RAPID DETECTION AND QUANTITATION OF FUNGAL SPORES FROM DUST SAMPLES USING REAL-TIME PCR

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ABSTRACT

Recent advances in real-time PCR have permitted accurate, rapid and quantitative identification of microorganisms in pure cultures regardless of viability or culturability. In this study, a simple sample processing method was investigated for rapid identification and quantitation of fungal spores from dust samples using real-time PCR. The proposed method was evaluated for susceptibility to interference from environmental dust samples. The extent of inhibition was calculated using real-time PCR reactions containing *Aspergillus fumigatus* spores specific primers and probe and various amounts of dust. No interference ($p<0.05$) was detected from 0.2 mg of four real-world dust samples. However, dusts weighing > 0.2 mg compromised the assay. The overall results suggest the potential usefulness of our method for monitoring indoor microbial aerosols containing dusts weighing ≤0.2 mg using real-time PCR.

INDEX TERMS: Quantitative PCR, Fungal spores, Dust samples, TaqMan

INTRODUCTION

Fungal aerosols are found in many types of indoor environments including homes, hospitals, and offices. They have been implicated in detrimental health effects. Therefore, accurate estimation of exposure to airborne fungi is important. Traditional detection methods have mainly relied on microscopic and culture-based techniques, both of which have limitations. Microscopy requires technical expertise and is highly subjective. Culture-based methods are biased towards identification of rapidly growing fungi and fungi in high concentrations, and there is no ideal medium for growth of all fungi. In addition, non-culturable/non-viable spores are not detected by culture, but can be allergenic causing health problems. Thus, there is a need for better techniques to identify environmental exposures to fungi.

Recent developments in molecular techniques such as nucleic acid amplification methods including polymerase chain reaction (PCR) have provided significant advances in rapid detection and characterization of microorganisms irrespective of their viability or culturability. PCR has been demonstrated to be robust, flexible, sensitive and accurate for detection of over a hundred of the most common indoor fungi (Kahane, *et al.*, 2002). The majority of studies on real-time PCR have been performed using pure cultures grown in the laboratory and have not considered the effect of contaminants that might be present in environmental samples. A few studies have focused on removal of inhibitory compounds from samples using commercially available nucleic acid extraction kits (Haugland, *et al.*, 2002; Cruz-Perez, *et al.*, 2001a, b). Each additional step adds time and complexity, and generally reduces the DNA yield. Some investigators have added enhancement agents to overcome PCR inhibition (Bickley, Hopkins, 1999) and others have simply used serial dilution (Fode-Vaughan, *et al.*, 2001).

Previous studies in our laboratory investigated a simple sample processing method using 20% nutrient broth to maintain the specificity and sensitivity of conventional PCR and to overcome PCR inhibition in various dusts collected from a poultry house, hospital rooms, and HVAC filters. Those results showed no interference from dusts weighing 0.2 mg with a 10-fold dilution. The focus of the present research was to expand that study to real-time PCR. The dusts were sieved to represent the aerosolizable fraction and were spiked with spores of *Aspergillus fumigatus*. This fungus was chosen as a test organism due to its adverse health effects in indoor environments, and availability of species specific primers and probe for real-time PCR (Haugland, *et al.*, 2002). Detection sensitivity and reproducibility of the method was evaluated.

METHODS

A total of four dusts were tested for interference; settled dust collected from inside a heater in a poultry house, settled dust on chairs from two hospital rooms, and dust on filters from a commercial HVAC system (roof-mounted filters). The dusts were sieved through a 425-µm mesh (Sieve Shaker, W. S. Tyler Inc., Mentor, OH) to represent the aerosolizable fraction of dust. The dust amount ranged from 0.2 to 2 mg/sample.

Aspergillus fumigatus 17-30-37 was provided by Janet Simpson (NIOSH, Morgantown, WV) and was cultured on Malt Extract Agar (MEA, Difco 325, Difco laboratories, Detroit, MI, U.S.A.) plates at 24°C at a relative humidity of 32-40% for 10 days. The fungal spores were collected by gentle rolling of moistened, sterile cotton swabs over the surface of the colonies. Amounts ranging from 2 x 10^3 to 2 x 10^5 spores/sample were added directly to a sterile Eppendorf tube con-

taining 20% nutrient broth, known amounts of dusts and zirconia/silica beads. After sample processing, 200 spores corresponded to 1 spore DNA equivalent/PCR reaction.

