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REVIEW

Models of evaluation of antimicrobial activity of essential oils in vapour phase: a promising use in healthcare decontamination

Juan Bueno*

Bioprospecting development and consulting, Bogotá, Colombia

*Corresponding author. Email: juangbueno@gmail.com

Abstract

Hospital-acquired infections caused by viruses, bacteria and fungi are a constant concern in the health system. In this respect the decontamination of hospital environments is a control measure which decreases the reservoirs of pathogenic microorganisms and their transmission. For that reason new products have been developed such as antimicrobial surfaces that prevent microbial contamination and cleaning vapour systems, in search of a potent disinfection method more friendly to the environment, less toxic, safer and biodegradable. In this way has been considered the use of microbicidal essential oils (EOs) in vapour-phase as an interesting alternative in the development of hospital decontamination devices. Although *in vitro* antimicrobial activity of EOs have been demonstrated, there are no standardized tests to evaluate these products in vapour-phase, the aim of this review is to present the different evaluation methods that have been used to establish the activity of the vapours of EOs and other disinfectants, with the purpose of provide a rational approach to the research, development and implementation of new biocide agents based on this natural product for cleaning in hospitals and healthcare.

Keywords: Nosocomial infections, decontamination, essential oil, antimicrobial activity, vapour phase

Introduction

Hospital-acquired (nosocomial) infections is a worldwide healthcare problem, with a general prevalence in developing countries of 15.5 per 100 patients in where patients in intensive care are the most affected with a global prevalence of 47.9 per 1000 patient-days (Allegranzi et al., 2010). The microorganisms isolated more often from patients and hospital environments are *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Escherichia coli*, *Clostridium difficile*, *Streptococcus* species, *Enterobacter* spp, *Acinetobacter* spp., *Klebsiella* spp., influenza virus and noroviruses (Gadi, Borkow & Monk, 2012). In addition the pharmacological treatment of nosocomial infection is compounded by the emergence of drug-resistant pathogens (Gady, Borkow & Gabbay, 2010). Also, microbial colonization and persistence in medical surfaces have been associated with the existence of intrahospital infections (Tétault et al., 2012; de Abreu et al., 2014). For that reason the constant cleansing using biocides (antiseptics and disinfectants) reduces the density of microorganisms in healthcare environments (McCoy et al., 2014). Equally surgical site infections can be reduced by use of skin antiseptics in a decolonization process or antiseptic wound lavage until 41% (Hakkarainen et al., 2014; Yokoe et al., 2014).

Between the strategies developed to reduce the environmental microbial burden through biocides is the vaporous decontamination, which consists in the application of a decontaminant agent in vapour (or gas) phase to decontaminate confined spaces and medical devices (Kačer et al., 2012). In the use of this decontamination method, it is very important to take into account that the efficacy depends of the control of following parameters such as biocide concentration, exposure time, temperature, humidity, and the contaminant conditions (Fraise, 2013). Vapour phase decontamination and sterilization techniques have been

applied in processes as pharmaceuticals manufacturing, equipment cleaning, cleaning healthcare rooms and foodstuffs (Arlene, Klapes & Vesley, 1990). In this way, biocides such as formaldehyde have been employed, but it is a human carcinogenic chemical so its use has been restricted (Johnston et al., 2005); currently, hydrogen peroxide vapour is the most advantageous biodecontamination method with activity against bacterial endospores, vegetative bacteria, viruses and mycobacteria (Hall et al., 2007).

An interesting choice in the use of biocide in vapour phase are essential oils (EOs) due to their content of volatile compounds with antimicrobial activity that have been considered as a promising alternative to biocides and antibiotics. This antimicrobial activity is determined by the synergistic action of functional groups present in the oil as phenols, aldehydes, ketones, alcohols, ethers and esters (Li et al., 2014). Equally EOs and their components have shown excellent results against multidrug-resistant (MDR) bacteria such as methicillin-resistant *S. aureus* (MRSA), which makes them important for the development of hospital biocides (Maria, Faleiro & Miguel, 2013). In addition, several studies have confirmed that EOs used in vapour phases are more potent antimicrobials if used in liquid form between them thyme, citrus oil, *Eucalyptus globulus*, *Melaleuca alternifolia* and lemon grass (Katie, Laird & Phillips, 2012; Nadjib et al., 2014).

