Review

On the Identification of Culturable Microorganisms for the Assessment of Biodiversity in Bioaerosols

Philippe Duquenne*

Department of pollutants metrology, Institut National de Recherche et de Sécurité (INRS), Vandœuvre-lès Nancy 54500, France.

*Author to whom correspondence should be addressed. Tel: +33-0-3-83-50-98-75; fax: +33-0-3-83-50-87-11; e-mail: philippe.duquenne@inrs.fr

Submitted 27 June 2017; revised 15 October 2017; editorial decision 17 October, 2017; revised version accepted 10 November 2017.

Abstract

The Annals of Work Exposures and Health recently published two interesting studies combining the use of culture and molecular methods. The method involves the cultivation of bioaerosol samples on agar media and the pick-up of grown colonies 16S rRNA gene amplification, subsequent cloning, sequencing, and identification of bacterial isolates through the assignment against known gene databases. The aim of the present paper is to discuss the contribution of the proposed method in regards with the already proposed approaches used for identification of cultured bacteria. It details the new proposed method and discusses its contribution to the existing culture-based identification methods. Such methods include macroscopic and microscopic observations, miniature biochemical tests (API® trips, VITEK 2® etc.), chemical methods such as the Fatty Acid Methyl Ester (FAME) and the Matrix-assisted laser desorption/ionization time of flight (MALDI-TOF) analysis as well polymerase chain reaction (PCR) followed by sequencing. The proposed method supplements the panel of existing biodiversity ones for cultivated bacteria, especially useful for infectious microorganisms, as well as culture-independent ones. As both culture-based and culture-independent methods could therefore be used for the characterization of the occupational environmental microbiome, further applications in other occupational environments as well as additional comparisons with both culture-based and culture-independent methods would complete its characterization.

Keywords: bioaerosol; culturable microorganisms; culture-independent method; identification; microbiome

Introduction

Microorganisms are microscopic living organisms that are ubiquitous in the environment. Microbial entities, including cells, propagules, spores, their components and metabolites can get airborne at occupational settings from process they are involved in or during handling of contaminated matters. Thus, exposure to airborne bacteria, fungi, viruses, endotoxins, mycotoxins has been shown in numerous occupational situations and
in diverse sectors such as health care, agriculture, food industry, waste management, etc. (Duquenne et al., 2013; Oppliger and Duquenne, 2016). These exposures were associated with several health disorders observed among workers including gastrointestinal, inflammatory and immuno-allergic symptoms (Walser et al., 2016). However, there are still no Occupational Exposure Limit values (OELs) for airborne biological agents and the interpretation of bioaerosol exposure data in term of biological risks is still uncertain.

The complex composition of bioaerosols in microbial species and compounds is one of the reasons cited for explaining the actual lack of OELs. Indeed, the health effects of airborne microorganisms depend on the species and the compounds to which workers are exposed to. For example, occupational infectious symptoms were observed among workers supposed to be exposed to airborne Legionella sp. (Hautemaniere et al., 2011) and the opportunistic Aspergillus fumigatus is known to cause severe invasive infections of immunosuppressed people (Pitt, 1994). The exposure to Penicillium nalgiovense, Penicillium camembertii and other allergenic fungi was associated to immuno-allergic symptoms such as hypersensitivity pneumonitis (Marchisio et al., 1999; Rouzaud et al., 2001; Eduard, 2006). The exposure to microbial compounds such as endotoxins found in Gram-negative bacteria was involved in occupational pathologies such as irritation of the respiratory tract, coughing and fever (Liebers et al., 2008). The understanding of health effects is complicated by multiple exposures and by the heterogeneity and non-specificity of the observed health effects. Thus, determining the composition in species and microbial compounds would provide a better understanding of biological risks due to bioaerosol exposures in occupational environments.

The Annals of Work Exposures and Health recently published two interesting studies dealing with the combined use of culture and molecular methods (PCR, cloning of PCR amplicons, and DNA sequencing) to assess microbial diversity in bioaerosol samples (Jäckel et al., 2017; Schäfer et al., 2017). Bioaerosol samples were collected in a duck hatchery, cultivated on agar media and grown colonies were taken and used with no further isolation step for 16S rRNA gene amplification, subsequent cloning and sequencing. The main aim of the study was to evaluate the suitability of the cultivation/cloning/sequencing approach for the investigation of biodiversity among airborne the bacterial communities using 16S rRNA gene sequence analyses after cultivation. This approach would avoid pure culture isolation in the future.

