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Application of Real-Time PCR to Identify Residual Bio-Decontamination of Confined Environments after Hydrogen Peroxide Vapor Treatment: Preliminary Results

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Abstract

This study was conducted to assess the effectiveness of Hydrogen Peroxide Vapor (HPV) to remove biological contamination in a confined environment and to evaluate real-time PCR assay as a technique for the evaluation of the decontamination efficiency. Decontamination after the dispersion of biological aerosol is a main issue from a civilian, public health and military perspective. Despite the effectiveness of aggressive substances, eco-friendly but still efficient methods for decontamination are a relevant demand and Hydrogen Peroxide Vapor (HPV) is among the most recent and promising technologies in this field. Another related issue is: when an environment can be considered fully decontaminated? The answer clearly depends on the objectives of the decontamination and this will affect the choice of the methodology.

Furthermore, classical microbiological and molecular biology techniques are commonly used to identify biological contamination and residual contamination, but many of them are time consuming and require advanced training for the operators who perform the analysis. This may represent a bottleneck, especially when a quick response to an emergency is needed (i.e. during an unconventional event like CBRNe ones). In this work, a combination of commercially available equipment for detection, identification and decontamination, was evaluated in partnership between the Italian Army, the Department of Industrial Engineering and the School of Medicine and Surgery of the University of Rome "Tor Vergata". The purpose of this work was to find a setup for equipment and methodologies for detection, identification and decontamination, to implement in case of biological events. Preliminary results show that, despite the death of the microorganisms, nucleic acids are not completely degraded by HPV treatment and, as a consequence, that real-time PCR may be the adequate, quick and easy method to verify the efficiency of bio decontamination when nucleic acid degradation represent the final objective.

Keywords: Hydrogen Peroxide Vapor; Real-Time PCR; Decontamination

Introduction

Contamination of confined environment with biological agents (i.e. toxins, vegetative bacteria, endospores, viruses, fungal and mold spores) is a main issue from both a civilian and military point of view.

From a "security" point of view, a biological event could be the result of the dissemination of biological agents due to a terrorist attack, routines operation in a healthcare setting [1-6] or to the outbreak of an infectious disease. In all those cases detection, identification and decontamination are among the main focuses. Key aspects, strictly related to this last aspect are: the bio-decontamination efficiency, the decontamination check system.

A biological event could affect a close environment, or in a wide open area, thus it becomes necessary to use an eco-friendly decontamination system: most available methods use substances, such as bleach or formaldehyde, which are each hazardous by themselves and produce further hazardous wastes. Advances in this field are represented by the use of hydrogen peroxide vapor (HPV) as surfaces decontaminant and sterilant [7] thanks to its anti-microbial effect due to the free radicals which acts on different biological molecules: lipids, proteins and nucleic acids [8].

HPV works well at low temperature and pressure, and is effective against a wide range of organisms including: bacterial endospores [9], vegetative bacteria [10] and viruses [11]. It is safer than other disinfectant gases such as formaldehyde and ethylene oxide, and decomposes to water vapor and oxygen [12]. For this reason, HPV is currently used to decontaminate laboratory and medical equipment, pharmaceutical manufacturing facilities, and healthcare settings [13-17], especially to reduce the risk of nosocomial infections [18-20].

Recent studies in this fields also proposes the use of HPV as

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decontaminant for hazardous chemicals showing that HPV is able to produce changes in the structures of several pharmaceutical substances, although changes strongly depended on the chemical groups in the substance [21].

Several systems for HPV production are commercially available, and each shows differences and similarities. The VHP system by Steris (Basingstoke, UK) operates as a dry system, reducing the relative humidity inside the enclosure, so that condensation does not form on surfaces. The HPV system, by Bioquell (Andover, Hants, UK), injects the hydrogen peroxide vapor into the enclosure at such a level that micro-condensation forms on the internal surfaces. Therefore, the environment control is imperative for both systems [22]. Other comparative studies of hydrogen peroxide-based systems for airborne decontamination showed differences in efficacy, efficiency, and safety [23,24], showing that technical aspects may strongly influence the decontamination results. All these aspects are to be considered when choosing the proper equipment for decontamination.

