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MICROBIOLOGICAL CHARACTERIZATION OF STABLE RESUSPENDED DUST

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Abstract
Objectives: Air quality in the stables is characterized by elevated level of dust and aeroallergens which are supposed to directly cause or exacerbate several respiratory disorders. The most often recognized problem is recurrent airway obstruction (RAO), previously known as chronic obstructive pulmonary disease (COPD). There is some indication that aeroallergens (among them endotoxins) may also cause inflammation in human airways and may exceed safe levels in stables. Monitoring studies have covered mainly the determination of the concentration of respirable particles and of culturable fungi and their toxins. However, these particles do not only directly affect the respiratory system, but might act as a carrier conveying toxic contaminants and biological agents such as bacteria. In a typical, 20-horse Hungarian stable, microbial community of respirable fraction of resuspended dust has been characterized to reveal if these particles convey hazardous pathogenic bacteria, posing risk to either horses or staff. **Material and Methods:** Resuspended dust was sampled using a mobile instrument. The instrument contains a PARTISOL-FRM model 2000 sampler that was operated at a flow rate of 16.7 l/min and a cyclone separator which collected the particulate matter with an aerodynamic size between 1 μm and 10 μm ($PM_{1–10}$) fraction. Microbial taxa were identified by culture-independent next generation sequencing (NGS) of variable 16S ribosomal ribonucleic acid (rRNA) gene regions. **Results:** In total, 1491 different taxa were identified, of them 384 were identified to species level, 961 to genus level. The sample was dominated by common ubiquitous soil and organic material-dwelling taxa. **Conclusions:** Pathogens occurred at low abundance, and were represented by mostly facultative human pathogens, with the prevalence of *Staphylococcus* species.

Key words:
Stable, Air quality, Dust, Microbial community, Next generation sequencing, Pathogens

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INTRODUCTION

Air quality in the stables is characterized by elevated level of dust and aeroallergens which are supposed to directly cause or exacerbate several equine respiratory disorders. The most often recognized problem is recurrent airway obstruction (RAO), previously known as chronic obstructive pulmonary disease (COPD) [1]. Recurrent airway obstruction is supposed to develop due to hypersensitivity to environmental allergens, principally those associated with stabling, resulting in inflammation and reversible obstruction of the lower airway.

As such, most studies have focused on respirable particle monitoring and determining the presence of aeroallergens. Particle monitoring techniques are reviewed by Millerick-May et al., 2011 [2]. There is some indication that aeroallergens (among them endotoxins) may also cause inflammation in human airways and may exceed safe levels in stables [3,4].

In general, there are 2 important sources contributing to airborne dust, feed and bedding. Of feed, hay has the highest contribution [5]. Clements and Pirie [6] found that feed had a greater influence on mean and maximum respirable dust concentration than bedding.

Apart from the direct effect airborne dust has on the respiratory system, these particles might act as a carrier conveying toxic contaminants and biological contaminating agents such as microorganisms [7]. In stables, for identification of microbial community being found on airborne particles, most often culture techniques are applied [4]. Though not specifically in stables, most recent studies use culture-independent methods such as real-time polymerase chain reaction (PCR) technique [8,9] or ribonucleic acid (RNA) gene sequencing [10,11].

In our study, microbial community of respirable fraction of resuspended dust was sampled in a typical, 20-horse Hungarian stable and microbial community was characterized by culture-independent next generation sequencing (NGS) of variable 16S ribosomal ribonucleic acid (rRNA) gene regions. Besides, giving a general description of the microbial community being found on respirable particles, the main target of the work was to reveal if these particles convey hazardous pathogenic bacteria, posing risk to either horses or staff.

MATERIAL AND METHODS

Sampling

The studied stable was an enclosed 20-stall building. Along a central aisle, 10–10 boxes are placed, with doors providing ventilation. Horses are kept in the pasture during the day whenever weather permits. During sampling, the horses were also kept in the pasture.

For sampling, a specific mobile sampling unit was used, which was developed to simulate the effects of traffic and wind conditions on road surfaces and collect the respirable fraction of resuspended road dust (PM_{10}) [12]. 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 and contains a cyclone separator which collects the $PM_{1–10}$ fraction (particles with aerodynamically equivalent diameters 1 μm and 10 μm) in bulk; the collected material is then transferred into pre-weighted cleaned vials. The sampler is mounted on a mobile platform and powered with a portable electrical power generator. Sampling was completed on 9 March 2013.

Deoxyribonucleic acid (DNA) extraction and PCR amplification

Deoxyribonucleic acid concentration was determined using the Qubit dsDNA HS Assay Kit with the Qubit 2.0 Fluorometer according to the manufacturer's (Life Technologies) instructions.

For fusion method-based, unidirectional Ion Torrent bacterial 16S rRNA sequencing, PCR amplification was carried

out, using the forward primer consisting 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') was linked downstream to the sample identifying Ion Xpress Barcode (Life Technologies) sequence, which was 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 using KOD Hot Start DNA Polymerase (Novagen) according to the manufacturer's instructions, except that 10 ng of template gDNA and 3% dimethyl sulfoxide (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. Polymerase chain reaction products were pooled and a 2-step purification using 0.5 volumes of Agencourt AMPure XP Reagent was performed.

