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Community analysis of dental plaque and endotracheal tube biofilms from mechanically ventilated patients

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Abstract

Purpose: Mechanically ventilated patients are at risk of developing ventilator-associated pneumonia and it has been reported that dental plaque provides a reservoir of respiratory pathogens that may aspirate to the lungs and endotracheal tube (ETT) biofilms. For the first time, metataxonomics was used to simultaneously characterise the microbiome of dental plaque, ETTs and non-directed bronchial lavages (NBL) in mechanically ventilated patients to determine similarities in respective microbial communities and therefore likely associations.

Material and Methods: Bacterial 16S rRNA gene sequences from 34 samples of dental plaque, NBLs and ETTs from 12 adult mechanically ventilated patients were analysed.

Results: No significant differences in the microbial communities of these samples were evident. Detected bacteria were primarily oral species (*e.g. Fusobacterium nucleatum, Streptococcus salivarius, Prevotella melaninogenica*) with respiratory pathogens (*Staphylococcus aureus, Pseudomonas aeruginosa, Streptococcus pneumoniae* and *Haemophilus influenzae*) also in high abundance.

Conclusion: The high similarity between the microbiomes of dental plaque, NBLs and ETTs suggests that the oral cavity is indeed an important site involved in microbial aspiration to the lower airway and ETT. As such, maintenance of good oral hygiene is likely to be highly important in limiting aspiration of bacterial in this vulnerable patient group.

Key words: Endotracheal tube biofilm, dental plaque, metataxonomics, mechanical ventilation

Introduction

Mechanical ventilation (MV) is an essential intervention in many critically ill patients and an endotracheal tube (ETT) is a key interface in this process. Unfortunately, patients receiving MV are at higher risk of nosocomial infection, and of particular concern is ventilator-associated pneumonia (VAP) [1].

VAP is the most prevalent hospital-acquired infection in critical care, with an incidence of 9-24% in patients who are mechanically ventilated for longer than 48 h [2, 3]. In addition, depending on patient group, VAP has a high attributable mortality of around 13% [4], and leads to longer hospital stay (mean 6.1 days) and increased healthcare costs [5]. A wide range of microorganisms may be present in the lower respiratory tract of hospitalised patients, and as part of VAP surveillance and diagnosis, specimens from the lower airways are frequently analysed to monitor pathogenic colonisation [6]. The least invasive interventions to obtain such specimens are non-directed bronchial lavages (NBLs), with the current diagnostic threshold based on microbial growth exceeding 10^4 CFU/ml [7]. These colonising microorganisms frequently exhibit multidrug resistance and if resistant microorganisms are involved in VAP, effective treatment becomes even more problematic with higher risk of treatment failure [8].

The microorganisms associated with VAP are wide ranging, but most frequently encountered are Gram-negative bacteria including *Pseudomonas aeruginosa*, *Stenotrophomonas maltophilia*, *Citrobacter* species, *Klebsiella pneumoniae*, and *Acinetobacter baumannii*. *Staphylococcus aureus* (including methicillin-resistant *Staphylococcus aureus*; MRSA) is the most prevalent Gram-positive bacterial species [9, 10].

The precise origin of VAP-causing bacteria is unclear, but upper airway colonisation by pathogens is considered a risk factor. It is thought that the microorganisms accumulate within subglottic secretions above the inflated ETT cuff and enter the lower airway below the cuff, either due to cuff displacement from the tracheal wall or via microchannels that develop within the cuff material [6]. Microorganisms may also be drawn into the inner lumen of the ETT, where they grow as biofilms, which may also serve as sources of VAP-causing agents, which are largely protected from host defence mechanisms [11].

Recent evidence has indicated that a microbial change may also occur in the dental plaque of MV patients [12]. Such a microbial 'shift' sees the colonisation of dental plaque with bacteria associated with VAP and these species are ones not normally found in 'healthy' mouths. As a

consequence and similar to ETT biofilms, the dental plaque itself would then be a source of VAP pathogens. The reason for the microbial change in dental plaque during mechanical ventilation is currently not known. Dental plaque contains many bacteria adept at biofilm development and these may assist recruitment of microorganisms less able to attach directly to the teeth, such as respiratory pathogens. Similarly, this same phenomenon could occur within the ETT lumen. To fully appreciate the potential role of oral microorganisms in promoting ETT biofilm formation and lower airway contamination, characterisation of the microbial populations at these sites is an important undertaking.

The majority of bacteria have often been considered unculturable using currently available microbiological media and methodology, and studies indicate that over 98% of environmental bacteria are currently unculturable *in vitro* [13]. In clinical samples, the numbers of unculturable bacteria, whilst lower, can still exceed 90% [14-16]. In the oral cavity, there are an estimated 500 to 600 species, and it is thought that approximately half of these have not yet been cultivated [17]. In contrast, a novel approach that combines whole-genome and metagenomic sequencing, with computational and phenotypic analysis recently showed that 90% of the gut bacterial microbiome are in fact culturable [18]. As such, it is impractical to fully determine the microbial composition of communities including those in the oral cavity, lower airway and ETT without use of culture-independent molecular techniques.

