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# Development and evaluation of a method for the quantification of airborne *Thermoactinomyces vulgaris* by real-time PCR

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ABSTRACT

Actinomycetes are ubiquitous and some can be potentially pathogenic for humans when present in the air of some working areas. It's notably the case for *Thermoactinomyces vulgaris* in composting facilities where aerial concentrations can reach high values of more than $10^7$ CFU·m$^{-3}$. Workers exposure to these inhalable bioaerosols can be the source of various diseases. The literature reveals a lack of knowledge about risk assessment: there is neither dose–effects relationship for most agents, or threshold limit value. The objectives of this study were to develop and standardize a method to quantify workers exposure to bioaerosols. We have developed and evaluated a method to quantify airborne *T. vulgaris* based on DNA extraction of aerial microbial communities and qPCR. Four DNA extraction protocols were compared, and primers and a hydrolysis probe were designed for specific amplification of the target species (*gyrB* gene). This method was compared to traditional methods based on viable or cultivable counting by epifluorescence microscopy or plating on selective media. The method was applied on environmental bioaerosols sampled under real exposure conditions in composting plants. We demonstrate that the method to quantify *T. vulgaris* in bioaerosols is specific, sensitive and repeatable. We demonstrate the occurrence and quantified *T. vulgaris* in the atmosphere of composting facilities with concentrations ranging from $3\times10^2$ to $3\times10^6\times$m$^{-3}$.
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## 1. Introduction

Actinomycetes are Gram positive bacteria producing branched hyphae and spores. They are ubiquitous in the environment and are associated with organic matter and biodegradation process. Actinomycetes can be present at high concentration levels in the air of numerous working areas, particularly those associated with agricultural activities and waste composting (Miyashita et al., 1982; Strom, 1985; Lacey, 1997; Le Goff et al., 2010). Actinomycetes (i.e. *Streptomyces albus*, *Micromonospora halophytica*, *Saccharomonospora viridis*) are able to produce small spores with aerodynamic diameters ranging from 0.9 to 1.5 μm (Grinshpun et al., 1997; Reponen et al., 1998; Madelin and Johnson, 1992). They are able to enter the respiratory track with a high probability and may deposit in the deepest part of the lungs (ICRP, 1994; CEN, 1993). Worker exposure to bioaerosols containing thermophilic actinomycetes spores may induce numerous health disorders, hypersensitivity pneumonitis (HP), being the most severe (Kagen et al., 1981; Kleyn et al., 1981; Pepys et al., 1990; Lacey and Dutkiewicz, 1994; Dalphin et al., 1998–1999; Jamey, 2008). Risks associated with occupational exposure to *Thermoactinomyces vulgaris*, i.e. dose–effects relationship and threshold limit value for occupational exposure, remain to be assessed.

Among thermophilic actinomycetes, *T. vulgaris* represents one of the main causing agents of HP, such as farmer's lung disease, mushroom-worker's lung disease and bagassosis, (Barboriak et al., 1972; Kleyn et al., 1981; Pepys et al., 1990; Reijula, 1993; Dalphin et al., 1998–1999). This species is widely present in high temperature biotopes like composts during the heating phase of the composting process (Millner et al., 1980; Lacey, 1997). It produces true endospores ranging in size from 0.5 to 1.5 μm diameter (Cross, 1970; Lacey, 1989; Reponen et al., 1998).

The most often used technique for analyses of microbiological aerosols containing actinomycetes is plate counts on agar media. Well-adapted for the investigation of new unknown environments, standard culture has however some drawbacks: the need for a huge diversity of culture media and incubation parameters and the underestimation of microbial concentrations in the samples. Plate counts only give access to cultivable microorganisms and colony masking may occur due to overgrowth of fast growing microorganisms on Petri dishes. Alternative culture-independent methods such

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as, epifluorescence microscopy (after DAPI or acridine orange staining), flow cytometry or fluorescence in situ hybridization (FISH), exist method but have been poorly assessed for the enumeration of actinomycetes.

Previous studies reported fungi in bioaerosols such as *Penicillium sp.* (Lignell et al., 2008), *Aspergillus sp.* (Haugland et al., 2002; McDevitt et al., 2004; Lignell et al., 2008; Pietarinen et al., 2008), *Cladosporium sp.* (Zeng et al., 2006; Lignell et al., 2008; Pietarinen et al., 2008) or *Stachybotrys sp.* (Haugland et al., 2002; Lignell et al., 2008; Pietarinen et al., 2008), *Fusarium circinatum* (Schweigkofler et al., 2004), or yeast-type fungi like *Candida albicans* species (Haugland et al., 2002). Several pathogenic bacteria were also reported in bioaerosols such as *Mycobacterium tuberculosis* (Chen and Li, 2005), *Legionella pneumophila* (Sirigul et al., 2006), *Bacillus anthracis*, *Brucella sp.* or *Yersinia pestis* (Makino and Cheun, 2003; Fykse et al., 2008).

The use of quantitative real-time polymerase chain reaction (qPCR) based protocols represents a very attractive alternative for the detection and the quantification of microorganisms in bioaerosols (Bustin et al., 2009). This method is increasingly used for environmental detection and quantification of microorganisms, on substrates (dust) as well as in indoor air (house, water-damaged buildings, working areas…). To date, mold specific quantitative PCR (MSQPCR) was designed for environmental detection of more than 100 molds, and combined results of the 36 of them were used to design the Environmental Relative Moldiness Index (ERMI) for mold exposure assessment of different patient groups (Reponen et al., 2011; Vesper, 2011). As well, qPCR was used for global assessment of bacterial load in aerosols from contaminated environments (Oppliger et al., 2008; Blais Lecours et al., 2012), and environmental studies targeting mainly *Streptomyces* species, in dust or building materials (Rintala and Nevalainen, 2006; Pietarinen et al., 2008). To date, implementation of species-specific or genus-specific qPCR for airborne actinomycetes detection is scarce, and was only recently developed to quantify *Saccharopolyspora rectivirgula* and *Streptomyces spp.* in bioaerosols (Schafer et al., 2011; Kaarakainen et al., 2011; Blais Lecours et al., 2012).