Currently, although vapour phase screening platforms for EOs have been described, there are no standardized tests to evaluate the antimicrobial activity of these vaporized products (Al-Yousef, 2014). The aim of this review is to present the different evaluation methods that have been used to establish the activity of the vapours of EOs and other disinfectants, with the purpose of provide a rational approach to the research, development and implementation of new biocide agents based in this natural product for decontamination in healthcare.

Vapour-phase Decontamination

The hospital environment is a constant source and reservoir of MDR microorganisms (Radhouani et al., 2014). Equally, there is an association between contamination of healthcare spaces and the increase of nosocomial infections (Chemaly et al., 2014). In addition, surfaces, medical equipment and other fomites are frequently contaminated after contact with patients or contaminated surfaces (Weber et al., 2010). For that reason the use of environmental disinfection strategies have the ability to reduce transmission (Steinberg et al., 2013). These strategies have two objectives, first the cleaning and disinfection of hospital rooms to reduce the risk of acquired pathogens from contaminated surfaces. Second, the disinfection of surfaces to reduce the risk of contamination and transmission (Donskey, 2013; Weber et al., 2013).

In fact, many methods of disinfection are unable to remove environmental MDR (Tuladhar et al., 2012; Passaretti et al, 2013). These microorganisms have the ability to persist on inanimate surfaces for days and months, increasing the risk of transmission (Table 1.)(Kramer et al., 2006; Chemaly et al., 2014). In this way, hydrogen peroxide vapour (HPV) has been demonstrated to be a microbicidal vapour-phase method that have the ability of destroy nosocomial pathogens *in situ*. Equally, HPV have eliminated environmental reservoirs of MDR more efficiently than other cleaning methods so it is now considered an election in hospital disinfection (Passaretti et al, 2013).

Table 1. Survival time of MDR microorganisms that causing nosocomial infections (Chemaly et al., 2014).

Microorganisms	**Survival time**
Methicillin-resistant *Staphylococcus aureus* (MRSA)	7 days to >12 month
Vancomycin-resistant *Staphylococcus aureus* (VRSA)	5 days to >46 months
Pseudomonas aeruginosa	6h to 16 months
Clostridium difficile	>5 months (spores)
Acinetobacter baumannii	3 days to 11 months
Norovirus (feline calicivurus)	8h to 7 days
Rotavirus	6-60 days

Antimicrobial Activity of Essential Oils in Vapour Phase

EOs have higher microbicidal potency in vapour phases more than their liquid phases (Imaël Henri Nestor, Bassolé & Juliani, 2012). Antimicrobial activity of various EOs in vapour phase have been described between them *Eucalyptus* (Tyagi et al., 2014), *Melaleuca alternifolia* (Carson et al., 2006) *Cymbopogon citratus* (Kumar, Tyagi & Malik, 2010; 2012) and *Thymus vulgaris* (Tullio et al., 2007). As well as major compounds as limonene and citral (Chee et al., 2009; Kumar, Tyagi & Malik, 2010). The antimicrobial action of these vapours depends of the presence in a gaseous state of functional groups in EOs, as well as their vapour pressure, allowing it to cross the microbial cell membranes (Belletti et al., 2007). This results in very low concentrations are very active (1.56–6.25 μg/ml) in inhibit bacterial growth, making them ideal for the development of vaporized biocides (Reichling et al., 2009).

Between the action mechanisms of these vapours has been found to produce in microbial cells shrinkage and partial degradation, observed by scanning electron microscopy/atomic force microscopy SEM/AFM of *C. albicans* under exposition of *Cymbopogon* oil in vapour-phase (Kumar, Tyagi & Malik, 2010). Equally, the EOs have the ability of modulate bacterial resistance mechanims (efflux pumps) by gas contact as oils obtained from *Zanthoxylum articulatum* and *Hyptis martiusii* Engler which makes them useful as adjuvants in antibiotic therapy and disinfection (Coutinho et al., 2010; Rodrigues et al., 2010; de Oliveira et al., 2014). Also ionized gaseous species of EOs from orange and thyme generated by candles shown antibacterial effects mediated by reactive oxygen species (ROS) that induce cell membrane disruption (Gaunt et al., 2005). Other interesting finding is the concomitant use of EOs in vapour-phase and heat that can produce hyphal damage of *Trichophyton mentagrophytes* at 27ºC, fact that can be taken into account when designing an atomiser (Inouye et al., 2007).

