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Assessment of airborne microorganisms by real-time PCR: optimistic findings and research challenges

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TABLE OF CONTENTS

1. ABSTRACT

Most airborne microorganisms are natural components of our ecosystem. Soil, vegetation and animals, including humans, are sources for aerial release of these living or dead cells. In the past, assessment of airborne microorganisms was mainly restricted to occupational health concerns. Indeed, in several occupations, exposure to very high concentrations of non-infectious airborne bacteria and fungi, result in allergenic, toxic or irritant reactions. Recently, the threat of bioterrorism and pandemics have highlighted the urgent need to increase knowledge of bioaerosol ecology. More fundamentally, airborne bacterial and fungal communities begin to draw much more consideration from environmental microbiologists, who have neglected this area for a long time. This increased interest of scientists is to a great part due to the development and use of real-time PCR techniques to identify and quantify airborne microorganisms. Even if the advantages of the PCR technology are obvious, researchers are confronted with new problems. This review describes the methodological state of the art in bioaerosols field and emphasizes the future challenges and perspectives of the real-time PCR-based methods for airborne microorganism studies.

2. INTRODUCTION

Quantification and identification of airborne microorganisms, such as bacteria, fungi and viruses, are of interest in several research areas, including public and occupational health, bioterrorism sciences and fundamental microbiological ecology. Indeed, these microorganisms have the potential to become aerosolized and inhaled by humans as their size is smaller than 10 µm. Pathogenic microorganisms may infect and reproduce in human tissues. Many non-pathogenic bacteria and fungi may have allergenic, irritant or toxic effects. Exposure prevention to particular microorganisms may sometimes be problematic because most of these microorganisms are able to travel long distance through airborne transport. Long distance transport has been demonstrated for viral particles such as the foot-and-mouth disease virus and the Aujezsky's disease virus (1, 2, 3). The survival of aerosolized viruses for relatively long periods of time is favoured by particular environmental factors, mainly temperature, humidity and the presence of surrounding organic material (4). Detection of airborne microorganisms in various environments have received lately increasing attention, especially with recent events like SARS (severe acute respiratory syndrome) and H1N1 pandemics and the risk of bioterrorism attacks such

as that with *Bacillus anthracis*, the bacteria causing the acute disease anthrax.

Different methodological tools are available to quantify and identify airborne microorganisms, depending on the aim of study objective. For example, to evaluate the total number of cultivable bacteria or fungi from a particular air sample, the culture-dependent methods consisting in the enumeration of growing cells on a particular culture medium, is often used. To identify viruses in air samples, inoculation of host culture cells by the particles isolated from air samples (infectivity test) is used. However, the vast majority (90% - 99%) of naturally occurring microorganisms cannot be cultivated using standard techniques (5, 6). Culture-independent methods have been developed since the culture-dependent methods lead to a large underestimation in the numbers of microorganisms present in aerosol. One of these methods is the microscopic enumeration of bacteria or fungal spores. The advantage of this method is to take into account cultivable and non-cultivable or dead cells. Indeed, non-cultivable or dead cells may also have allergenic, irritant or toxic properties and are therefore relevant to the occupational or public health assessment of total microorganisms load. To identify microorganisms at species level in air samples, other methods are more appropriate such as the molecular techniques - PCR methods (review 7, 8, 9) - or the immunological techniques - ELISA (10), fluorescent *in situ* hybridization (11, 12) or flow cytometry with fluorochrome (13). However, these methods are often not sufficiently accurate for the quantification of specific microorganisms. The classic PCR methods give only semi-quantitative results and the immunological techniques are not always species specific.

Quantitative real-time PCR (Q-PCR) method allows for accurate quantification of all particles from specific bacterial and fungal (cultivable, non-cultivable and dead) or virus species (infectious and non infectious). This method uses amplicon sequence non-specific fluorescent dyes or sequence-specific fluorescent probes for counting the copy number of a target DNA sequence in an aerosol sample (14, 15). The cycle threshold, at which the fluorescent signal is detected over background, is used to calculate the initial amount of DNA present in a sample. Q-PCR standard curves, generated using known amounts of DNA, enable the quantification of DNA in unknown samples. The ability to analyze easily time-integrated samples provides sensitive and representative assessment of a specific airborne microorganism.