The aim of this study was to develop a standardized method, based on qPCR, for the survey of worker exposure to bioaerosols contaminated with actinomycetes. *T. vulgaris* was used as a model microorganism because of its wide distribution in the environment and its potential health hazard. A qPCR based method was designed and then validated for the quantification of *T. vulgaris* in bioaerosols. This method has been compared with traditional plate count method and validated under laboratory conditions using pure cultures and experimental bioaerosols. Then, it is has been evaluated in real exposure conditions to monitor the occurrence of *T. vulgaris* in the atmosphere of five composting plants.

## 2. Materials and methods

### 2.1. Origin, maintenance and growth conditions of microbial strains

The strains used for sequence alignment and oligonucleotide specificity testing are listed in Table 1. They were used to design specific primers and a hydrolysis probe for the specific quantification of *T. vulgaris* by qPCR. Collection strains were from DSMZ (German Collection of Microorganisms and Cell Cultures), CIP or UMIP (Pasteur Institute Collection), IHEM (Biomedical Collection of the Scientific Institute of Public Health) and ATCC (American Type Culture Collection). Strains were cultured according to the protocols given by the supplier. Strains from the environment were isolated from hay at the University Hospital of Besançon (Jamey, 2008) and from bioaerosols sampled in composting plants by filtration (37 mm closed-face cassettes). Environmental strains were isolated and identified by 16S rDNA sequencing at the University Hospital of Besançon. The model microorganism chosen for the rest of the study is the type strain of *T. vulgaris* (ATCC 43649). Stock culture was prepared by inoculation of the strain in 750 mL cell culture flask containing 90 mL of BBL™ Trypticase™ Soy Agar TSA

**Table 1**
Type of wastes treated in the five composting plants where environmental bioaerosols were sampled. Green waste corresponds to plant wastes (garden trimmings, wood…). Biowaste corresponds to the biodegradable fraction of municipal solid waste (after selective sorting).

| Composting facility designation | Type of waste treated | | | |
|---|---|---|---|---|
| | Green waste | Sewage sludge | Biowaste | Municipal solid waste |
| 1 | x | x | x | |
| 2 | x | | x | x |
| 3 | x | x | | |
| 4 | x | | | x |
| 5 | x | | x | |

(Becton Dickinson, USA) and incubation at 50 °C for 15 days, under high relative humidity.

### 2.2. Design of the primers and probe for qPCR

We targeted the gene *gyr*B which encodes the beta subunit of DNA gyrase (accession number AB242203) in *T. vulgaris*. *gyr*B is often used as a target gene for qPCR in bacteria (Kasai et al., 2000; Shen et al., 2005). *gyr*B gene is considered as a single copy gene present in all bacteria (Dauga, 2002). However, Rodrigues and Tiedje (2007) reported from the analysis of 194 genomes from the Joint Genome Institute (http://genome.jgi.doe.gov/) that 11.8% of the bacterial species harbored 2 to 3 copies of the *gyr*B gene. In the absence of complete genome data for *T. vulgaris* we cannot exclude the occurrence of multiple copies of this gene in *T. vulgaris*. *gyr*B sequences from several actinomycetes species (*Thermoactinomyces* sp., *Laceyella* sp., *Mechercharimyces* sp.…) were retrieved from GenBank and aligned with ClustalW (through the bioinformatic platform Mobyle, Pasteur Institute). From the alignment, variable regions of *gyr*B were selected and used to design primers and a hydrolysis probe specific for *T. vulgaris* using the Primer Express software (Applied Biosystems, USA). The specificity of the primers and probe was tested "in silico" against the GenBank database using BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) on the National Center for Biotechnology Information (NCBI) website. Selected primers were: forward primer Tvu-F751 (5′-GCC GAT CAT TTC CAA ACC TAT TT-3′), reverse primer Tvu-R824 (5′-GCC ATC ACG GCT TTG TTA ATA ATC-3′) and the hydrolysis probe was Tvu-S776 (5′-FAM-ACG AGC ATC CCG CGG AAG CG-TAMRA-3′). The corresponding PCR product has a 74 bp length. All purified oligonucleotides (Reverse-phase cartridge Purification for primers and High Performance Liquid Chromatography for probes) were obtained from Sigma-Aldrich (USA).

### 2.3. Genomic DNA extraction

Four protocols for genomic DNA extraction from bioaerosols were tested and compared. The first protocol is based on the FastDNA® SPIN kit for soil (MP Biomedicals, USA). The second protocol is based on the UltraClean® Microbial DNA Isolation Kit (MO BIO Laboratories, USA). The two last protocols tested are based on the DNeasy® Blood and Tissue Kit (Qiagen®, Netherlands); DNA was extracted following the supplier recommendations, using either the Gram negative bacteria specific protocol or Gram positive bacteria specific protocol. In preliminary experiments, we determined that the FastDNA® SPIN kit for soil associated to the FastPrep automated homogenizer (MP Biomedicals, USA) and following the manufacturer's protocols was the most efficient method to extract DNA from environmental bioaerosols (higher DNA yield). This DNA extraction method was chosen and used for both experimental and environmental bioaerosols as well as for DNA extraction from pure cultures of *T. vulgaris*.

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