In case of intentional or unintentional release of biological agents, military forces and fire brigade are equipped and trained for detection identification and decontamination procedures of personnel and technical equipment. Since they have also molecular biology capability, an efficient decontamination of the equipment and the surrounding environment is paramount to avoid cross contamination issues [25].

The aim of this study was to simulate the diffusion of a biological aerosol in a confined environment, and to evaluate the efficiency of HPV as bio-decontaminant, with particular attention to the ability of HPV to degrade nucleic acids. With this aim, real-time PCR was proposed as a quick and easy technique to verify the residual biological contamination including that deriving from nucleic acids.

Materials, Instruments and Methods

The rationale of the trials is presented here. A solution containing *Saccharomyces cerevisiae* is aerosolized within a sealed chemical hood. Aerosol samples and samples of the Biological Indicator (BI), *Geobacillus stearothermophilus*, were collected before decontamination as positive control of the experiment. Two experiments were performed to evaluate differences in decontaminating with HPV a) immediately after the release of the aerosol or b) after 18 hours from the release.

At the end of the decontamination procedures, samples were collected and analyzed for cell viability; *S. cerevisiae* samples were further analyzed by real-time PCR to evaluate the presence of amplifiable target sequences.

Strains

A solution containing food grade *S. cerevisiae* lyophilized powder (Bertolini), at a concentration of 7 g/L, was aerosolized at a constant temperature of 25°C within a sealed chemical hood (1.5 m^3 volume) by means of an automatic atomizer. This atomizer guarantees a flux of 0-70 mL/min with a particle size in the range between 5 and 20 micron and the dispersion of the biological aerosol lasted for 3 minutes. It has to be clarified that *S. cerevisiae* is an "unofficial" biological warfare agent (BWA) substitute but it was chosen to guarantee safety of operators because of its non-pathogenicity, and also because of its structural characteristics which are in between prokaryotic and eukaryotic organisms (cell wall, nucleus, double strand linear chromosome). Besides, all the experiments were conducted using "Saccharomyces training kits" (Idaho Technology, Inc. now supplied as Bio Fire Diagnostics, Inc), which are manufactured to safely perform laboratory routines in the same experimental conditions as using BWA samples.

G. stearothermophilus spores (purchased from BIOQUELL) have been added as positive control of decontamination. *G. stearothermophilus* spores are commonly used as biological indicator for decontamination due to their high resistance to heat and vapor [26,27]. *G. stearothermophilus* spores inoculated on steel carriers (10 mm ø) to form a population of more than 1×10^6 CFU/carrier. This BI`s were placed on the most representative spots of the chemical hood i.e., the surface and the front glass of the hood for a total of 6 slides.

Hydrogen peroxide vapor bio-decontamination

Hydrogen peroxide vapor was produced and decomposed by the ClarusTM 'S' system (BIOQUELL). HPV was introduced within the hood by means of a double axis distribution system which guarantees an even distribution of HPV on the different surfaces; a schematic representation of the apparatus is shown in Figure 1. After 30 minutes of exposition, HPV was catalytically converted to water vapour and oxygen in the conversion phase which lasts 60 minutes, before continuation of operations.

Sampling methods

Samples from air and surfaces of the hood were collected before and after the decontamination procedure with HPV. Two different methods were used to sample the biological aerosol: a) active sampling of aerosol in air by means of a portable sampler (Fido 1 (FLIRTM), and b) passive sampling by positioning Petri dishes on the worktop of the hood.

The active sampler Fido 1 (FLIRTM) has a sampling flow rate of 200 liters per min and can collect particles whose size ranges between 0.5 and 10 microns.