Increasing number of scientific publications propose species-specific Q-PCR protocols for a routine quantification of a species. Based on these species specific adjusted protocols, a routine monitoring of these corresponding species may easily be perform in any molecular lab in a short time and for a relatively low price. The expertise of molecular biologists is needed for the set-up in the laboratory of Q-PCR protocol since some steps may require particular attention. For instance, the extraction efficiency of DNA (or RNA for some viruses), the inclusion of an internal positive control to detect inhibition of DNA amplification and the choice of standard curve, could be critical for accurate quantification.

3. AIRBORNE BACTERIA, FUNGI AND VIRUS: STATEMENT OF FACT

3.1. Bacteria

Detection and quantification of airborne bacteria using Q-PCR technology was demonstrated for the first time in 2002. In response to a bioterrorism attack, a group of American scientists (16) investigated air samples by Q-PCR methods in order to detect and quantify *Bacillus anthracis*. Recently, the experimental detection of five bioterror agents (*B. anthracis, Brucella melitensis, Coxiella burnetti, Francisella tularensis, Yersinia pestis*) by the Q-PCR method in an artificial air sample containing eight other natural environmental bacteria, was successfully performed (17). The Q-PCR method has also been used in detecting a large number of pathogenic bacteria such as *Mycobacterium tuberculosis* in health care settings (15), *Legionnella spp.* and *Mycobacterium spp.* from dental unit waterlines during dental treatment (18) and different pathogenic airborne bacteria in animal confinement houses. *Campylobacter sp.* is a fastidious bacteria that was detected and quantified successively by Q-PCR in broiler flocks and during the slaughter process (19). Interestingly, the comparison of the results obtained by the Q-PCR method to that obtained by cuture-dependent method for *Salmonella sp.* in livestock stables, showed a 85-fold better detection efficiency by Q-PCR (20).

To quantify total airborne bacterial load, others have used Q-PCR in swine confinement building (21), poultry houses (22) and sewage treatment plants (23). Hovewer, quantifying total bacteria by using universal 16S rRNA is not very precise because the determination of bacterial load by real-time PCR in a multi-species population is influenced by the variation in the number of rRNA operons in a given species (24) at a given time (depending on the metabolic status of the bacteria). This is the main limitation to determining total bacteria by real-time PCR based on 16S rDNA. However, in a variety of complex environmental, industrial and health-care settings in which multi-species populations are sampled along with impurities, or where the bacteria are internalized within a matrix, other methodologies are likely to be far less sensitive or precise (25).

3.2. Fungi

Q-PCR using amplicon sequence non-specific fluorescent dyes (26, 27) or sequence-specific fluorescent probes (28, 29, 30) is at present the best method for the detection of specific fungal species in air samples. This method is rapid, sensitive and specific and there is no for post-PCR electrophoresis (31, 32, 33, 34). Quantification may be performed at the species or at the taxon levels depending on the target DNA sequence specificity. The rRNA genes, both bacterial and fungal, contain variable sequences suitable for species discrimination. Whereas in bacteria the 16S gene is the most commonly used locus for taxonomic identification, in fungi the two internal transcribed spacer regions, ITS1 and ITS2, and an

intergenic spacer, IGS are the most commonly targeted loci. Primer sites need to be variable enough to allow specific amplification of the targeted taxon, and at the same time conserved enough to allow amplification of all species or strains contained within the targeted taxon. Primers that are not sufficiently specific may lead to false positives through amplification of non-target species. Several airborne fungal groups and species have been quantified by Q- PCR with the TaqMan fluorogenic hybridisation probe system (35, 36, 27). Because their recognised allergenic and occasionally invasive properties, *Aspergillus* and *Penicillium* species are among the taxons for which the most sequence-specific fluorescent probes have been developed. These Q-PCR protocols have been used to survey these species in indoor air (37, 38, 39), water-damaged homes (40) and hospital environments (41). Fungi producing mycotoxins have also been quantified by Q-PCR, however correlation were not found between mycotoxins load of the sample and genome copy number (40).