To compare PCR amplification from crude extract and purified DNA of *A. fumigatus* spores, both preparations were made from the same stock of spores. The crude extract of spores with and without dust was prepared by the general method of bead-beating as described by Zhou, *et al.*, (2000) with slight modification. The modifications included addition of 0.3 g of 0.1 mm zirconia/silica beads (Biospec Products, Bartlesville, OK, U.S.A.) to 2.0 ml screw-cap tubes, 0.6 ml of 20% nutrient buffer, 0.1 ml of spore suspension of known concentration, and known amounts of dust. Standard curve was prepared using crude extract from 2×10^2 to 2×10^6spores/ml in sample. Four independent reactions were performed to determine the correlation between cycle number and initial copy number of templates [log (spore DNA equivalents)].

DNA was purified by FastDNA kit (Bio 101, Vista, CA) using the manufacturer's protocol with slight modifications to correspond to the protocol of crude extract preparation. The lysing matrix from the kit was substituted by the same amount and type of beads as mentioned before and added to similar 2.0 ml screw-cap tubes. The volume of CLS-Y solution was reduced to 0.6 ml from 1.0 ml. The samples were homogenized in a Mini-Bead Beater (Biospec Products, Bartlesville, OK, U.S.A.). The remaining steps were followed as mentioned in the manufacturer's protocol with respect to DNA purification using the spin filters and catch tubes in the kit.

The TaqMan assay was prepared and performed using previously designed primers and probes for *A. fumigatus* as described by Haugland, *et al.*, (2002). PCR inhibition was calculated by comparing the threshold cycle (or Ct value which indicates the fractional cycle number at which the amount of amplified target reaches a fixed threshold) obtained from samples with and without dust to an external standard curve linear over a 5 log scale. Each experiment was replicated twice, with PCR reaction from each sample being performed in duplicates.

Statistical methods were used to evaluate reproducibility of the experiments. The data analysis for this study was performed using SAS/STAT software, Version 8.2 of the SAS System for Windows (SAS Institute, Cary, NC). The standard curve regression equation was generated with Proc Reg utilizing the Ct values and natural log of the known concentration of spores. Differences between treatment groups were analyzed using a 3 (Spore concentration) by 5 (dust concentration) mixed model analysis of variance (Proc Mixed). Differences were considered significant at $p < 0.05$. To determine when the concentration of dust begins to inhib-

it the recovery of spores from the reaction, 95% confidence intervals were calculated.

RESULTS

The sensitivity of previously developed primer and probe set to detect *A. fumigatus* DNA in a crude DNA preparation was evaluated by two independent real-time PCR experiments. In one experiment, crude DNA extract was diluted ten fold serially. In the other experiment, crude DNA extract was directly obtained without dilution from specific number of spores. Each experiment was replicated twice, with PCR reaction from each dilution being performed in duplicates.