The aim of the present paper is to discuss the contribution of the proposed method in regards with the already proposed approaches used for identification of cultured bacteria in bioaerosols.

Presentation of the New Method

The different step of the method proposed by Schäfer et al. (2017) is presented in Fig. 1. The method is based on the collection of bioaerosol samples in a given working environment and their cultivation on agar nutrient media. The morphologically different grown colonies are then selected, picked, and individually transferred into a DNA-free reaction tubes. The Genomic DNA from picked bacterial colonies is extracted and amplified by PCR using 16S rRNA gene universal primers. An amplicon-mixture of each bioaerosol sample is prepared by mixing equimolar DNA amounts of PCR product from each selected colony (Fig. 1). Then, the amplicon-mixture is used to generate clone libraries that are sequenced; the gene sequences are finally aligned against the NCBI database for bacterial identification. The method was first evaluated with an experimental microbiome prepared by mixing pure bacterial cultures grown in laboratory conditions (Schäfer et al., 2017) and then tested with bioaerosol samples collected in a ducks hatchery (Schäfer et al., 2017).

Overview of Published Culture-Based Methods

The biodiversity of airborne microbial population in occupational environments was mainly assessed by cultivation of microorganisms on nutrient media prior to identification. The culture-based methods used for the assessment of biodiversity in bioaerosols generally involve a first culture step of the sampled microbial population, a subsequent sub-culture step of the different types of grown colonies and then an identification process of each selected colony (Supplementary Table S1).

The criteria used for the identification microbial isolates included the classical description of macroscopic and microscopic phenotypes as well as biochemical, chemical, and bio-molecular features. Microbial identification processes of cultured microorganisms begin with a macroscopic observation of colonies that help for the selection of different morphological types to be investigated. This also allows the collection of morphological criteria that are useful for further classification. The medium used for microbial cultivation as well as the incubation conditions orientate the microbial groups that are cultivated (Rahkonen, 1992; Danneberg et al., 1997; Alwis et al., 1999). Macroscopic observations are usually amended with microscopic ones, especially used.
at the workplace for the characterization of fungal structures such as hyphae and spores (Tolvanen, 2001; Xu and Yao, 2011; Simon and Duquenne, 2014).

In addition with growth conditions, the ability to grow on specific media, standard biochemical tests used in classical microbiology (catalase, oxidase, oxygen tolerance, etc.) have been used for decades in order to classify airborne microorganisms at occupational settings (Kurup and Fink, 1975). For example, bacterial isolates are gram-stained, for differencing Gram-positive and Gram-negative bacteria, before identification. Miniature biochemical tests such as the API® trips, the Microscan, the VITEK 2® or the BIOLOG Microstation® Systems were introduced, providing a wide range of parameters to be tested, an automation of the analysis as well as a gain of place and time (Bochner, 1989; Bullock and Aslanzadeh, 2013). These miniature tests investigate the ability of microorganisms to oxidize different carbon sources and to exhibit diverse metabolic activities providing metabolic profiles that are compared to a database containing profiles of known microbial isolates. Such methods were successfully used to assess bacterial diversity in bioaerosols from waste sorting plants (Rahkonen, 1992), composting facilities (Byeon et al., 2008), eggs production facilities (Wang-Li et al., 2013), medical care units (Yu et al., 2015), and swiftlet houses (Sien et al., 2012).

Chemical methods are also used to identify microbial isolates in bioaerosols. Few bioaerosol studies focused on the use of Fatty Acid Methyl Ester (FAME) analysis for the identification of microbial isolates (Byeon et al., 2008; Madonna et al., 2001). Matrix-assisted laser desorption/ionization time of light (MALDI-TOF) mass spectrometry has recently been proposed for microbial identification (Singhal et al., 2015). The MALDI-TOF identification process is based on the characterization of the proteome of microbial isolates (from intact cells as well as cell extracts) and its comparison against a known database.

**Figure 1.** Analytical process of the new proposed method for the assessment of bacterial biodiversity in bioaerosols. ¹: selection of colony based on morphological criteria; ²: PCR amplification with universal primers ³: equimolar mix of each PCR amplicon.
database. The technique has been used to identify bacterial and fungal isolates in bioaerosols collected during grass seeds handling (Madsen et al., 2015), waste collection (Madsen et al., 2016), and in the subway (Dybwald et al., 2014).