The quality and quantity of the ca. 530-bp products were assayed by DNA 1000 Kit on Agilent Bioanalyzer 2100 instrument. Twenty pM of total DNA were amplified in an emulsion PCR followed by target enrichment by an Ion OneTouch 400 template kit, whilst sequencing of the pooled library was performed using an Ion Torrent Personal Genome Machine (PGM) system and a 316v2 chip with the Ion Sequencing 400 kit according to the Life Technologies' protocol.

From an 80 m^2 area, 39.57 mg $PM_{1–10}$ fraction were collected. Due to the low sample quantity and the potential presence of PCR inhibitors, the determination of the effectiveness of the applied DNA extraction method was necessary. A sterile spicemen of rock was crushed in a ball-mill to $PM_{1–10}$ fraction, and different quantities of *Bacillus subtilis* were added to 20 mg of samples. Deoxyribonucleic acid extraction was performed, and qualities of samples were evaluated. With the slight modification of the recommended procedure, and the optimization of the dry weight/buffer rate, successful amplification of 16S rRNA gene was accomplished from samples containing 20–30 cells each.

Bioinformatic analyses

In order to classify reads covering the variable regions (V3, V4) of 16S rRNA gene up to species level, the bioinformatic pipeline described by Eiler et al. [13] was slightly modified. The operating system was Ubuntu, BioLinux 7 and the open-source software 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, homopolymers 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, Ribosomal Database Project (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 were used to generate the OTU-based table to count the number of sequences per OTU per sample.

RESULTS

About 20% of the reads could be identified on the species level, 49% on the genus level and 17.5% on the class level. Among the identified bacteria, Gram-negative organisms were present at 59.4%, and Gram-positive organisms at 40.1% of relative abundance. Half percent of the taxa belong to phyla known only from sequencing data, and not classified according to their cell wall structure.

The relative abundance of the most common phyla is presented in Figure 1.

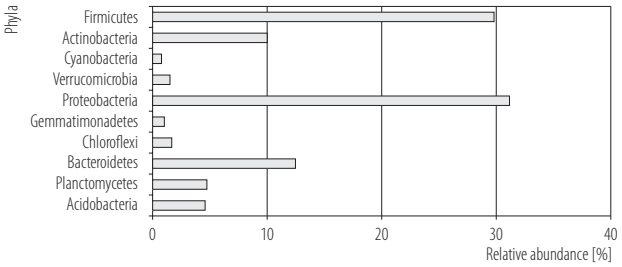

Fig. 1. Relative abundance of the most abundant phyla

DISCUSSION

In fact, the majority of the genera observed are ubiquitous soil dwelling bacteria, such as *Pedobacter*, with 28 species/strains (total read count of *Pedobacter* species was 275, amounting to 1.934%) or *Rhizobium* sp. Occurrence of pathogenic bacteria in our sample was rather low, and almost exclusively restricted to species isolated from human specimens. *Staphylococcus* genus was the most dominant (total read count 1064, amounting to 7.42%). Coagulase-negative staphylococci (CoNS) are normally considered as part of the animal microbiota, inhabiting the skin, skin glands and mucous membranes of various mammals and birds [14]. *Staphylococcus* cells can be maintained for relatively long periods of time on various surfaces and can survive in aerosols for extended periods. Aerosol transmission of staphylococci dissemination is far under-estimated root of colonisation [15,16].

Though CoNS are generally regarded to be of low virulence, several *Staphylococcus* species occurring in the sample have been isolated from (human) clinical specimens: *S. succinus* (the most dominant species with read count 711, amounting to 4.957%) [17]; *S. equorum* (read count 118, amounting to 0.823%) [17,18]. *Staphylococcus xylosus* (read count 14, amounting to 0.098%) has been shown to play a role in infections of the urinary tract in humans and more rarely, in pneumonia [19]. The prevalence of *Staphylococcus* species is not surprising: in a study conducted in a poultry house, *Staphylococcus* sp., including *S. lentus*, *S. xylosus*, *S. sciuri*, and *S. chromogenes* accounted for 42% of all isolated microorganisms [20].

The pathogenicity of the abundant *Streptococcus infantarius* subsp. *infantarius* (read count 195, amounting to 1.36% of the total sample) is not clear: the species was differentiated from the *Streptococcus bovis* / *Streptococcus equinus* complex isolated from human samples and associated with non-colonic cancer [21,22].

Acinetobacter ursingii (read count 66, amounting to 0.46% of the total sample) was isolated from human clinical samples [23] and reported to cause bacteremia in a patient with a pulmonary adenocarcinoma [24].

Amongst rare bacteria (taxa represented by only 1 read count [25]), 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 [26].

CONCLUSIONS

The overall objective of this study was to obtain a snapshot of the culturable microbiota of respirable fraction of stable resuspended dust using Ion Torrent sequencing of 16S rRNA gene. Analysis of the microbial community of the PM_{1-10} fraction of resuspended dust within the stable revealed that its major components are taxa naturally present on soil particles and on organic material such as hay or straw. Somewhat against our starting hypothesis, these particles have not conveyed pathogens posing hazard to horses' health; however, some human pathogens have been identified.

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