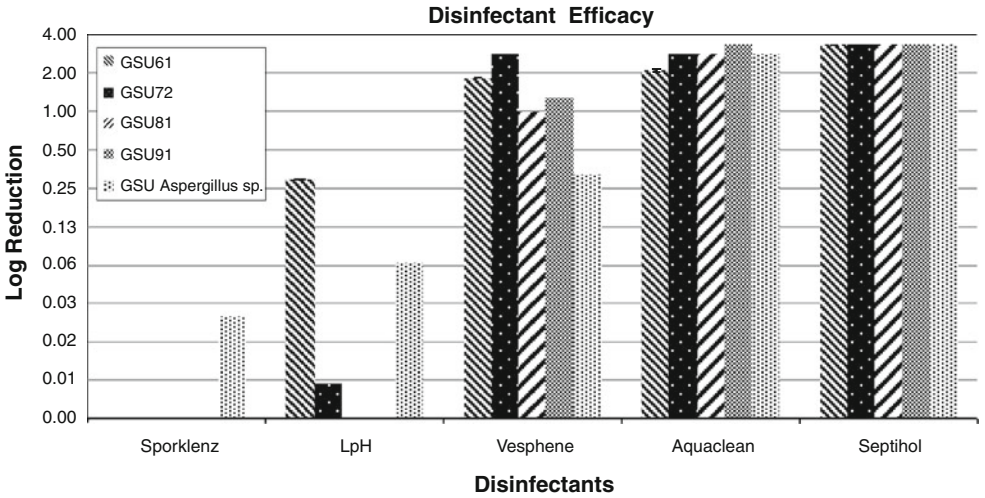


Fig. 6 Disinfectant efficacy comparison with the BRF *Aspergillus* spp. using an environmentally derived non-BRF *Aspergillus* sp. as a control (GSU *Aspergillus* sp.). See "Materials and methods" for details on experimental conditions

efforts, and two disinfectants used in response to contamination for their ability to inhibit spore germination. An exposure time of 1 min was used to model the surface wipe down carried out at the BRF. Vesphene, Aquaclean, and Septihol exhibited greater than two log reductions in all trials (Fig. 6). On the other hand, Sporklenz exhibited less than 0.03 log reductions in all trials. LpH demonstrated the greatest degree of variability amongst the disinfectants with log reductions ranging from 0.01 to 0.25 (Fig. 6).

Following the initial investigation, the BRF elected to replace the stoppers used to hold temperature sensors on a monthly basis. The new plugs were silicone based. Samples of the new silicone plugs were obtained from the BRF in August 2010. No fungal growth was visible on the new stoppers; attempts to culture any viable organisms were unsuccessful (Fig. 2b).

Discussion

This study exemplifies how microenvironments in industrial facilities have the potential to harbor recalcitrant microorganisms and initiate recurrent contamination. Although stringent management of classic transmission routes such as HVAC systems, municipal water supplies, and personnel traffic [7, 13, 15, 17] are essential for ensuring process fidelity, acute awareness of microbial ecology, particularly the ability of diverse microorganisms to grow as biofilms, is needed to ensure all potential sources of contamination are identified and mitigated. Facilities maintenance programs can be undermined by the presence of microenvironments that serve as havens for contaminant strains, particularly those that are adept at biofilm formation. *Aspergillus* contamination of hospitals is a well-documented problem, and many methods have

been developed to reduce the introduction of this potential pathogen [5, 8]. In contrast, cases of *Aspergillus* contamination in industrial facilities are not well documented. The presented work provides a much needed example that can assist industrial microbiologists in preventing the occurrence of industrial *Aspergillus* contamination.

Diverse fungi including *Aspergillus* are known to form robust biofilms [3, 14, 16]. These surface-attached communities may contain a single species or be polymicrobial and in many cases, biofilms show increased resistance to both antibiotics and antimicrobial agents [14]. Within the clean room, the rubber stoppers equipped with temperature sensors were identified as the primary reservoir of *A. fumigatus* and served to protect resident biofilms from disinfectant treatment and UV exposure. The resulting biofilms resemble the current model of biofilm formation for *Aspergillus* spp. (Fig. 5) [3, 16]. These results, together with the condition of the rubber stoppers indicated that biofilm growth by *Aspergillus* spp. was an important factor in the persistent contamination of clean-room incubators. Moreover, spores from *A. fumigatus* strains isolated from the incubators did not show any broad spectrum resistance to the employed disinfectants (Fig. 5). These results indicate the presence of microenvironments harboring resistant biofilms, such as the incubator plugs, were principally responsible for persistent fungal colonization, rather than issues with the efficacy of disinfectants applied to the incubator surface. From the case study presented here, the BRF QC department expanded the standard facility maintenance to include routine replacement of the rubber stoppers from clean room incubators. Subsequent reports from the BRF since the completion of this study indicate that routine replacement of stopper used in clean room incubators has eliminated fungal contamination in the clean room.

This study successfully employed well-established techniques from phylogenetics for forensic analysis of industrial contaminants. This application of RAPD PCR should be considered a valuable tool in industrial quality control efforts and will be beneficial for assessing the long-term efficacy of disinfectant protocols and contamination response efforts. Future work in industrial contamination should be focused on the identification of microenvironments capable of harboring contaminant strains and the physiology, particularly the ability to form biofilms, of microorganisms capable of filling the niches made available by these microenvironments.

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