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Rare airborne bacteria identified in resuspended dust in stables by next generation sequencing

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Abstract

Air quality of stables in Europe is a critical issue, as many horses are kept in stables quite often even for 23 hours per day. Inhalation of airborne dust and aeroallergens in stables is supposed to directly cause or exacerbate several respiratory disorders, the most often recognized problem being recurrent airway obstruction (RAO), previously termed chronic obstructive pulmonary disease (COPD). Monitoring studies have covered mainly the determination of the concentration of particles with diameter of 0.5−5 μm (particles of that size may interfere with lower parts of the airway). Also, as RAO is associated with the presence of aeroallergens, many studies have been targeted to determine the concentration of culturable fungi and their toxins. However, the $PM_{0.5-5}$ fraction (particles with the diameter of 0.5−5 μm) does not only cause direct effect on the respiratory tract, but these particles might act as carrier, conveying toxic contaminants and biological agents such as bacteria. As part of a large-scale study, microbial community of respirable fraction of resuspended dust has been characterised by culture-independent next generation sequencing (NGS) of variable 16S rRNA gene regions. Sample was collected in a typical Hungarian stable, using a mobile resuspended road dust PM_{10} sampling unit which induces resuspension and collects particles on-line directly from surfaces. The sampling unit includes a PARTISOL-FRM MODEL 2000 sampler which collects resuspended PM_{1-10} samples in a cyclone separator and PM_1 samples on filters. Apart from common, mostly ubiqituos soil and organic material-dwelling bacteria, rare airborne species have been identified, such as *Variovorax ginsengisoli*, previously isolated from Korean ginseng fields or *Exiguobacterium sibiricum*, isolated from the Siberian permafrost. Their potential source and transport are discussed.

Keywords: Stable; resuspended dust; microbial community; next generation sequencing.

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1. Introduction

Air quality of stables in Europe is a critical issue, as many horses are kept in stables quite often even for 23 hours per day. Inhalation of airborne dust and aeroallergens in stables is supposed to directly cause or exacerbate several respiratory disorders, the most often recognized problem being recurrent airway obstruction (RAO), previously termed chronic obstructive pulmonary disease (COPD). As such, air quality assessment in stables most often implies measurement of airborne dust concentration (ADC) (e.g., Kirschvink et al., 2002; Clements and Pirie, 2007) and/or monitoring the availability of aeroallergens, collecting and identifying viable airborne spores (e.g., Samadi et al., 2009). Particles with diameter of 0.5−5 μm may interfere with lower parts of the airway. Apart from the direct effect airborne dust has on the respiratory tract, these particles might act as a carrier, conveying toxic contaminants and biological contaminating agents such as bacteria (Fleming et al., 2008). Monitoring studies for example revealed the airborne transmission of the pathogen *Rhodococcus equi* (Muscatello, 2012).

As part of a large-scale study, microbial community of respirable fraction of resuspended dust has been characterised by culture-independent next generation sequencing (NGS) of variable 16S rRNA gene regions, taking a quite typical Hungarian stable as an example. Besides the overall characterisation of the bacterial community, the main aim of the study was to assess the risk of transport of specific horse pathogens by respirable particles. However, NGS revealed the presence of rare and unique bacteria, which have most possibly been transported from other geographical regions and remained viable.

2. Materials and methods

2.1 Sampling

The stable used for the study was a rather typical 20-stall building. Boxes are placed along a central aisle, which provides ventilation as well. Normally, whenever weather permits, horses are kept during the daytime on the pasture.

For sampling, a specific mobile sampling unit was used, which was developed for the simulating the effects of traffic and wind conditions on road surfaces and collecting the respirable fraction of resuspended road dust (PM_{10}) (Jancsek-Turóczi et al., 2013). The instrument uses a leaf blower to mobilize dust from paved surfaces, simulating very windy conditions (wind speeds ~65 km/h). The key unit in the apparatus is a PARTISOL-FRM model 2000 sampler that is operated at a flow rate of 16.7 L min^{-1} and contains a cyclone separator which collects the PM_{1-10} fraction (particles with aerodynamically equivalent diameters between 10 μm and 1 μm) in bulk form which is transferred into pre-weighted cleaned vial. The sampler is mounted on a mobile platform and powered with a portable electrical power generator. Sampling was completed on 9th March, 2013.