In this order ideas EOs in vapour-phase have been used in formulations for environmental decontamination as BioScent™ that contains oils of lemongrass and geranium and being dispersed using the ST Pro™ machine (Scent Technologies Ltd) was able to inhibit MRSA after 20 hours of exposure (Doran et al., 2009). Also, Citri-V™, that contains oils from orange and bergamot in concentrations of 1:1 v/v had antimicrobial activity against *Enterococcus* sp. and *S. aureus* on stainless steel surfaces as well as bacterial biofilms (Laird et al., 2012). Which demonstrates the potential of these natural products used as biocides in hospital environments.

Methods in Vapour Phase Evaluation

These methods determine the minimal inhibitory dose (MID), which is a measure of the vapour antimicrobial activity that permits to know vapour concentration and absorption for inhibit microbial species, also MID can be established in function of time for determine if EOs are more effective in high or low vapour concentration as well as in short or long exposure time (Inouye et al., 2001).

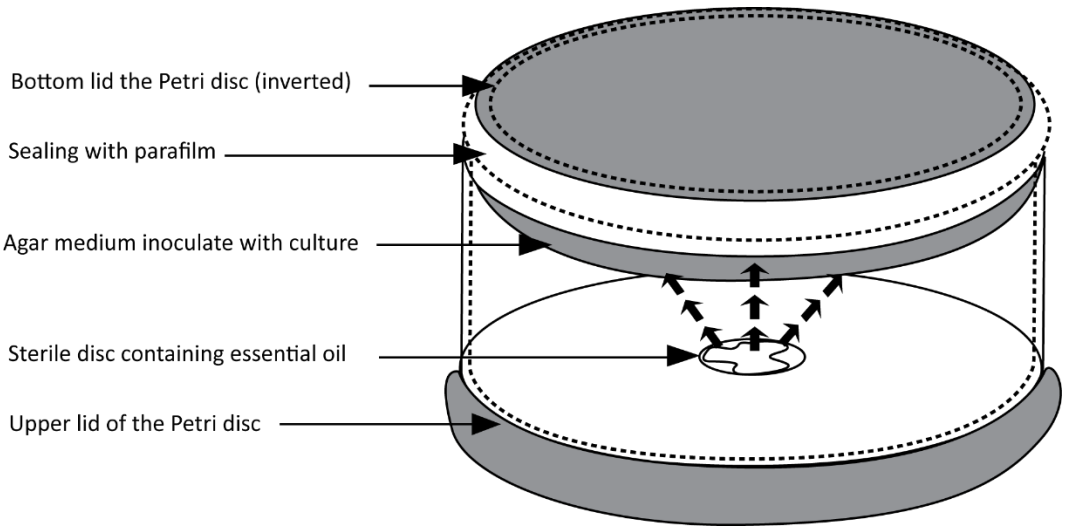
Disc Volatilization Assay

This assay require a culture agar plate inoculated with 100 μL, of microbial suspension containing 10^6 colony-forming unit (CFU)/mL inserted upside down on top of a container (Figure 1.). A paper disc (diameter 6mm) is deposited at the bottom of the container with 10 µL of different dilutions of EO. The plates inoculated and the disc should be sealed with parafilm to prevent the steam outlet. Plates should be incubated at 30°C for 24h (also can be observed in function of time) and measure the resulting inhibition (Kumar, Tyagi & Malik, 2012).

A variant of this method for antibiofilm activity of EOs in vapour phase can be developed using a paper disc (1 cm diameter) soaked with EO or terpene and fixed on the cover lid of 96 multiwell plate with the biofilm. LIVE/DEAD BacLight Viability kit can be employed for measure the biofilm mass after exposure (Nostro et al., 2009; Bueno, 2014).

Disc volatilization assay is the simplest assay for antimicrobial evaluation of EOs in vapour-phase and can be used as primary screening for to choose the most promising oil, terpene or their combinations.