Duration of sampling can be set by the operator according to the preset sampling duration: 5, 15, 30 and 60 min. In this work, a sample

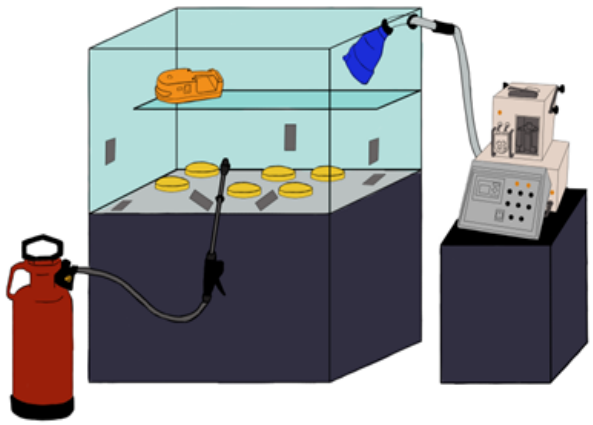

Figure 1: Representation of the experimental apparatus showing the chemical hood, the automatic atomizer (for the dispersion of *S. cerevisiae*) and the HPV production system. The system is sealed and no hydrogen peroxide vapor is allowed to escape. Petri dishes (in yellow) were placed on the worktop of the hood together with the automatic air sampler (orange), to collect samples of *S. cerevisiae*. Stainless steel indicators (grey rectangles) inoculated with *G. stearothermophilus* was placed on the worktop and on the internal walls of the hood.

time of 5 minutes was chosen after considering the volume of air contained in a 1.5 m^3 hood.

A 5 minutes sample duration, with a flow of 200 liters per min (for a total of 1000 liters of air) guarantees that the same air volume is not collected twice during the same sampling operation. Collected samples are then diluted in a water volume of 5 ml.

Passive sampling was performed by swabbing the 6 opened Petri dishes positioned on the worktop of the hood and plating swab on Agar Sabouraud [28].

Air and surface samples were collected at time intervals of an hour, and after about 24 hours from the release.

All samples were collected once, thus, the outcomes of this work are the result of single experiments.

Culture media

S. cerevisiae samples (0.1 mL from *S. cerevisiae* samples collected immediately after aerosolization; 0.1 mL solution from air sampler and swab from the Petri dishes) were plated on Agar Sabauraud [28] and incubated at a temperature of 37°C for 48 hours.

G. stearotermophilus samples were inoculated in vials containing 10 mL of Tryptic Soy Broth (TSB) (BIOQUELL) and incubated at a temperature of 65°C for 7days.

Real-time PCR

DNA extraction from the samples has been performed using the "IT 1-2-3DNA Sample purification kit" (BioFire Diagnostics, Inc).

Real-time PCR assay was conducted using the Ruggedized Advanced Pathogen Identification Device. The R.A.P.I.D.TM Bio Detection system (Idaho Technology Inc, now supplied as Bio Fire Diagnostics, Inc) is a ruggedized, portable real-time PCR designed to identify biological agents especially for mobile analytical labs and fields hospital.

The "*S. cerevisiae* Detection Kit for Hybridization Probe assay" (Idaho Technology, Inc., now supplied as Bio Fire Diagnostics, Inc.) was used for the identification of the agent; the kit consists of lyophilized reagents including primers which have specificity for *S. cerevisiae* and are validated according to the GMP (Good Manufacturing Practice) standards.

A fragment of the target DNA is amplified using specific primers. The amplicon is detected by fluorescence using a specific pair of hybridization probes. These probes consist of two different short oligonucleotides that hybridize to an internal sequence of the amplified fragment during the annealing phase of the reaction cycle. One probe is labeled at the 5' end with LC Red 640. To avoid extension on the 3' end, it is modified by phosphorylation. The second probe is labeled at the 3' end with fluorescein.

Only after hybridization to the template DNA do the two probes come in close proximity, resulting in fluorescence resonance energy transfer (FRET) between the two fluorophores.

The fluorescence emitted by the LC Red 640 dye is measured in channel 2 of the R.A.P.I.D. instrument.

The fluorescent signal from the unknown sample is compared to the signals from the positive and negative control samples.

Real-time PCR reactions were optimized at a temperature of 90°C for 5 sec, 35 amplification cycles at a temperature of 60°C for 15 sec. The melting curve analysis has been performed reaching a temperature of 90°C with a ramp of 0.2°C/sec.

For real-time PCR cycles settings, melting curve settings and sample preparation, protocols have been implemented according to the manufacturer's instructions.

Results

Real-time PCR assay, inoculation in liquid media of *G. stearotermophilus* spores and plating of *S. cerevisiae* were conducted for: a) decontamination with HPV immediately after the biological aerosol dispersion,

b) decontamination with HPV after 18 hours from the biological aerosol dispersion.