2.2 DNA extraction and PCR amplification

DNA concentration was determined by using the Qubit dsDNA HS Assay Kit with the Qubit 2.0 Fluorometer according to the manufacturer's (LifeTechnologies) instructions.

For fusion method-based, unidirectional Ion Torrent bacterial 16S rRNA sequencing, we carried out PCR amplification using the forward primer consists of the Ion Torrent adapter region (trP1: 5'-CCTCTCTATGGGCAGTCGGTGAT-3') fused to the 5' end of 16S rDNA target sequence (Bakt_341F: 5'-CCTACGGGNGGCWGCAG-3'). In reverse

primers, Ion A adapter region (5'-CCATCTCATCCCTGCGTGTCTCCGACTCAG-3') is linked downstream to the sample identifying Ion Xpress Barcode (LifeTechnologies) sequence, which is fused to the 5' end of the 16S rDNA target sequence (Bakt_805R: 5'-GACTACHVGGGTATCTAATCC-3'). Triplicate reactions were carried out in 20 µl volumes by using KOD Hot Start DNA Polymerase (Novagen) according to the manufacturer's instructions, except that 10 ng of template DNA and 3% DMSO were present in all reactions. Cycling conditions involved an initial 2-min denaturing step at 95 °C, followed by 25 cycles of 30 s at 95 °C, 30 s at 54 °C, and 30 s at 70 °C and a final elongation step of 5 min at 70 °C. PCR products were pooled and a two-step purification using 0.5 volumes of Agencourt AMPure XP Reagent was performed, respectively. The quality and quantity of the ca. 530-bp products were assayed by DNA 1000 Kit on Agilent Bioanalyzer 2100 instrument. 20 pM of total DNA were amplified in an emulsion PCR followed by target enrichment by using an Ion OneTouch 400 template kit, whilst sequencing of the pooled library was performed using an Ion Torrent PGM system and a 316v2 chip with the Ion Sequencing 400 kit according to the Life Technologies' protocol.

2.3 Bioinformatic analyses

In order to classify reads covering the V3 and V4 regions of 16S rRNA gene up to species level, the bioinformatic pipeline described by Eiler et al. (2012) was slightly modified. The operating system was Ubuntu, BioLinux 7 and the open-source softwares MOTHUR 1.31.2, R 3.0.1, Qiime 1.7.0, Cytoscape 2.7.0 and Krona excel template were applied. Out of 1.1 million total reads, sequences having an average quality number under 25, containing ambiguous bases, homopolimers longer than 8 bases, having more than 1 mismatch to the barcode sequence, more than 2 mismatches to the primer sequence or being shorter than 400 bp and chimeric sequences were discarded. The unique sequences were aligned to the appropriate reference small subunit databases (Greengenes, RDP and Silva). For operational taxonomic unit (OTU) calculations, a 97% similarity cutoff was used, and the OTU assignment data and sequence to sample mapping are used to generate the OTU-based table to count the number of sequences per OTU per sample.

3. Results

In total, 1491 different taxa were identified, with total read count of 14 382. For the purpose of this report, rather arbitrarily, those taxa are identified as 'rare' which were represented by only 1 read count. As such, 658 rare taxa were isolated.

Of them, 442 were identified to genus level, 199 to species level. Among the identified rare bacteria Gram negative organisms were present at 67, and Gram positive organisms at 32% of relative abundance. 1% of the rare organisms belong to the Candidate division TM7 group, a phylum known only from sequencing data, closely related to Clorococci, but it is not classified according to its cell wall structure. Distribution of identified Phyla is shown in Table 1.

Table 1. The relative abundance of the rare taxa.