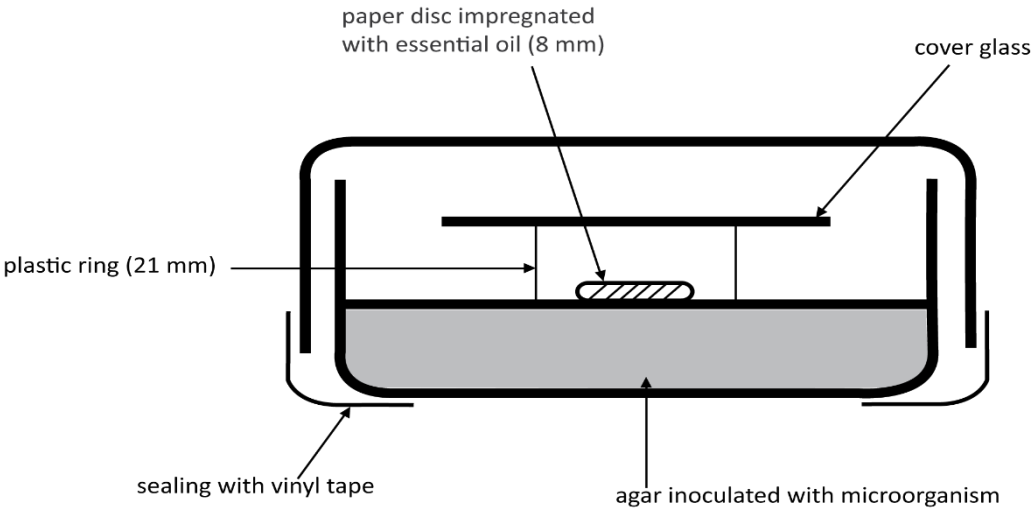
Figure 1. Disc volatilization assay



Agar Vapour Assay

Agar vapour assay is other assay useful as primary screening, and have the ability of control the evaporation reducing the place of exposure. In this technique, a paper disc of 8mm containing 30 µl of different dilutions of EO is placed over agar medium inoculated with microbial suspension containing 10^6 CFU/mL surrounded by a plastic ring and covered by glass in a Petri dish, later the plate should be turned upside down and incubated at 30°C for 24h (also can be observed in function of time) for measure of inhibition zone and obtain MID of the EO vapours, the advantage of this method is the control of evaporation and the measure of antimicrobial activity in a limited space (Figure 2)(Inouye et al., 2006).