Figure 1(A). Comparison of Ct versus Log (Spore) for purified and crude DNA

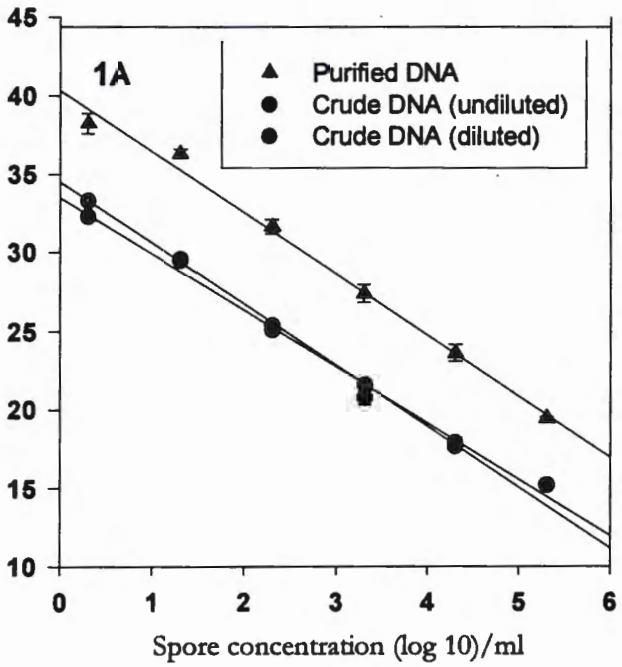


Figure 1(B) Comparison of Ct versus Log (Spore) for purified and crude DNA

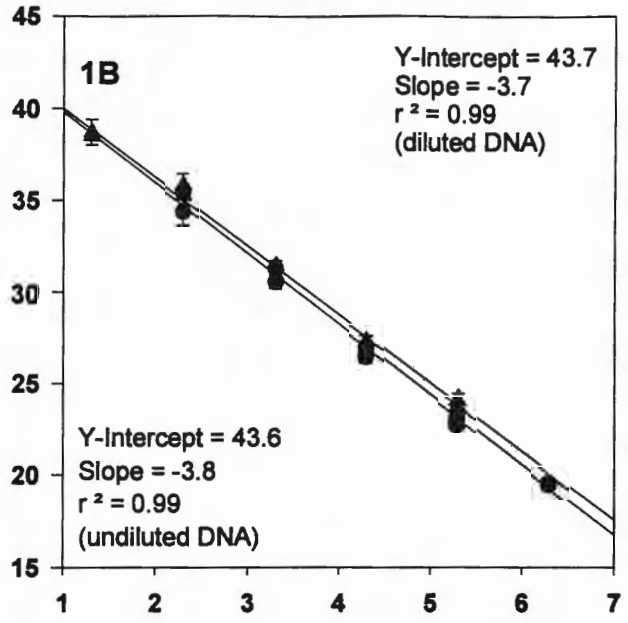


Figure 1A demonstrates that there is a correlation between target DNA concentration and changes in fluorescence over six orders of magnitude ($y = 34.5\text{-}3.8x$, $r^2 = 0.999$ for serially diluted crude DNA; $y = 33.5\text{-}3.6x$, $r^2 = 0.991$ for undiluted crude DNA). The standard curve from purified DNA was also linear over six orders of magnitude and parallel to that of crude DNA. However, the Ct values at each spore concentration were higher, indicating lower sensitivity ($y = 43.6\text{-}3.8x$, $r^2 = 0.99$ for undiluted DNA).

For comparison of real-time PCR in the presence or absence of dust, another standard curve was obtained using crude DNA from spores ranging from 2×10^2 to 2×10^6 spores/ml. These concentrations were chosen to encompass the range of spores normally encountered in indoor air. The results in Figure 1B show linearity over 5 orders of magnitude and reproducibility of results with replicate data points very close together. A 10-fold dilution of the samples mentioned above also resulted in a linear standard curve, with a sensitivity of detection of 0.1 spore DNA equivalent/PCR reaction.

A detailed study using real-world samples collected from a poultry farm was performed to investigate interference on real-time PCR. Dust samples ranging in

weight from 0.2 to 2.0 mg were spiked with 2 x 10^3 to 2 x 10^5 *A. fumigatus* spores/ml in sample.

Figure 2. *A. fumigatus* spore concentration in dust based on standard curve from real-time PCR.

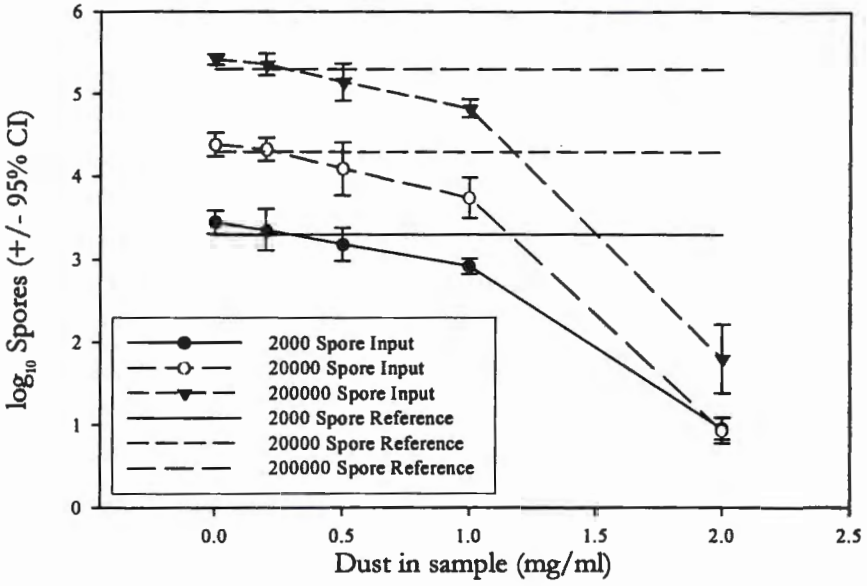
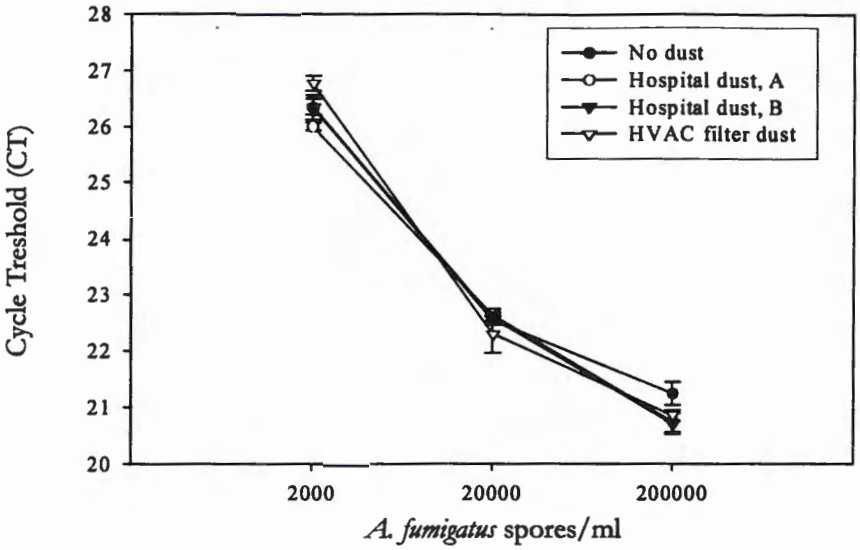