3.3. Virus

Currently, Q-PCR is the best detection method for virus, as many viruses cannot be cultured and sampling methods often decrease the infectivity potential of viruses. Q-PCR was used successively to detect several virus species in hospital air environments. Booth et al. confirmed that the virus causing SARS can spread through air, not just by direct human contact, as previously believed (42). Interestingly, culture-dependant methods produced negative results whereas PCR techniques allowed virus detection. Using Q-PCR, the presence of airborne influenza viral particles was detected in a health care environment (43) and an urgent care environment (44). The possible airborne transmission of the influenza virus, which can be infectious at very low airborne concentration (45), was revealed by Q-PCR in these medical environments. Influenza and avian influenza viruses were detected by Q-PCR in a poultry market, which is a wet and warm environment, charged with high levels of particles (46). Another study detected up to 10^7 genomes of porcine circovirus 2 per cubic meter of air from swine confinement buildings (47). Interestingly, a correlation was observed between airborne dust concentrations and virus concentrations but further investigations are required to demonstrate the infectivity potential.

4. SAMPLING OF AIRBORNE MICROORGANISMS

Collection of particulate matter on filters with subsequent laboratory analysis is one of the preferred methods used to evaluate aerosols for exposure assessment (48). This method can be used with high-volume pumps for stationary area sampling or with lightweight, battery-powered pumps for personal sampling. Thus, use of filters for sample collection offers an advantage over many commonly used culture sample collection methods that are not well characterized or convenient for field use (49). Filters vary with respect to material, pore size, pore type, nominal sampling flow rate, reactivity, and hydrophilicity or hydrophobicity (50, 51, 52). The use of cellulose ester, polycarbonate, fluoramide and gelatin filters has been cited as filter media suitable to collect bioaerosols (53, 54, 55, 56). Membrane filters housed in polypropylene cassettes have been shown to have 100% collection efficiency for particles up to 20 μm aerodynamic equivalent diameter (well within the size range of many fungal conidia), and filters are amenable to sampling over long periods of time and for drawing large volumes of air (57).

For fungi, some of the sampling characteristics will favor air sample collection and particle retention, while others may favor conidia extraction from the filter. Concerning viruses, recovery efficiency of viral particles depends strongly on the selected sampling method. Sampling methods frequently used for virus sampling and their efficiency are extensively reviewed in Verreault et al. (58). Filters are commonly used to recover viruses from large air volumes but they are known to cause decrease of viral infectivity. Since the viability is not an important parameter for Q-PCR, filters can be used to sample airborne virus. The sampling process could be responsible for the particle integrity loss because it often induces a strong change in environmental conditions, e.g. a drying effect. Alternative sampling methods, like high volume liquid based cyclonic impinger (16), are possible to circumvent drying effects. This cyclonic impinger concentrates airborne bacteria in 10 ml of liquid surfactant that allows to quickly collect high volumes of air (10-15 m^3) and removes the problem of DNA recovery inherent to sampling air with filters devices, but they were reported to be less efficient in recovery rates than filters. Nevertheless, liquid based cyclonic impingers are more efficient than filters in the recovery of infectious particles. Loss of viability induced by sampling process was demonstrated for viruses (59), but also for airborne bacteria and fungi, which are commonly more resistant than viral particles (60, 15). The effect of the sampling process on the viability of the microorganisms is still unclear and needs further investigation.

Nucleic acids are detected by Q-PCR even if the viral particle has been damaged and is consequently not infectious. In consequence, nucleic acids amount detected by Q-PCR do not always reflect the number of viable viral particles in the considered environment. If the virus should be completely absent from the environment (e.g. in the case of bioterrorism), detection of residual nucleic acid in air samples provides evidence for the recent presence of viral particles. However, when considering common viruses (like flu viruses), detection of residual nucleic acid is difficult to interpret since Q-PCR results represent the sum of non-viable and viable particles numbers. This overestimation of viable particles is a problem for exposure studies or risk establishment. How long nucleic acids in damaged viral particles or particle-free nucleic acids last in air environment is unknown at present. Moreover, the effects of sampling process on virus viability are not yet clearly defined. Further investigations are required to establish correlations between nucleic acids detection levels and number of viable particles for each virus present in air samples.