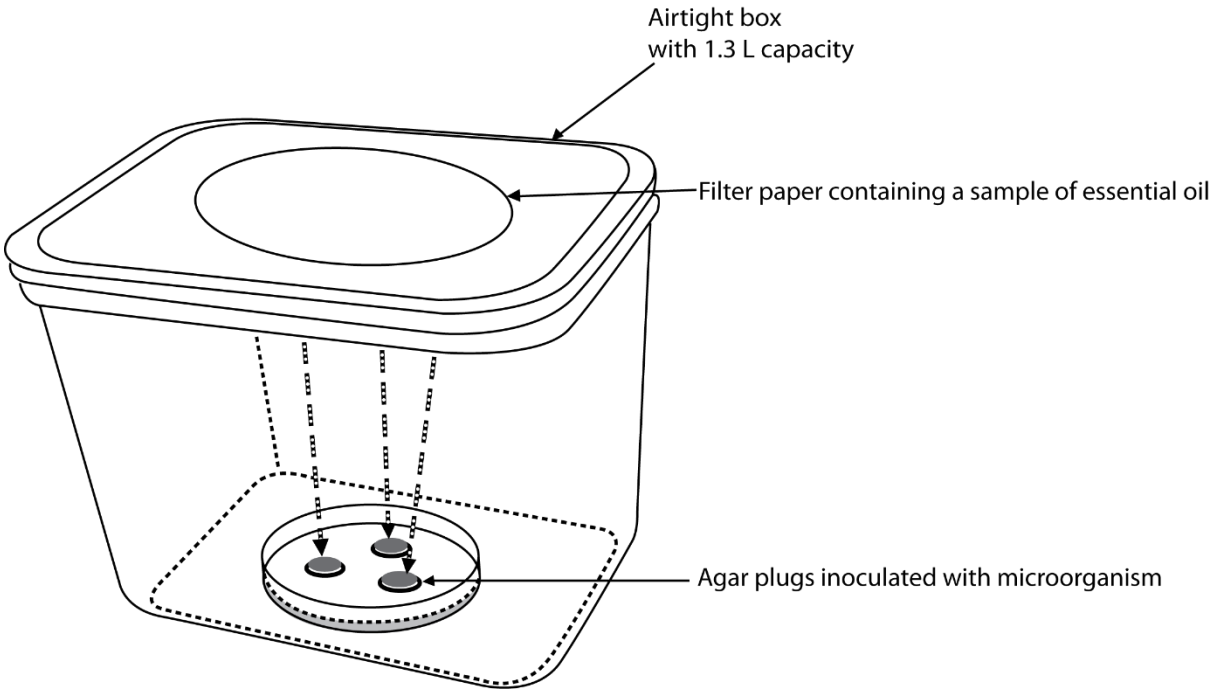
Figure 2. Agar vapour assay



Airtight Box

This is another assay that can be useful to determine the antimicrobial activity of the vapours in function of space using plastic boxes of known volumes. This method uses Petri dishes with culture media or agar plugs inoculated with 10^6 CFU/mL of microbial suspension and placed in 1.3 L airtight boxes recovered with aluminium foil for prevent the plastic absorption of EO and protect boxes of the contamination. EOs in different dilutions can be inserted in the top of airtight box on filter paper of 9 cm in diameter (Figure 3.). The boxes should be incubated to 37ºC for 24 h (also can be observed in function of time to 0, 2, 4, 6, 8, 12 and 24 h) and the MID determined in mg of EO/L of air (mg/L) is established where the EO concentration did not allow microbial growth (Inouye et al., 2000; Inouye et al., 2001a; Inouye et al., 2001b). This assay have the advantage to permit the use of inoculated material as plastic and steel to evaluate surface decontamination.