Phylum	Relative abundance
Armatimonadetes	0.46
Chlamydiae	0.46
Chlorobi	0.15
Elusimicrobia	0.15
Fibrobacteres	0.15
Lentisphaerae	0..3
Nitrospirae	0.61
Acidobacteria	3.34
Planctomycetes	5.62
Bacteroidetes	13.37
Chloroflexi	3.19
Gemmatimonadetes	0..3
Proteobacteria	34.19
Verrucomicrobia	3.65
Cyanobacteria	1.22
Deinococcus-Thermus	1.22
Actinobacteria	14.89
Firmicutes	15.35
Candidate_division_TM7	1.06

4. Discussion

Considering species diversity, number of rare taxa represents 44%. Most of them are most possibly of local origin, representing ubiqituous soil or organic-material dwelling species such as *Rhizobium leguminosarum* that nodulates Fabaceae species or soil-dwelling *Bacillus cereus*. Though to rather low extent, pathogens or facultative pathogens have also been identified, such as *Bacillus anthracis*, an (uncultured) *Legionella sp*. (identified only to genus level) or *Aerococcus viridans* which has rarely been reported to cause endocarditis or septic arthritis (Taylor & Trueblood, 1985).

However, in the scope of this paper, those bacteria are the most interesting which are rare and unique: have been isolated in a geographically distinct area and most possibly have been transported by aerosol particles. Some species are highlighted here and given as an example:

- *Brevundimonas mediterranea*: Gram-negative, rod-shaped, non-spore-forming bacterium isolated from the Mediterranean Sea (Fritz et al., 2005)
- *Deinococcus reticulitermitis*: Gram-negative, spherical, non-motile, non-sporulating bacterium, isolated from a termite gut (Chen et al., 2012)
- *Exiguobacterium sibiricum*: Gram-positive, rod-shaped, facultative aerobic, motile bacterium, isolated from Siberian permafrost (Rodrigues et al., 2006)
- *Flavisolibacter ginsengisoli*: Gram-negative, aerobic, non-motile, rod-shaped bacterium, isolated from ginseng cultivating soil in Korea (Yoon & Im, 2007)
- *Mesorhizobium alhagi*: Gram-negative, rod-shaped, non-spore-forming, aerobic, motile bacterium, forming symbiotic root nodules on (Chinese) *Alhagi sparsifolia* (Chen et al,, 2010)
- *Mucilaginibacter ximonensis*: Gram-negative, irregular rod-shaped, non-motile bacterium, isolated from Tibetan soil (Luo et al., 2009)
- *Nocardioides exalbidus*: Gram-positive, non-endospore-forming, non-motile, irregular coccoid to rod-shaped bacterium, isolated from lichen in Japan (Li et al., 2007)

- *Paenibacillus xylanexedens*: Gram-positive, rod-shaped, motile bacterium, isolated from Alaskan tundra (Nelson et al., 2009)
- *Pedobacter composti*: Gram-negative, aerobic, rod-shaped, non-motile, non-spore-forming bacterum, isolated from compost in Korea (Lee et al., 2009a).
- *Pseudomonas xanthomarina*: Gram-negative, rod-shaped, motile bacterium, isolated from marine ascidians *Didemnum* sp. and *Halocynthia* sp. (Romanenko et al., 2005)
- *Streptomyces sedi*: Gram-positive, elliptical/rod-shaped bacterium, isolated in China from roots of *Sedum* sp. (Li et al., 2009)
- *Variovorax ginsengisoli*: Gram-negative, aerobic or facultatively anaerobic, non-spore-forming, motile, rod-shaped bacterium, isolated from soil of a ginseng field (Im et al., 2010)

It is a well-known fact that bacteria are constantly being transported through the atmosphere, which may have implications for their dispersal (Burrows et al., 2009). Airborne bacterial communities are especially frequently monitored during dust events (e.g., Lee et al., 2009b). However, species-level characterisation mostly concentrates on bacteria having human health implications (e.g., Kaushik and Balasubramanian, 2012).

As in our study resuspended dust was sampled within the stable, some of the species mentioned above might have distribution not restricted only to the region where they were isolated from, especially those isolated from soil such as *Flavisolibacter ginsengisoli*, *Mucilaginibacter ximonensis, Paenibacillus xylanexedens* or *Pedobacter composti*. On the contrary, those species which are specific in terms of occurrence, such as *Deinococcus reticulitermitis* which was isolated from a termite gut or *Mesorhizobium alhagi* which is a symbiont of *Alhagi sparsifolia* or *Pseudomonas xanthomarina* which was isolated from marine ascidian (none of these co-existing taxa can be found in Hungary) most possibly can be regarded as of distinct origin.

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