5. NUCLEIC ACID EXTRACTION, PCR INHIBITORS & TECHNICAL SOLUTIONS

False negative results and reduced sensitivity are possible if samples contain environmental contaminants named 'PCR inhibitors', such as phenols, humic and fulvic acids from soil, polyphosphates in fungi, heavy metals, some plant acidic polysaccharides, and even high concentrations of non-target DNA (61, 62, 63). Samples from different environments may vary in chemical and organic composition, and affect assay sensitivity differentially. PCR inhibitors may be removed by including a purification step in the extraction procedure (36, 64). DNA extract dilution is known to attenuate the inhibition effect, but also reduces sensitivity (31, 10). Several commercial kits are now available to minimize co-purification of inhibitors from environmental samples. The sources of different DNA polymerases could also influence their resistance to inhibitors and they should be chosen with care. Moreover, filters of cellulose and nitrocellulose, but not polycarbonate, may inhibit PCR (65).

Possible inhibition may be tested either by spiking the processed sample with known amount of target DNA (internal amplification control (IAC)), or spiking the unprocessed sample with known amounts of target spores (internal positive control (IPC)). The pros and cons of each method are reviewed by Halstensen (66). In the majority of published Q-PCR methods, the control is an IAC which is a known nucleic acid added in the reaction mix. Spiking with unrelated nucleic acids - that is not expected to be found in the samples - may be very reliable as an IAC (67). However, detection methods include the steps of sampling, nucleic acid extraction and Q-PCR. Without any control in each of these steps, a negative detection result might be interpreted as the absence of the microorganism or as a technical failure. The inclusion of an internal positive control (IPC) directly at the beginning of the whole process will be very beneficial for the quality and the reliability of the detection. An efficient IPC is a control microorganism, which is included very early and will go through all the steps.

6. DETECTION SENSITIVITY

One parameter, which can strongly affect Q-PCR sensitivity, is the genetic variability of the microorganisms. Primers and probe are designed purposefully for the target region of the microorganism. Variations in the target region can lead to a decrease in amplification efficiency. As an extreme, such variations can lead to a complete absence of amplification. Genetic variability is consequently a source of false negative results. For this reason, it is important to consider a large number of alleles for the target sequence when designing primers and sequence-specific fluorescent probe. A multialignement of these alleles will favour the localization of the motifs that are still conserved within the species but are different from all the other species. These motifs have to be used as template for primers and probe design. Virus genomes are often highly variable since they reproduce at a high rate in a short time. Furthermore, reverse transcriptase (or RNA polymerase for some RNA virus) has a much larger error rate (10^{-5}) than DNA polymerases (10^{-7}) (68). For this reason, it can be much more difficult to find three conserved motifs in the same region of a virus genome compared to a bacterial or fungal gene when designing the two primers and the probe. Variability in DNA extraction efficiency may also strongly impact detection sensitivity, in particular for fungi, where DNA is much more difficult to extract than that of bacteria or viruses. Indeed, it was reported that the detection sensitivity may vary 100-1000 fold in fungi (36). This variation in detection sensitivity is crucial when DNA is extracted from a low spore density (69). Detection sensitivity can be improved either by extracting DNA from a large amount of spores followed by DNA dilution or starting with a spore suspension dilution followed by DNA extraction (70).

7. EVALUATION AND INTERPRETATION OF DATA FROM MOLECULAR ANALYSIS

The data output from the Q-PCR are the cycle threshold (CT) values that reflect the PCR cycle number when the specific signal is detected above a certain threshold value. For bioaerosol exposure assessment in general, the DNA concentration should preferably be converted to a unit that is applicable to public health or occupational measures (66). For example, the genes copy number has to be converted to a number of cells per cubic meters of air (cells/m^3). To do this conversion, the CT/m^3 and the gene copy number present in each fungal or bacterial species have to be taken into account. Indeed the target gene used in some Q-PCR methods for fungi and bacteria, is a multicopy gene, the rDNA locus, that frequently vary in copy number per individual from one species to the other. This problem does not exist when the target is a single copy locus. Most viruses possess one single copy genome embedded in a viral particle and most genes are present as single copies within the genome. The conversion of genes copy number to a number of particles per cubic meters of air is therefore easy to achieve.