Identification of culturable microbial isolates based on DNA studies aroused a growing interest during the last decades (Supplementary Table S1). In the published bioaerosol studies, DNA was extracted from a unique grown colony picked up from Petri dishes, amplified by PCR using universal gene primers and then the purified PCR products were sequenced and genes sequences compared with existing sequences in available databases. For examples, this approach was performed for studying bioaerosols in ducks houses (Fallschissel et al., 2010; Martin et al., 2010), in Swiftlet houses (Sien et al., 2012), and in stables (Normand et al., 2009). Other studies proposed a cloning step of purified PCR products from a unique colony before sequencing DNA extraction, construction of 16S rRNA gene clone libraries, and subsequent sequencing (Supplementary Table S1). The 16S rRNA gene is the mostly targeted gene for bacterial identification in bioaerosols.

**Contribution to the Existing Culture-Based Methods**

The method proposed by Schäfer et al. (2017) involves an original approach with the mixing of PCR products obtained by amplification of genomic DNA from individual colonies and a cloning step of the mixed PCR products for further sequencing. It supplements the panel of existing methods for the identification of cultivated bacteria, suffering from some limits of the culture-based technics while overcoming others. The assessment of biodiversity in bioaerosols with culture-based techniques suffers from several biases. The first category of biases is related to the first step of culture of bioaerosol samples. Indeed, dead cells and microorganisms at viable but non-cultivable (VBNC) state as well as microorganisms unable to grow upon the selective pressure of culture media and culture conditions escape from the enumeration by culture. Several, previous studies reported the microbial concentration measured in bioaerosols by cultivation of microorganism was up to 1000 times lower that the ones of total microorganisms measurement by culture-independent methods such as fluorescent microscopy and quantitative PCR (qPCR) (Albrecht et al., 2007; Nehmé et al., 2008; Duquenne et al., 2012; Lehtinen et al., 2013). Studies carried out in laboratory and in real exposure conditions also showed that the type agar media used for cultivation had an impact on biodiversity measurements in environmental samples (Martin et al., 2010; Lutton et al., 2013; Medina et al., 2017). The technics used to cultivate microorganisms were also shown to affect the measured biodiversity microbial populations (Schoenborn et al., 2004). Furthermore, the ability of a given airborne microbial population to cultivate on nutrient media is known to be deeply affected by the physiological and physical stress and injuries induced by the aerosolization process, the sampling methods, and other factors that affect microbial cells during their transport at airborne state (Heidelberg et al., 1997; Zhen et al., 2013). This increases the VBNC state problematic for bioaerosols. As a consequence, the culture methods underestimate the total number of microorganisms and the real microbial taxon richness in environmental samples (Amann et al., 1995; Heidelberg et al., 1997). Furthermore, cell debris, and microbial components, which may also have toxic and/or allergenic properties, as well of viruses are not detected by culture. As the method proposed by Schäfer et al. (2017) involves the primary cultivation of microorganisms from bioaerosols samples, it does not overcome such limits.

The second category of biases is related to the sub-cultivation of grown colonies and the subsequent identification process. Sub-cultivation of bacterial and fungal colonies has the same limitations than those discussed above and a selected colony may not grow due to the physiological phenomena that were previously discussed. The inability of microorganisms to grow when sub-cultivate will induce a loss of colonies and may impair the representativeness of biodiversity measurements. Purity of selected colonies is also a fundamental requirement for their accurate identification (Krieg, 2001). The first step of cultivation is traditionally performed by spreading an aliquot of a bioaerosol sample eluate on the surface of the nutrient agar medium. The eluate may contain microbial single or aggregated cells that are supposed to be evenly spread on the surface of agar. As a consequence grown colonies after incubation may not be pure and contain cells from different taxa that were able to grow or statd at the VBNC state (Shrestha et al., 2013). This will impair the correct identification of colonies with chemical, biochemical as well as molecular identification methods and requires a purification checking, which is laborious as well material and time consuming. The method proposed by Schäfer et al. (2017) does not involve any subculture step, it overcomes the problems of losses due to sub-cultivation. Furthermore, selected colonies are directly submitted to PCR amplification and DNA of multiple bacteria from non-pure colonies may lead the detection of all the bacteria in the following step of the identification process (Fig. 1). By the way, data from
the use of the method in a ducks hatchery support this advantage as they suggest the occurrence of non-pure selected colonies (Jäckel et al., 2017).