Decontamination immediately after the release

Real-time PCR assay on *S. cerevisiae* samples was conducted before and after the decontamination procedures. Results are shown in Table 1.

Decontamination efficiency has been tested on *S. cerevisiae* by plating samples collected both with the active and passive method (see section Materials, instruments and methods) on Agar Sabouraud and incubating the plates at a temperature of 37°C for 48 hours. After that the presence or the absence of microorganisms on the plate was evaluated. Results are shown in Table 2.

Decontamination efficiency was also tested on *G. stearothermophilus* spores used as biological indicator inside the hood. Steel carriers were collected after decontamination with HPV and inoculated in TSB. Table 3 shows results after an incubation period of 7days at a temperature of 60°C.

Decontamination after 18 hours from the release

Real-time PCR assay on *S. cerevisiae* samples was conducted before

	Sampling method	Real-time PCR assay
Before decontamination	SWAB	Positive
	Fido 1	Positive
After decontamination	SWAB	Positive
	Fido 1	Negative

Table 1: Real-time PCR results before and after decontamination with HPV.

Time (min)	Swab	Air sampler	*S. cerevisiae* culture after Atomization (positive control)
0	/	/	growth
5	growth	growth	/
135	no growth	no growth	/

Table 2: Swab from Petri dishes on the worktop and 0.1 mL of solution from air sampler plated on Agar Sabouraud at different timing during decontamination with HPV.

Samples	Bacterial growth
Positive control (Non treated)	Positive
Hood internal wall	Negative
Hood worktop	Negative
Negative control (TBM not inoculated)	Negative

Table 3: *G. staerothermophilus* growth on TSB after decontamination with HPV.

and after the decontamination procedure which, in this case, started 18 hours after the dispersion of the yeast in the hood. Results are shown in Table 4.

Decontamination efficiency on organisms vitality was tested on *S. cerevisiae* by plating samples collected both with the active and passive method on Agar Sabouraud and incubating the plates at a temperature of 37°C for 48 hours. Results are shown in Table 5.

Steel carriers inoculated with *G. stearothermophilus* spores were collected after decontamination with HPV (18 hours after the release of biological aerosol) and inoculated in TSB. Results after an incubation period of 7 days at a temperature of 60°C are shown in Table 6.

Discussion

According to the data collected from these preliminary trials, several aspects emerged. Decontamination with HPV showed similar results either when performed immediately after the release of *S. cerevisiae* and with a delay of 18 hours from the release, when almost all the aerosol is deposited on the worktop of the hood.

As a matter of fact, contamination evaluated by inoculation of samples (before and after the decontamination procedures) of *S. cerevisiae*, from active or passive sampling, shows the absence of microbial growth independently from weather decontamination is performed immediately after the release of the aerosol, or after 18 hours from the release. This result is also supported by the evaluation of bacterial growth after treatment with HPV of *G. stearotermophilus* in the same experimental conditions. No growth is observed from samples placed in the hood internal walls or work top when inoculating the steel carriers in TSB.

Controversial results were obtained from real-time PCR analysis. Real-time PCR on *S. cerevisiae* samples collected when decontamination is performed immediately after the dispersion of the yeast, show positive results (i.e. amplification of the sample) for the swab and negative results for air samples.

On the contrary, in case of decontamination after 18 hours from the dispersion of *S. cerevisiae*, amplification is present either in the sample collected from the petri dishes and from air.

This unexpected result may suggest that HPV is partially effective in degradation of *S. cerevisiae* nucleic acids when the yeast is still in the air fraction despite the fact that HPV is a surface sterilant; furthermore this would be true only if the positive result from decontamination after 18 hours can be attributed to a re-suspension of nucleic acids from the worktop of the hood. This last point may be explained considering the high sampling capacity of the air sampler versus the small volume of the air in the hood.

Nevertheless, real-time PCR seems to be a promising tool to detect residual contamination from biological agents (with the exception of toxins) since positive results were associated with negative growth on culture media for *S. cerevisiae* plated on Agar Sabouraud.