In recent years, molecular approaches have been developed that combine the sensitivity of PCR with the specificity of sequencing in order to gain detailed information and understanding of microbial species interactions in particular communities. Microbiomics is a collective term, which encompasses the molecular tools available to achieve profiling of microbial communities, including uncultivable components [19]. One aspect of this toolkit uses inventories of 16S rRNA genes to provide a 'snapshot' of bacterial diversity and relative abundance within a sample [20, 21].

In this present study, we report for the first time the metataxonomic characterisation of the bacterial microbiota at three separate, but potentially linked sites within individual MV patients. Analysed clinical samples were dental plaque, ETT biofilms and non-directed bronchial lavages (NBLs). It was envisaged that through this investigation, insight into microbial links between the oral cavity, the ETT and the lower respiratory tract would be achieved. Should components of the oral microbiota contribute to the ETT biofilm, and/or

respiratory pathogens found to colonise the dental plaque, further supporting evidence for maintaining high levels of oral hygiene in MV patients will have been provided.

Materials and Methods

Collection and processing of clinical specimens

Ethical approval for collection of clinical samples was obtained from the Research Ethics Committee for Wales (Reference # 08044240). A total of 21 patients were recruited (Trial registration: Clinical Trials.Gov NCT01154257 14th June 2010) [22] and following receipt of informed and written consent from patients or relatives complying with the Mental Capacity Act 2005, these patients provided dental plaque, NBLs and ETT samples for analyses. Inclusion criteria were that patients had to be aged >18 years, had >20 teeth, and an expectation of mechanical ventilation with an endotracheal tube for >24 h. At the start of the study, oral hygiene status was assessed using a plaque and gingival index [23]. During the study, routine oral care was delivered by mechanical tooth brushing using the modified Bass technique [23] with each side of the mouth cleaned for 1 min. Chlorhexidine was not part of the routine oral care in the critical care unit and was not administered to these patients. Samples from 12 patients (7 male and 5 female, aged 23 to 70 years old; Table 1) were selected for next generation sequencing. Patients were recruited for the study for between 2 and 7 days.

Dental plaque was obtained from the upper and lower first molars, first bicuspid and central incisors on each side of the mouth using sterile endodontic paper points (size ISO45). Sampling was initiated at the distal part of the buccal aspect of the tooth with 1 mm of paper point placed into the gingival sulcus. Using a slow and continuous motion, the paper point collected dental plaque by being drawn towards the operator. Paper points were placed in 1 ml of microbiological transport medium (Reduced Transport Fluid) [24] prior to processing for DNA extraction.

NBL specimens were obtained twice weekly from patients and the ETT itself was recovered for analysis after extubation. The ETT was wrapped in a sterile paper towel dampened with saline solution and sealed; the samples were all processed within 4 h. The central part of the ETT was cut to provide a 1 cm section from which the biofilm was recovered by scraping with a sterile surgical blade. Recovered biofilm was resuspended in 1 ml of phosphate saline buffer (PBS). All samples were stored at -80°C prior to DNA extraction.

DNA extraction

DNA was extracted from all samples using the Gentra Puregene® Yeast/Bacteria kit employing the Gram-positive bacterial protocol as described by the manufacturer (Qiagen, Manchester,

UK). Pre-treatment of highly viscous NBLs and ETT biofilms involved addition of an equal volume of Sputasol® (Oxoid, Altrincham, UK) to the specimen, which was incubated at 37°C whilst being rotated at 100 rev/min for 2 h. Samples that remained viscous following Sputasol® treatment had 50 µl sterile glass beads (425-600 µm in diameter, Sigma) added and were homogenised for 30 s in a mini bead beater (Stratech Scientific, Newmarket, UK) before proceeding with the DNA extraction protocol. Purified DNA was stabilised in a DNA eluting solution (Qiagen).

DNA extraction was confirmed by PCR amplification of the bacterial 16S rRNA gene using the bacterial primers GAGAGTTTGATYMTGGCTCAG (D88) and GAAGGAGGTGWTCCARCCGCA (E94) [25]. The PCR mixes (25 µl) contained 12.5 µl of PCR master mix (Promega), 1 µl of DNA template, and 0.5 µl of each forward and reverse primers at 50 µM. PCR thermal cycling parameters were an initial denaturation step of 95°C for 1 min, followed by 26 cycles of 94°C for 45 s, 50°C for 45 s and 72°C for 90 s (Thermocycler G-Storm). Amplicons were visualised by agarose gel electrophoresis.

MiSeq sequencing

DNA sequencing was undertaken by Research and Testing Laboratory (Austin, USA) using the Illumina MiSeq. Bacterial primers (GAGTTTGATCNTGGCTCAG [28F] and TGCTGCCTCCGTTAGGAGT [388R]) were used to generate multiple sequences of approximately 250 base pairs, overlapping within the V4 region of the 16S rRNA gene.

