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Major Article

Aerobiology of the built environment: Synergy between *Legionella* and fungi

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Background: The modern built environment (BE) design creates unique ecological niches ideal for the survival and mutual interaction of microbial communities. This investigation focused on the synergistic relations between *Legionella* and the fungal species commonly found in BEs and the impact of these synergistic relationships on the survival and transmission of *Legionella*.

Methods: A field study was conducted to identify the types and concentrations of fungi in BEs. The fungal isolates purified from BEs were cocultured with *Legionella* to study their synergistic association. Cocultured *Legionella* cells were aerosolized in an air-tight chamber to evaluate the efficacy of ultraviolet (UV) to inactivate these cells.

Results: *Aspergillus*, *Alternaria*, and *Cladosporium* were the most common fungi detected in samples that tested positive for *Legionella*. After coculturing, *Legionella* cells were detected inside fungal hyphae. The microscopic observations of *Legionella* internalization in fungal hyphae were confirmed by molecular analyses. UV disinfection of the aerosolized *Legionella* cells that were cocultured with fungi indicated that fungal spores and propagules act as a shield against UV radiation. The shield effect of fungal spores on *Legionella* cells was quantified at >2.5 $\log_{10}$.

Conclusions: This study provides the first evidence, to our knowledge, of *Legionella* cell presence inside fungi detected in an indoor environment. This symbiotic relationship with fungi results in longer survival of *Legionella* under ambient conditions and provides protection against UV rays.

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BACKGROUND

The evolution in the architectural designs of living spaces and lifestyle changes has resulted in a significant shift of human activities from open-air environments to indoor. Modern living spaces are designed for energy efficiency and controlled air conditions.[1,2] Conditions in the built environments (BEs) used for office or residential purposes are impacted by building design, maintenance operations, and occupant activities. A compromise on these factors can potentially result in sick building syndrome, adversely impacting the health of occupants. The severity of the adverse impact on health appears to be linked to the time spent in such buildings.[3,4]

Indoor air pollution originates from nonbiologic and biologic sources. Bacteria, fungi, pollen, viruses, insect body parts, human squames, animal dander, and bird droppings are the most common sources of biologic contamination in BEs.[5,6] In the indoor environment, fungi are a serious public health threat.[7,8] Fungal species belonging to Ascomycetes, Basidiomycetes, and the anamorphic fungi (Deuteromycota) are the most common causes of allergic reaction in occupants.[8,9]

Legionella pneumophila is the most serious bacterial pathogen associated with indoor environments. Since the discovery of Legionnaire disease in 1976, bacteria of the genus *Legionella* have become a leading public health concern.[10] *Legionella* can be found in air as part of aerosols generated from contaminated sources. It is known to cause Legionnaire disease and Pontiac fever, a milder and self-limiting respiratory infection.[11] Aerosolization is an important component of *Legionella* transmission to the human respiratory system,[12] and it can be accomplished by aerosol-generating systems,

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such as cooling towers, evaporative condensers, plumbing equipment (eg, faucets, showerheads, hot water tanks), humidifiers, respiratory therapy equipment (eg, nebulizers), and whirlpool baths.[12] Continually rising incidences of *Legionella*-associated diseases, and the variety of sources of transmission,[13] highlight the need for understanding the survival and transmission of *Legionella* in BEs.

Factors supporting the survival and growth of fungi and bacteria in BEs have been extensively studied.[14-17] Fungi are able to grow on almost all natural and synthetic materials, especially if they are hygroscopic or wet, and accumulations of dust further facilitate microbial colonization. In addition, increases in temperature and humidity not only facilitate microbial growth but also affect the release of volatile organic compound (VOC).[18-20] The BEs have unique features that separate them from the natural world where microorganisms have evolved over time. Enrichment of particular bacterial and fungal species has been repeatedly shown in BEs under specific conditions.[21-23] The materials, chemical compounds, and physical conditions found in BEs not only provide favorable conditions for selective growth of specific bacterial and fungal species, they also provide niches for unusual interactions among different groups of microorganisms that may not occur in the natural environment.

Although *Legionella* is most prevalent in aquatic environments in BEs (ie, premise water fittings), they have been isolated from nonsaturated media, such as moist soil, dust and potting mixtures.[12,24] In the environment under less than optimal conditions, *Legionella* can survive as parasites of single-celled protozoa (ie, species of *Acanthamoeba* and *Naegleria*).[12] In addition to their ability to live and survive as parasites, they have been shown to develop symbiotic relationships with other microorganisms in aquatic ecosystems[12]; however, no such evidence has been documented for nonaquatic ecosystems. This study focuses on the interaction of *Legionella* with common fungal isolates in BEs and how these interactions impact their mutual survival and dissemination to a susceptible human population.