Figure 2 shows that the extent of inhibition by poultry dust on real-time PCR varies with amount of dust. For 0.2 mg of tested dust, no statistically significant differences were detected with undiluted or 10-fold diluted samples ($P > 0.05$). For 0.5,1 and 2 mg dusts, reduction in spore number (inhibition) compared with values determined in samples without dust was statistically significant ($P < 0.05$). However, the number of spores recovered from 0.5 mg dust was within 95% confidence intervals.

A preliminary study using 0.2 mg of 3 additional real-world dust samples collected from two hospital rooms and commercial HVAC filters were studied to evaluate the pattern of inhibition. No interference was detected from the 3 additional dusts with undiluted samples at p-value > 0.05 (Figure 3).

Figure 3 Comparison of Ct versus Spore concentration for 0.2 mg of 3 dusts.



DISCUSSION

A simple sample processing method for real-time PCR assay has been developed and evaluated in our laboratory. It has the potential for monitoring microbial aerosol in indoor environments in conjunction with rapid, sensitive and specific real-time PCR. The method includes bead-beating in the presence of nutrient broth, six times in excess of spore volume, a PCR enhancer, and a primer-probe set for real-time PCR.

The presence of contaminants in environmental samples can interfere with real-time PCR assay to varying degrees leading to false negatives or underestimation of microbial load. Most of the studies employing real-time PCR reported recently in the literature focused on purification of DNA from the samples (Haugland, *et al.*, 2002; Cruz-Perez, *et al.*, 2001a, b). DNA purification methods affect the detection limits of PCR-based methods either due to inefficient DNA extraction or loss of DNA during the process as seen by the variability in results (Poussier., *et al.*, 2002). Also, despite elaborate purification steps, sometimes it is necessary to dilute the DNA preparations (Cruz-Perez, *et al.*, 2001a, b).

In this study, we used crude DNA extracted in the presence of nutrient broth and bovine serum albumin (BSA) to maintain the integrity of samples with minimal manipulations. Nutrient broth, six times in excess of spore volume served as a dilu-

ent to overcome inhibition. Proteins from beef extract and peptone in the nutrient broth and BSA presumably act as scavengers for inhibitory compounds, preventing them from binding to and inactivating Taq DNA polymerase. Both, peptone and BSA have been reported earlier as enhancers of PCR (Bickley, Hopkins, 1999). These proteins may also nonspecifically increase PCR efficiency by preventing released fungal DNA from sticking to the beads used to lyse spores. Our results showed no interference using 0.2 mg of four different dusts.

For bioaerosols collected from environments with dust concentrations of < 100 $\mu g/m^3$ (typical of indoor environments) using a sampling flow rate of 2 l/min for 6 hour sampling time less than 0.072 mg of total dust is collected. Because 0.2 mg of various dusts did not show any inhibition, our method has the potential to monitor bioaerosols from indoor environments collected using low-volume samplers over a period of hours. The number of target organisms evaluated in dust samples was based on calculations using average fungal spore level in indoor environments as described by Zhou, *et al.*, (2000). The sensitivity of detection was as low as 200 spores/sample corresponding to 1 spore DNA equivalent/PCR reaction using a 10 fold dilution.

CONCLUSIONS AND IMPLICATIONS

The method proposed in this study has a sensitivity of detecting low levels of spores with sample processing achievable within one hour, followed by real-time PCR performed in three hours which includes set up of PCR-reactions. Our study used four different types of dusts spiked with *A. fumigatus* spores. This technique was tested using duplicate and replicate samples and shown to be highly reproducible. The sample processing method can be used in conjunction with any previously designed species specific primers and probe sets for real-time PCR. Thus the work can likely be extended to all the fungi in indoor environments that have been implicated in adverse health effects as long as the dust sample size is ≤ 0.2 mg.

