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Normalization of test and evaluation of biothreat detection systems: Overcoming microbial air content fluctuations by using a standardized reagent bacterial mixture

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ABSTRACT

Test and evaluation of engineered biothreat agent detection systems ("biodetectors") are a challenging task for government agencies and industries involved in biosecurity and biodefense programs. In addition to user friendly features, biodetectors need to perform both highly sensitive and specific detection, and must not produce excessive false alerts. In fact, the atmosphere displays a number of variables such as airborne bacterial content that can interfere with the detection process, thus impeding comparative tests when carried out at different times or places. To overcome these bacterial air content fluctuations, a standardized reagent bacterial mixture (SRBM), consisting in a collection of selected cultivable environmental species that are prevalent in temperate climate bioaerosols, was designed to generate a stable, reproducible, and easy to use surrogate of bioaerosol sample. The rationale, design, and production process are reported. The results showed that 8.59; CI 95%: 8.46–8.72 log cfu distributed into vials underwent a 0.95; CI 95%: 0.65–1.26 log viability decay after dehydration and subsequent reconstitution, thus advantageously mimicking a natural bioaerosol sample which is typically composed of cultivable and uncultivable particles. Dehydrated SRBM was stable for more than 12 months at 4 °C and allowed the reconstitution of a dead/live cells aqueous suspension that is stable for 96 h at +4 °C, according to plate counts. Specific detection of a simulating biothreat agent (e.g. *Bacillus atrophaeus*) by immuno-magnetic or PCR assays did not display any significant loss of sensitivity, false negative or positive results in the presence of SRBM. This work provides guidance on testing and evaluating detection devices, and may contribute to the establishment of suitable standards and normalized procedures.

1. Introduction

Dangerous bacteria, viruses and toxins, have been classified into biological agent categories depending on their potential for adverse public health impact and their dissemination potential (Rotz et al., 2002). The aerosol route of dissemination is a great concern for public health and biosecurity worldwide (World Health Organization, 2004). Current aerosol detection concepts in biodefense, from real time nonspecific trigger to confirmatory diagnosis, have been reviewed (Demirev et al., 2005). Although some of these systems have been used to detect and/or monitor potential release of biological agent, it remains challenging to test and evaluate these systems and therefore to validate their impact and benefits.

Biological agent detection is complex compared to chemical or radiological agents, owing to the natural occurrence of microorganisms. Typically, biological detection systems surveying environmental aerosols comprise at least five major functions, providing increased level of confidence in data from detection systems: (i) triggering using a device that continuously monitors any change in the air content by using a size-segregated aerosol sampler, (ii) collecting the agent by sampling the bioaerosol and collecting it into liquid or onto a filter (bio-collectors), (iii) determining by more sophisticated means whether the alarm is of biological origin or not (e.g. fluorescence labeling, ATP tests), and (iv) identifying the biological agent by detection of specific antigenic and/or genetic signatures (i.e. immunoassays, nucleic acid-based assays). Such systems operate in successive steps, with each function acting as a gateway for the subsequent step.

Abbreviations: CF, concentration factor; CIP, Collection de l'Institut Pasteur; IMS-ECL, immuno-magnetic separation-electro-chemiluminescence; LOD, limit of detection; SRBM, standardized reagent bacterial mixture; T & E, test and evaluation; TSA, trypticase soy agar.

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Detection of a target in trace amounts in a changing complex background is demanding, and highly sensitive and specific detection is required (from detectors) under any conditions (Lim et al., 2005). Further, the quantitative and qualitative fluctuations of natural bioaerosol backgrounds may inhibit any attempt to evaluate and compare objectively the different systems available on the market. Recently, an improved method to help with the evaluation of real-time biological aerosol detection technologies has been designed to offer dynamic aerosol generation capabilities. This system uses background aerosol components such as road dust, sea salt, bacterial or fungal species to produce an artificial ambient aerosol under controlled laboratory settings (Ratnesar-Shumate et al., 2011). However, this system does not use standardized ready-to-use reagents, and therefore, does not allow easy comparison of different tests and evaluation procedures of biodetectors.

The environmental background is complex and dynamic; its components (physical, chemical and biological parameters and/or constituents) can alter the ability to detect the biowarfare agents (Stetzenbach et al., 2004). Besides being composed of debris from eukaryotes (mainly but not exclusively from plants), air samples contain numerous unicellular microorganisms that may be present in large excess compared to the targeted biological agent. As a result, true performances of biodetectors cannot be easily predicted before field trials are carried out. Furthermore, an in depth validation is often achieved after a long period of field trials prior to being put into service.

The microbial community living or transiting in the air has been extensively studied by culture techniques during the 20th century (Burrows et al., 2009; Stetzenbach, 1996). Recent advances in DNA technologies, such as next generation sequencing, has provided new insights in the bioaerosol diversity (Gandolfi et al., 2013). Globally, metagenomic data from airborne bacterial communities are dominated by sequences assigned to *Proteobacteria*, *Firmicutes* and *Actinobacteria*, whilst other phyla to subphyla are also identified at a lower extent. However, singular signatures have also been known to arise at occupational sampling sites as exemplified by the identification of a high content of *Bacillus*, *Micrococcus* and *Staphylococcus* at a subway station arising as a likely consequence of anthropogenic sources (Dybwad et al., 2012). Data in the literature indicates that the bacterial concentration in the atmosphere fluctuates around 10^6 cfu/m^3, and higher values by 1 to 3 orders of magnitude have also been documented for the total number of particles (live and dead cells).