Concerning exposure assessment, it is important to evaluate what is quantified and what is relevant to public or occupational health. In highly fungi-exposed working populations, lung function decline, respiratory symptoms and airway inflammation appear at 10^5 spores/m^3 whereas the same symptoms appear at 10^4 spores/m^3 for asthmatic patients allergic to *Penicillium sp*. or *Alternaria alternata*. Mycotoxin-producing and pathogenic species have to be detectable at an even lower level, because of their higher toxicity (71). The actual Q-PCR protocols may easily and reproductively detect this low amount of spores. Although the usage of the Q-PCR method may be important for the detection of a mycotoxin producing species, this method is not informative concerning the level of exposure to mycotoxins as the mycotoxin production is not linearly correlated to the fungus number of cells. Thus, for each of these species, the mycotoxins or mycotoxin metabolites have to be detected and quantified by immunological (ELISA) or

chemical (HPLC-MS) methods from air samples (72, 73, 74).

The standard curve is a critical step in the protocol set-up. For bacteria and fungi, standard curves are usually prepared by serial dilutions of the genomic DNA isolated from a pure bacterial or fungal culture (9, 75). This approach does not take into account the DNA extraction efficiency that can differ depending on the microorganism concentration. An experimental study that compared several standard curve preparation methods (76) showed that standard curves used for Q-PCR needed to be prepared using the same environmental matrix and procedures as handling of the environmental sample in question. Indeed, reliance on standard curves generated with cultured bacterial suspension may lead to substantial underestimation of microorganism quantities in environmental samples.

8. CONCLUSION AND FUTURE PROSPECT

Q-PCR is a cheap and very efficient tool that enables fast, accurate and sensitive detection of airborne microorganisms. It has been successfully applied for detection of bacteria, viruses and fungi in bioaerosols. Airborne microorganisms quantification studies have shown that Q-PCR is as a more sensitive technique than other quantification methods like culture-dependant methods. The limitation for Q-PCR-based methods is that they need to be set-up for each species and even more for each particular environment (e.g. wet or dry, dusty or clean environment). The absence of inhibitors (depending on the environment) needs to be demonstrated by measuring the amplification efficiency of IAC or IPC in Q-PCR. These controls are also necessary to confirm the quality of the detection process.

For bacteria and fungi, quantification by Q-PCR is suitable for assessment of the total number of a specific microorganism, independently of its viability. For virus quantification, since non infectious particle could be considered as inert dust without health or ecological importance, it is relevant to correlate the detected amount of nucleic acid to the number of airborne infectious microorganism units.

Q-PCR can be used to detect several (five to eight) airborne microorganism species at the same time when using multiplexed Q-PCR. To screen for the presence of a large number of species within the same air sample, DNA microarray is a more adequate tool for parallel detection and quantification of multiple DNA sequences in one single experiment (77). An oligonucleotide microarray has been developed recently for the detection of a large number of potential mycotoxigenic fungi (78). Detection of bacterial pathogens in municipal wastewater was also performed with an oligonucleotide microarray (79). The authors also tested Q-PCR at the same time and found microarray less sensitive than Q-PCR. Once that the species have been identified with the microarray method, species specific Q-PCR reactions are still necessar to be performed for the validation of the quantification data proposed by the microarrays. The ability of microarray to detect microorganisms in bioaerosols has not yet been investigated.

Currently, possibilities to use Q-PCR methods to detect airborne bacteria, fungi and virus have been demonstrated in several studies. Hovewer, other biological material potentially present in bioaerosols could also be detected by Q-PCR depending on interest (e.g. unicellular algae, protozoa, plant fragments, pollen, animal fragments). Plant pollen is often a highly volatile element which can travel long distance and can reach high concentration in air. Detection of specific pollens is very important as pollens could be strongly allergenic, may carry transgenic material or may be a great source of information in forensic biology. Detection of allergenic pollen was recently achieved using Q-PCR providing a fast and high-throughput method compared to traditional observation methods (80).

Thus, Q-PCR technology is a promising tool to detect and quantify airborne microorganisms. This tool needs to be developed and has the potential to be applied in a broad range of bioscience domains.

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