ACKNOWLEDGEMENTS

The authors wish to acknowledge Drs. M. Harper, D. Lewis and D. Weissman at NIOSH for their advice and review of this paper. Poultry dust was provided by Steven Lenhart (Cincinnati); Hospital rooms and HVAC dusts were provided by Carol Rao (NIOSH, Morgantown, WV).

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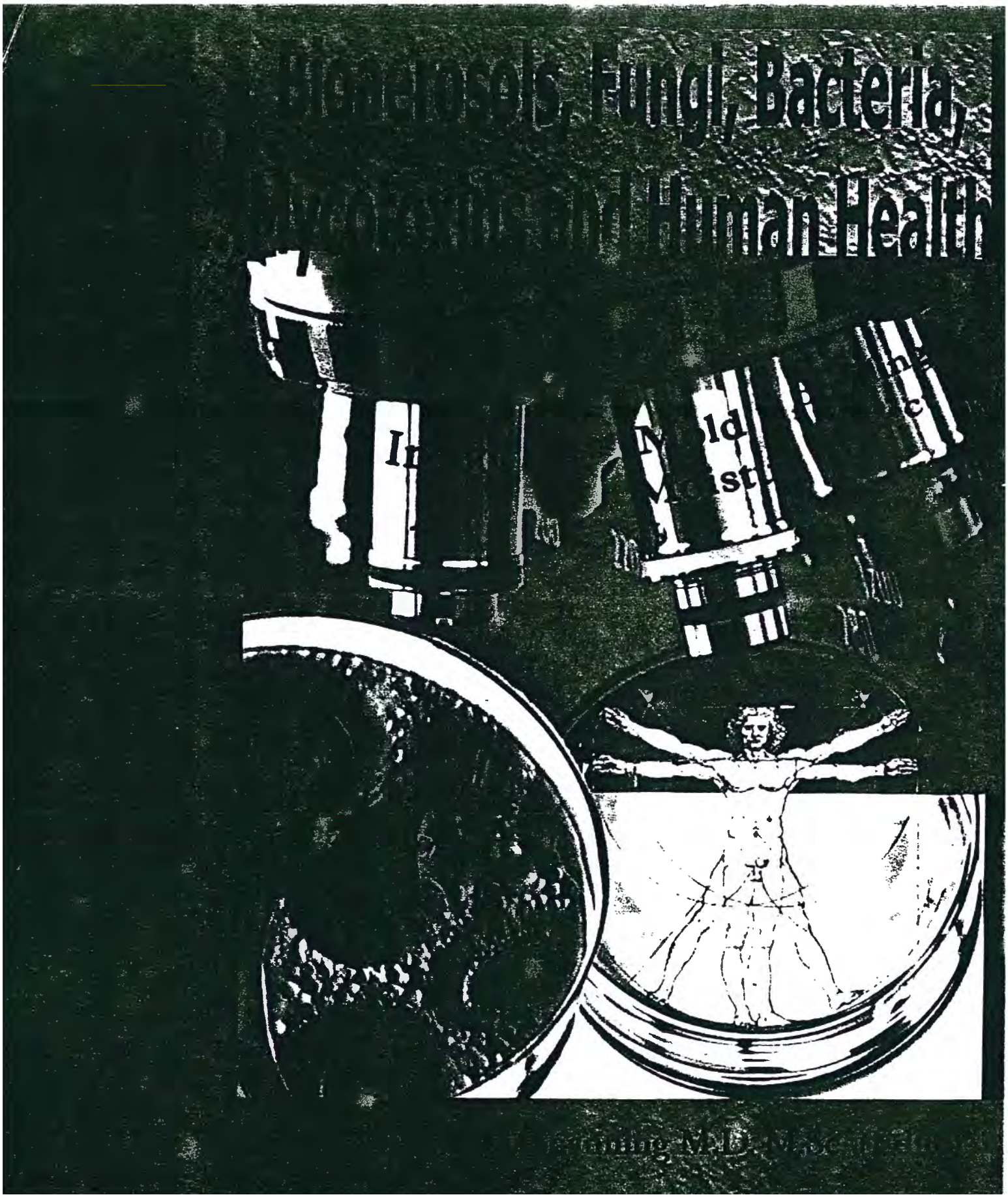
Human Health

Bioaerosols, Fungi, Bacteria, Mycotoxins and Human Health: Patho-physiology, Clinical Effects, Exposure Assessment, Prevention and Control in Indoor Environments and Work

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