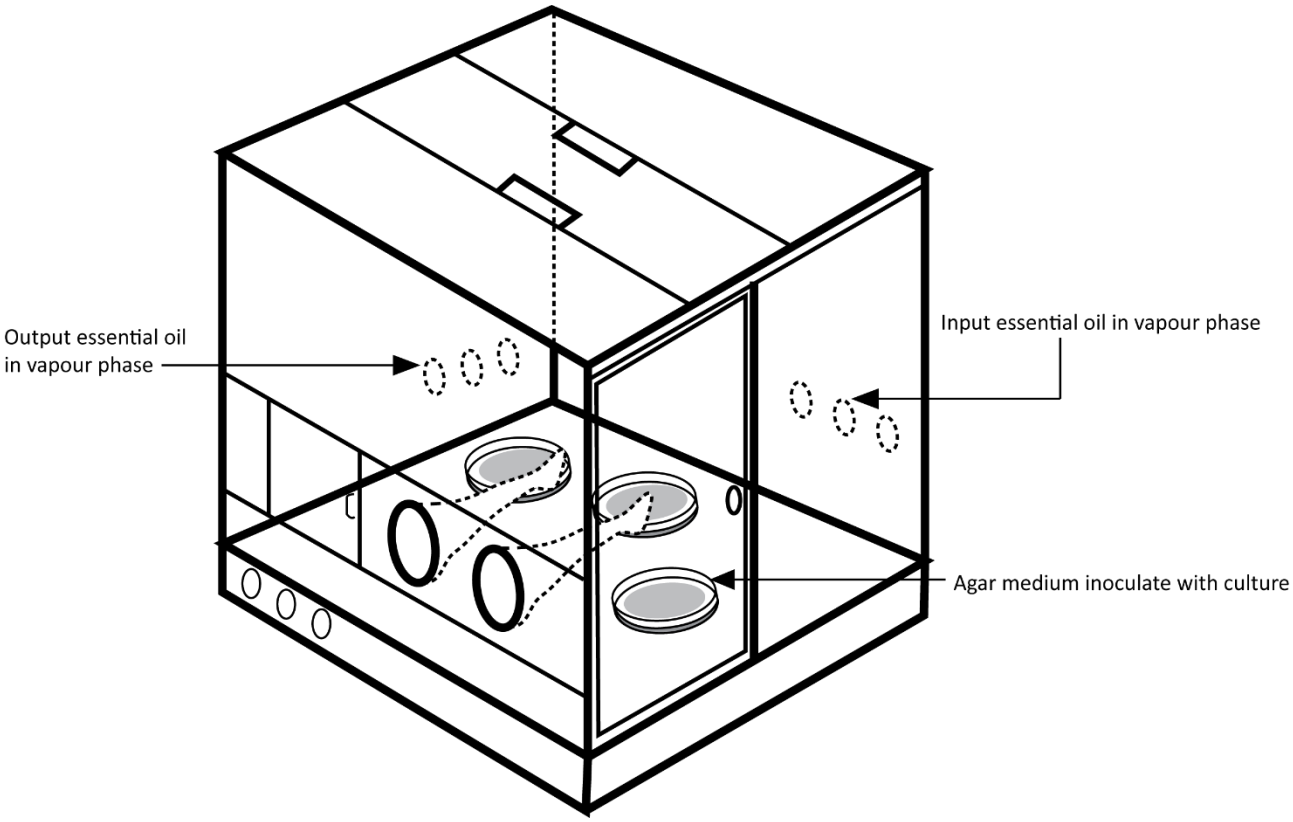
Figure 3. Airtight box assay



Bio-clean space

After primary screening is necessary to determine the decontamination activity of vapours of EOs in laboratory spaces as safety cabinets and bio-clean rooms. In this way bio-clean space decontamination is a useful test. This technique uses Petri dishes inoculated with microorganisms to concentration of 10^6 CFU/mL in agar medium or glass, cotton, plastic and steel material introduced in a sealed safety cabinet or in bioclean room with inlet and outlet fumes (Figure 4.) (Masaoka et al., 1982). With this assay is possible run several experiments in function of time with various types of microorganisms and require control of vapour pressure, temperature and relative humidity, after performing exposure, the Petri dishes should be closed and the materials suspended in sterile saline solution for inoculation in agar plates and incubation, with the aim of assess the degree of decontamination by oil spray (Moat et al., 2009).

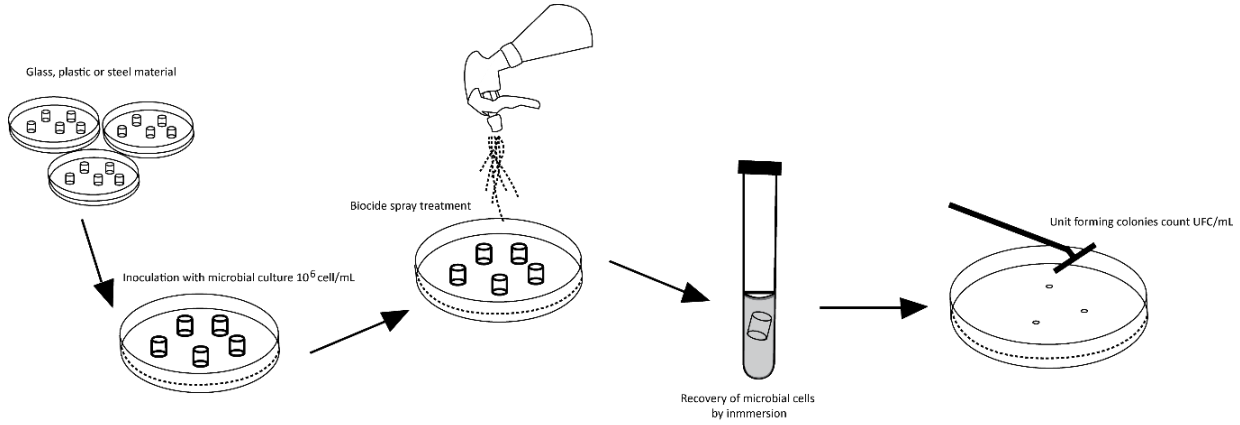
Figure 4. Bio-clean room assay in a safety cabinet



AOAC method 961.02 (Germicidal Spray Products as Disinfectants)

This is a standard method used for evaluation of products as a germicidal for disinfecting surfaces such as glass, metal, plastic and fibres (Pines et al., 2013). Several germicidal spray composition containing EOs have been evaluated under this technique against reference microorganisms as are *Salmonella cholerasuis* ATCC 10708, *Staphylococcus aureus* ATCC 6538, *Pseudomonas aeruginosa* ATCC 15442, *Escherichia coli* ATCC 8739, *Streptococcus pneumonia* ATCC 49619, and *Listeria monocytogenes* ATCC 19113. This Association of Official Analytical Chemists (AOAC) procedure is an accepted method for disinfectant evaluation. First, the microorganisms grown for 48 h in nutrient broth are inoculated on the test material and exposed to germicidal spray with EOs by time intervals of 1, 2, 5 and 10 minutes, the germicidal activity must be neutralized using letheen broth and the microorganisms should be recovered in sterile saline solution (Bowker, 2009). The use of different materials (carriers) allows evaluate the test substance and viability of microbial cells in a specified contact time (DeAth, 2008). Germicidal formulations containing thyme oil have been evaluated with this assay (Samuel, DeAth & Weiss, 2012).