MATERIALS AND METHODS

Sample collection

During the study period (2014-2015), 52 room-air and 38 vent-air samples were collected from homes and offices in Arizona and California. The room-air samples were collected from the middle of the facility or living room at breathing height, and the vent-air samples were taken from bathrooms and kitchens by positioning an air sampler (PBI SAS-Super ISO Air Sampler; PBI, Milano, Italy) approximately 30.5 cm from the air vent of the surveyed facility or room. Airborne microorganisms settled directly onto Petri plates containing a nutrient medium. The plates were exposed at the sampling points for a specified period of time. The number of microorganisms, expressed as colony forming units (CFU) per cubic meter, was estimated according to the following equation[25]:

$$\mathrm{CFU/m^3} = a \times 10{,}000 / p \times t \times 0.2$$

where a is the number of colonies on the Petri plate, p is the surface of the Petri plate (cubic meters), and t is the time of Petri plate exposure (seconds).

For each sampling, the air sampler was actuated to collect the specified air volume (eg, 50 or 100 L of air) from the sampling location. After each sample, the agar plate was removed from the sampler and placed in an incubator at 36°C ± 1°C for 18-72 hours. The bulk sampling of materials, such as settled dust in vents and visible microbial growth on bathroom surfaces, was collected and analyzed as described elsewhere.[26,27]

Identification of isolates

The fungal colonies were identified based on their macroscopic and microscopic characteristics. Macroscopic characteristics included colonial morphology, color, texture, shape, diameter, appearance, and lactophenol staining. Microscopic characteristics included septation in hyphae, presence of specific reproductive structures, shape and structure of conidia-spores, and presence of sterile mycelia.[28] The bacterial cultures were identified based on their colony morphology and growth on differential culture media, and gram-stain reaction.

Legionella *culturing*

All samples were processed, and *Legionella* isolates were cultured according to previously described methods.[29] *Legionella* was cultured on Buffered Charcoal Yeast Extract Agar (Diagnostic Systems, Sparks, MD) medium supplemented with glycine, polymyxin B, vancomycin, and cycloheximide.[30] Culture plates were incubated at 36°C ± 1°C for 72 hours, with an additional 96 hours, if necessary, for full colony formation. Colonies were presumed to be *Legionella* based on morphology.

Microscopy

All dust and biofilm samples were collected carefully without deformation of their surface features. For light microscopy, wet mounts were prepared and observed using an Olympus BX600 microscope (Olympus, Tokyo, Japan). For transmission electron microscopy, samples were embedded in resin and sectioned using a Reichert Ultracut E Microtome. The sections were stained with uranyl acetate and lead citrate and examined using a JEOL 1200EX transmission electron microscope (JEOL, Tokyo, Japan) equipped with a SIA model L3C CCD camera (Scientific Instruments and Applications, Duluth, GA).

DNA extraction and molecular analysis

Legionella samples were analyzed using polymerase chain reaction (PCR) as described elsewhere.[30] DNA was extracted from environmental samples using a ZYMO Research yeast/bacterial DNA extraction kit (Zymo Research, Irvine, CA), and PCR was performed using primers (LpneuF and LpneuR) specific for the *mip* gene. The PCR amplification reaction consisted of 12.5 µL Promega GoTaq Green MasterMix (Promega Biosciences, San Luis Obispo, CA), 10 µL DNA template, and 0.13 µM of each primer to result in a final reaction volume of 25 µL. Gel electrophoresis was performed in a 1% agarose gel containing 0.05 µL/mL of 10,000X Invitrogen SYBR Safe DNA Gel Stain (Life Technologies, Carlsbad, CA) to detect PCR products.

Preparation of spiked samples for aerosolization of Legionella

Pure cultures of *L pneumophila* (ATCC 33153) were obtained from ATCC (Manassas, VA). These cultures were propagated and maintained as recommended by ATCC. The titer of bacteria in each spray replicate was held constant at approximately 10^6 CFU/mL in 0.5× phosphate buffered saline (PBS). Stock concentrations were confirmed by plating a series of 10-fold dilutions of working stock on selective agar and examining for growth after 72 hour of incubation.

Study of synergy between Legionella *and fungi*

All fungal isolates were cultured on plates of Potato Dextrose Agar (BD Difco, Sparks, MD) incubated for no less than 1 week for

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