The identification begins by the selection of the different type of colonies grown on the agar medium. This arises the question of the selection strategy that need to be employed for an accurate representation of the actual biodiversity. First, regarding the morphological criteria used for selecting colonies and their pertinence for allowing a robust differentiation between different types of colonies. Indeed, previously published works reported that colony morphotyping underestimated taxonomic diversity (Lebaron et al., 1988). Secondly, regarding how many colonies should be sub-cultured and tested for each type to be representative of the abundance of the different taxa. This issue was not in the scope the published method (Jäckel et al., 2017; Schäfer et al., 2017) but should be taken into account for any methods involving the selection of microbial isolates grown on agar media (Altekruse et al., 2003).

As for the majority of published methods used for the identification of microbial isolates, the method proposed by schäfer et al., allowed the characterization at the specie level in a ducks hatchery and a higher diversity in bioaerosols as compared to previous published works (Jäckel et al., 2017). One of the major limitations of biochemical and chemical fingerprint technologies is that the identification of the isolates is limited to the metabolic profiles existing in the reference database. The databases also refer to known cultured microorganisms. Furthermore, the increment of databases with new microbial profiles, when possible, needs a robust validation. PCR and sequencing approach provide huge databases that are incremented regularly by the scientific community and contain known cultured microorganisms as well as known and unknown uncultured ones. They allow the identification at the specie level providing the sequence length of the amplicons is high enough.

Complementary Inputs From Culture-Independent Methods

Culture-independent methods were also proposed as alternatives to culture-based ones and were increasingly used in bioaerosols biodiversity studies during the last decades. Such methods are based on the direct study of microbial nucleic acids without cultivating microorganisms. They mainly involve the direct extraction of DNA from bioaerosol samples, followed by a PCR amplification of target genes. For example, PCR with oligonucleotides designed to target specific microbial taxa were used for the detection of Thermoactinomyces vulgaris, Saccharopolyspora rectivirgula in bioaerosols from composting facilities (Betteli et al., 2013; Schäfer et al., 2011), L. pneumophila and Legionella spp. in bioaerosols surrounding cooling towers (Chang and Hung, 2012) and Staphylococcus aureus in bioaerosols from pig farms (Masclaux et al., 2013). Sets of universal primers and probes also allowed the quantification of total microorganisms at occupational settings (Martin et al., 2010; Oplilger et al., 2008). Cloning of PCR products followed by sequencing is also a technique used to assess microbial diversity in workplace bioaerosols (Angenent et al., 2005; Nehmé et al., 2009; Han et al., 2013; Guo et al., 2014; Ding et al., 2016). Recent development of Next-generation sequencing (NGS) provided new insights for biodiversity in bioaerosols from coal mines (Wei et al., 2015), wastewater treatment plants (Lin et al., 2014), composting facilities (Partanen et al., 2010), and swine houses (Kumari and Choi, 2015) as compared to culture-based methods. The mostly targeted genes for the study of microbial communities in bioaerosols were the 16S rRNA gene for bacteria, the 18S, and ITS genes for Eukaryotes and Fungi.

Advantages and drawbacks of culture-independent methods were recently reviewed (Yoo et al., 2017). Indeed, these powerful molecular methods provide a description of microbiomes in bioaerosols to an extend that was never reached before, revealing uncultured taxa, new ones as well as a higher biodiversity as compared to the one found with culture-based methods. NGS allows the detection of non-preponderant taxa but also suffer from biases such as inefficient DNA extraction from microorganisms, formation of chimeric sequences during PCR runs, preparation of the sequencing libraries, preferential amplification of microbial taxa due to primers design and sequencing errors (Bálint et al., 2016). The shortness of length of the sequences produced by NGS runs may also lead to identification errors or do not allow identifying microorganisms at the specie rank (Nilsson et al., 2006; Ovaskainen et al., 2010). Furthermore, qPCR-based methods provide information about viable and non-viable microorganisms without distinguishing between both groups which is not totally suitable when infectious microorganisms are investigated. Thus, molecular-based methods were developed to assess alive microorganisms and were applied to bioaerosols. Fluorescent in situ hybridization (FISH) involves fluorochrome-labeled nucleic acid probes to target rRNA within microbial cells and provides information on both the identity and viability of individual cells in situ. FISH was used to assess the total microbial cell concentration and viability in swine buildings (Chi and Li, 2005). Other methods such as ethidium or propidium monoazide-qPCR and flow cytometry were developed to target DNA of
microorganism based on the integrity of cellular membranes. They were also proposed to investigate undamaged microorganisms in bioaerosols (Chang and Hung, 2012).