Figure 5. AOAC method 961.02 (Germicidal Spray Products as Disinfectants)



Biofilm Surface Decontamination Tests with Vaporized Biocides

Although the polysaccharide matrix components of biofilms decrease gas penetration (Epstein et al., 2011), it is very important to evaluate the activity of EOs in vapour phase against biofilm formation. A choice is the use of plastic coupons with biofilms in a isolator chamber joined to a vapour generator for to evaluate surface decontamination (Eterpi et al., 2011), these coupons can be cultured in a FC270 flow-cell system for flow biofilm study before the exposure (Bueno, 2014). Equally 96 multiwell plate with a biofilm in a vapour chamber or airtight box at 37ºC can be used and revealed by the crystal violet method or LIVE/DEAD BacLight Viability kit to determine the mass of the biofilm (Laird et al., 2012; Bueno, 2014).

ATP Bioluminescence Systems

Currently, it is very important to verify state of disinfection of laboratory and hospitals environments, due to the little evidence collected about the association between nosocomial infections and hospital surfaces contamination (Dancer, 2008). In this way the most used technique to assess the hospital cleaning for new biocidal products is ATP bioluminescence that has been proposed both to monitor the effectiveness of the cleaning agents as well as cleaning programs (Moore et al., 2010). The method takes a sample from the area to be tested after disinfection procedure with a swab that is placed in a detection device in presence of luciferase and luciferin (Dancer, 2014). Later using a luminometer the reaction is revealed and the light produced is proportional to the amount of ATP, the disadvantages of this method are the variable sensitivity between the available systems, the inefficacy to detect gram-negative bacteria because performs a incomplete lysis of this microorganisms and the lectures can be confounded by residues as plastics and microfibres (Turner et al., 2010; Dancer, 2011; Dancer, 2014).

Conclusions and Perspectives

Cleaning has two purposes, first is maintaining the appearance and function as well as to prevent spoilage; second is decreasing the microbial burden (Dancer, 2008). The reduction of microbes reduce the risk of transmission in hospital environments and the association between microbial surface contamination and the emergence of infectious diseases (Dancer, 2008). For that reason, the development of innovative biocidal

products in collaboration with infection control personnel, researchers and industry is necessary (Dancer, 2011).

Although applications in clinical treatment of EOs have been limited, they have been successfully used topically in creams and lotions as well as in liposomal formulations. Equally, EOs have shown to possess the ability to enhance penetration of antiseptics and block the resistance mechanisms in MDR microorganisms (Fortino, Solórzano-Santos & Miranda-Novales 2012).

On the other hand, for the product development of disinfectants and sanitizers agents based on EOs it is necessary to conduct Human Repeat Insult Patch Test (HRIPT) and the *in vitro* Dermal Irritection® Assay according to standardized methods with the aim to obtain safe and sustainable products as is the example of thyme oil based disinfectants (Bondi, 2011).

Equally, for designing new decontamination systems using EOs is necessary to determinate the physical chemistry of the decontamination process using this formulae:

mg/liter=P(Mol Wt)(1000 mg/g)/RT

In where the concentration of gas is expressed in mg/liter,

P: represents atmospheric pressure,

Mol Wt: symbolizes molecular weight of the gas,

R: typify ideal gas constant,

T: represents temperature (°K)

With this equation it is possible to determine the gas concentration, the pressure, the relative humidity and temperature in the decontamination chamber (Hultman et al., 2007).

Finally, the use of aerosol delivery technology for the development of biocides for surface decontamination is an interesting approach in innovation to increase the antimicrobial potency of EOs because using liquid droplets suspended in gas can destroy airborne bacteria and spores. This kind of formulations have been proved in disinfectants such as sodium hypochlorite, peroxyacetic acid and quaternary ammonium (Thorn et al., 2013).

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