These results also suggest that, despite the ability of HPV to act as an antimicrobial agent, it cannot fully degrade nucleic acids. As a matter of fact, results show that the specific real-time PCR primers still recognize their target sequences of the microorganism.

Another relevant aspect that has to be better investigated is that the vitality of microorganisms may be affected by the atomization process and also by the sampling methods especially by the automatic ones.

The first aspect may not be a major issue since atomizers are used to simulate the atmospheric dispersion of biological agents in laboratory conditions, and our preliminary results show that *S. cerevisiae* samples collected before the decontamination procedures are fully able to grow on culture media but the sampling methods may significantly affect the results of the analysis.

Passive sampling efficiency strongly depends on the aerodynamic properties of the particles, which affect their deposition velocity, and the movement of air in the environment.

This method is not quantitative: it is not possible to correlate the number of microorganisms detected to the sampled air volume. The sensitivity is also low: this could explain the negative result of the real-time PCR on the sample collected by swabbing the petri dish before decontamination, although an error in the sampling preparation cannot be ruled out.

This observation is supported by the positive, result from real-time PCR performed before decontamination in the second trial (i.e. when decontamination is performed 18 hours after the aerosol release) which, can be considered a duplicate of the experiment.

Active sampling is more efficient and allows for correlation between the amounts of detected microorganisms to the sampled volume of air. On the other hand, it may mechanically stress the microorganisms, especially if they are not spores.

For this reason, a conventional microbiological analysis may not be able to identify viable but non-culturable [29] microorganisms.

In this last case, the contribution of real-time PCR techniques appears to be even more useful to identify a biological contamination and then, to verify the efficiency of the decontamination procedures.

Conclusion

An efficient decontamination in case of biological threats (both in

	Sampling Method	Real-time-PCR assay
Before decontamination	SWAB	Negative
	Fido 1	Positive
After decontamination	SWAB	Positive
	Fido 1	Positive

Table 4: Real-time PCR results before and after decontamination with HPV.

Time (hours)	Swab	Air sampler	*S. cerevisiae* culture after atomization (positive control)
0 (aerosol release)	/	/	growth
5 h	growth	growth	/
18 h	growth	no growth	/
	Decontamination START		
20 h	Decontamination STOP		
20.75 h	no growth	no growth	/

Table 5: Swab from Petri dishes on the worktop and 0.1 mL of solution from air sampler plated on Agar Sabouraud at different timing before and after decontamination with HPV.

Samples	Bacterial growth
Positive control (Non treated)	Positive
Hood internal wall	Negative
Hood worktop	Negative
Negative control (TBM not inoculated)	Negative

Table 6: *G. staerothermophilus* growth on TSB after decontamination with HPV (18 hours after the release of biological aerosol).

civilian and military contest) is a key issue for the safety of population and operators. In case of biological event, it is essential to have quick and easy tools to detect, identify and neutralize the biological threat. In this work the authors evaluated the efficiency of commercially available equipment to produce HPV, a more eco-friendly decontamination technique. This technique was used to decontaminate a confined environment after the dispersion of *S. cerevisiae*. Samples collected before and after the decontamination procedures showed that cellular growth on Agar Sabouraud is absent after a treatment with HPV for 30 minutes (independent on whether the decontamination procedure started immediately, or 18 hours after the aerosol dispersion). This efficacy is also achieved for *G. stearothermophilus* spores which are intrinsically more resistant to HPV.

Real-time PCR assay performed before and after decontamination showed positive results for the amplification of specific target sequences on *S. cerevisiae* genome. This suggests that treatment with HPV is not sufficient to degrade nucleic acids since they are still amplifiable by real-time PCR.

For this reason, in order to guarantee an efficient decontamination from nucleic acids and to prevent PCR carryover contamination, and contamination of samples and equipment, HPV decontamination should be supported by other decontamination techniques.

Real-time PCR assay revealed to be a quick, easy and effective method to identify contamination from biological agents, but also to verify the efficiency of the decontamination procedures when degradation of nucleic acid is required.

Further studies, including cost benefits analysis will be required to assess whether real-time PCR assay may be an implementation of the conventional techniques for the identification of residual biological contamination.

This approach is a clear example of a dual-use technique that can be translated from military to civilian world.

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