Thus, the culture-independent methods do not overcome all the limits of culture-based ones. Indeed, the isolation of microorganisms from bioaerosol samples remains of a crucial interest for bioaerosol studies. It’s one way to investigate the physiology and the ecology of individual living specie, especially for the unknown ones and those that are difficult to cultivate such as Archea and anaerobes (specific growth requirement). Furthermore, molecular typing of isolates is also helpful for understanding of the transmission routes of infectious agents. New wild microbial isolates also complete the available collection of strains for further studies about the causative origins of occupational diseases due to bioaerosols exposure for example, by identifying allergens, toxins, and other cellular compounds. Finally, both culture-based and culture-independent methods progressively increment the knowledge about the structural and functional aspects of microbial communities in bioaerosols. Thus, microbial isolates also provide potential surrogates for the reconstitution of realistic and complex experimental bioaerosols of known composition as model microbiomes for laboratory studies.

The isolation or new and rare culturable microorganisms may be possible from bioaerosols as this was has been reported in several studies carried out with samples from other origins (Shade et al., 2012; Lutton et al., 2013; Tanaka et al., 2017). In the latter studies, the corresponding culturable microorganisms were not always found with culture-independent methods. Indeed, the recovery of cultivable microorganisms from bioaerosols samples would be improved by changing and multiplying culture conditions (Alain and Querellou, 2009; Martin et al., 2010; Lutton et al., 2013; Medina et al., 2017). While NGS generally allows a higher observed biodiversity in bioaerosols samples than culture-based methods, it is increasingly acknowledged that culture-based and culture-independent methods are complementary.

As a consequence, their still be an interest in proposals of new culture-based methods such as the one proposed by Shäfer et al. (2017) for bioaerosol studies. However, comparative studies should be carried out with both culture-based and culture-independent methods in order to complete knowledge about their respective scopes of application.

Conclusions

The *Annals of Work Exposures and Health* recently published two interesting studies combining the use of culture and molecular methods to assess microbial biodiversity in bioaerosols (Jäckel et al., 2017; Schäfer et al., 2017). The method involves the cultivation of bioaerosol samples on agar media and the pick-up of grown colonies 16S rRNA gene amplification, subsequent cloning, sequencing, and identification of bacterial isolates through the assignment against known gene databases. The method involves the mixing of PCR products obtained by amplification of genomic DNA from individual colonies and a cloning step of the mix PCR products allowing the separation of genes sequences and the recovery of all the existing bacteria in samples after the analysis. The method does not provide any improvement of the biases due the first step culture of bioaerosols samples, known to quantitatively and qualitatively underestimate microbial populations. It overcomes the limitations of sub-cultivation such as purity check of colony which is time and material consuming. The subsequent molecular identification process allowed the identification at the specie level. The application of the method in a ducks hatchery provided promising results and revealed a higher biodiversity than the one previously published in the same environment with culture-based methods. It suplements the panel of existing methods for the identification of cultivated bacteria, especially useful for infectious microorganisms, and complementing to the existing culture-independent ones. Further applications in other occupational environments as well as additional comparisons with both culture-based and culture-independent methods would complete it characterization.

Supplementary Data

Supplementary data are available at *Annals of Work Exposures and Health* online.

Declarations

Funding for this project was provided by the National Institute for National Research and Safety Institute for the Prevention of Occupational Accidents and Diseases (INRS). The author declares no conflict of interest relating to the material presented in this article. Its contents, including any opinions and/or conclusions expressed, are solely those of the author.

References


Lehtinen J, Tolvanen O, Nivukoski U et al. (2013) Occupational hygiene in terms of volatile organic compounds (VOCs) and bioaerosols at two solid waste management plants in Finland. Waste Manag; 33: 964–73.


Shrestha PM, Nevin KP, Shrestha M et al. (2013) When is a microbial culture “pure”? Persistent cryptic contaminant escapes detection even with deep genome sequencing. MBio; 4: e00591–12.