Challenges of detection systems using a large batch of natural aerosol sample, even kept frozen into divided fractions, would neither be acceptable due to storage and long life constraints. However, this could be overcome by using normalized biological background versions which may provide a common means to assess detection systems, allowing for reproducible results independent of time, users, and trial sites.

Our goal was to develop such a ready-to-use normalized reagent as a surrogate of bioaerosol, meeting the performance specifications expected by those involved in test and evaluation processes. The interfering effect of this surrogate was evaluated by means of antibody- or PCR-based techniques, with spores of *Bacillus atrophaeus*.

2. Material and methods

2.1. Bacterial strains, conservation and culture conditions

SRBM strains, *Arthrobacter oxydans* str.070340, *Bacillus megaterium* str.030290, *Bacillus simplex* str.070532, *Cellulosimicrobium cellulans* str.030551, *Micrococcus luteus* str.030236, *Pseudomonas fluorescens* str.060137, *Pseudomonas jessenii* str.040138, *Pseudomonas lurida* str.060147, *Pseudomonas putida* str.070129 and *Pseudomonas rhizosphaerae* str.060111, belong to the bacterial culture collection of DGA Maîtrise NRBC and have been originally isolated from the outdoor environment during the evaluation of various detection devices. The species were identified based on their 16S rDNA nucleotide sequence and phenotypic characteristics (data not shown). *B. atrophaeus* str.930029 was received from the Collection de l'Institut Pasteur (CIP77.18, alias ATCC 9372) and was used for spore preparation, extraction of the reference DNA (batch no. 299), and the production of specific polyclonal antibodies (data not shown). All strains were routinely grown at their optimal temperature on trypticase soy agar (TSA) plates or TS broth (respectively, ref. 42101 and ref. 42100, Biomérieux, Marcy l'Etoile) for 18 to 72 h, and were cryopreserved in TS/70 Protect-Plus beads (TSC Ltd., Lancashire, UK) at −80 °C.

2.2. Spore preparation

Spore production of *B. atrophaeus* was adapted from Kim and Naylor (Kim and Naylor, 1966). Overnight culture in TS broth at 37 °C was used to inoculate fresh TS broth (8% v/v). After incubation at 37 °C with shaking at 100 rpm during 6 to 8 h, 5 ml of this preculture were spread over the surface of 100 ml of NBY agar media (Nutrient Broth 0.8%, DIFCO 0003-17-8; Yeast extract 0.3%, DIFCO 0127-01-07; agar 0.28%, DIFCO A4076161-01-09) poured in a BD Falcon™ 225 cm^2 tissue culture flask with vented cap (ref. 353138, BD Biosciences). The excess of preculture was discarded and the flask was subsequently incubated at 37 °C in a humidified chamber for approximately 15 days, until 90% of the cells had sporulated as determined by microscopic examination. The flask was allowed to cool at room temperature before adding 10 ml of ice-cold distilled water and ten 3-mm sterile glass beads (ref. CENT5748, VWR International, Strasbourg) to harvest spores by washing. The resulting spore suspension had a concentration ranging from 10^9 to 10^{10} cfu/ml and was conserved at +4 °C, before its use for detection assays. The cultivable spore concentration was determined by CFU assay as described below, after heat treatment of an aliquot at 80 °C for 10 min in a water-bath.

2.3. Production of SRBM

A production flowchart is available as Supplementary data (Fig. S1). A detailed step-by-step protocol of the production of SRBM and other methods used for quality controls, reconstitution procedure, and calculation of bacterial cell concentration can be found in the Supplementary material. The stability of SRBM over time is shown in Supplementary data in Figs. S2 and S3.

2.4. Immuno detection assays

Detection of spores of *B. atrophaeus* was carried out on the Bio Veris® Detection System (BVDS) (Formerly Bio Veris Corp., Gaithersburg, MD, USA). Briefly, the instrument combines antigen capture by immuno magnetic separation (IMS) and signal detection by electro chemiluminescence (ECL), with antigen-specific antibodies conjugated either to biotin or ruthenium(II) bipyridine [$Ru(bpy)_3^{2+}$] to allow capture or detection, respectively. All reagents used to run the BVDS were provided by the manufacturer (Cell Cleaner – 4 × 1 l – Ref 110007, Assay Buffer – 4 × 1 l – Ref 110012), or otherwise are described below. Rabbit polyclonal antibodies (L030.496.1) directed against spores of *B. atrophaeus* have been obtained previously (unpublished data), and were labeled either with biotin using sulfosuccinimidyl-6-(biotinamido)hexanoate (BV Biotin-LC-Sulfo-NHS Ester, #110015, Bio Veris Corp., Gaithersburg, MD, USA), or with ruthenium using ruthenium (II) tris-bipyridine, N-hydroxysuccinimide (BV-TAG-NHS Ester, #110034, Bio Veris Corp., Gaithersburg, MD, USA), according to the manufacturer's instructions. Suspension of spores of *B. atrophaeus* (10^5, 10^4, 5.10^3, 10^3, 5.10^2 and 10^2 spores/ml) and the negative control (no spores) was prepared in PBS-Tween (0.15 M phosphate buffered saline pH 7.4, 0.01% (v/v) Tween 20®), or in SRBM suspension adjusted to 10^4 cfu/ml. Sandwich assays were carried out by mixing simultaneously 175 µl of capture solution containing 0.1 µg of biotin-conjugated