Phylogenetic identification and data analysis

The 16S rRNA gene sequences were analysed using the bioinformatics software package Mothur [26] and the MiSeq SOP Pipeline. 16S rRNA gene sequence reads were quality checked and normalised to the lowest number of reads in Mothur. To maintain normalisation and minimise artefacts, singletons and any Operational Taxonomic Units (OTUs), which were not found on more than 454 occasions in any sample, were collated as OTU singletons and OTU_rare phylotypes. Using the Vegan package of the R statistical package (R Development Core Team, 2008), analysis was performed on the datasets contained within the files generated by Mothur (all OTUs were defined using a cut off value of 97%). The Unifrac weighted distance matrix was analysed in R using non-metric multidimensional scaling (NMDS) ordination and the shared OTU file was used to determine the number of times that

an OTU was observed in multiple samples, and was used for multivariate analysis in R. OTU taxonomies (from phylum to genus) were determined using the RDP MultiClassifier script to generate the RDP taxonomy [27]. Alpha and beta indices were calculated from these datasets with Mothur and R using the Vegan package.

Results

From the 12 participating patients, 34 samples were obtained (Table 2) with one dental plaque sample from each patient, 12 ETTs from 10 patients, and 10 NBLs from 7 patients. There were samples from all three sites for 5 patients (P05, P10, P14, P20 and P21). The number of raw sequence reads was 2248956 and this was subsampled down to 9385 per sample. The number of OTUs for plaque was 127, for ETTs was 125 and for NBL was 83.

Evaluation of microbiome diversity between samples sites

Chao, Shannon, and ANOVA were used to measure diversity in the whole microbiome of dental plaque, NBLs and ETTs. Analyses revealed no significant differences in the microbiomes at the three different sites (Shannon $P=0.306$, Chao $P=0.685$; Fig. 1). Additionally, NMDS was used to visualise the position of each sample's community in a multidimensional space and showed clear overlaps between the microbial communities of dental plaque, NBLs and ETTs (Fig. 2).

Similarities between samples

Similarities between samples were analysed using the Bray-Curtis index. A constructed dendrogram (Fig. 3) showed identification of four major sample clusters, and each cluster contained dental plaque, ETT and/or NBL samples. There were 10 sub-clusters of five pairs, where the microbial composition of dental plaque and either NBL or ETT from the same patient was indistinguishable (P28, P03, P24, P27). Moreover, the same level of similarity was observed for samples from different patients on 7 occasions, forming pairs between dental plaque and NBLs ($N=2$), dental plaque and ETT ($N=1$), ETT and NBL ($N=1$), and grouping same site samples from different patients NBLs ($N=2$) and ETT ($N=1$). One cluster of three 'identical' dental plaque samples (P10, P14, P20) and a further 4 pairs of closely related samples from the same patient were apparent. Interestingly, samples from all three sites for patients 10, 14 and 21, appeared distantly related.

Bacterial composition

Analysis of the most representative species was based on the 100 most abundant species; with 58.5% of species identified at a cut off value of 97%. Analyses of the top 20 species showed that overall, the most commonly detected species was *Staphylococcus aureus*,

followed by *Pseudomonas aeruginosa*, *Streptococcus pneumoniae* and *Haemophilus influenza*. Importantly all of these species are potential respiratory pathogens. This group was followed by species normally associated with the urinary (*Enterococcus hirae*) and gastrointestinal (*Shigella dysenteriae*) tracts. The remaining 13 species were typical oral microorganisms.

The top 100 species were further analysed per sample site. In dental plaque (Fig. 4), *S. pneumoniae* was the most abundant species followed by *E. hirae*, (although most OTUs for this species originated from only one patient; P27) and *Fusobacterium nucleatum*, an oral microorganism recognised for its role in bacterial coaggregation. Importantly, the respiratory pathogens *S. aureus* and *P. aeruginosa* were also abundant in dental plaque samples. For ETTs, *S. aureus*, *H. influenzae* and *S. pneumoniae* were the three most abundant species, but the presence of oral microorganisms in these biofilms was also evident (Fig 5). Finally, in NBLs, the most prevalent species were *S. aureus* and *P. aeruginosa* and similar to the ETTs, typical oral species such as *F. nucleatum*, *S. oralis* and *P. melaninogenica* were detected (Fig. 6).

The top 20 species per individual patient was analysed by heat maps, and revealed that most microorganisms were simultaneously present in all available samples. Interestingly, in some cases (*S. aureus* in P14, P20 and P21) the OTUs were higher in the NBL and/or the ETTs than in dental plaque (data not shown).

Discussion

Amongst critically ill patients who are mechanically ventilated for more than 48 h, there is a risk of developing VAP (between 4 and 13 cases per 1000 ventilator days; [28]) of VAP. VAP also has an associated attributable high mortality, extends hospital stay, and increases economic burden [29-31]. Multiple factors contribute to the occurrence of VAP [32] and included amongst these is the oral microbiome. The oral microbiota could play a role in VAP in several ways. Firstly, microorganisms commonly encountered in the mouth could directly instigate VAP following aspiration to the lungs [33]. In addition, accepted respiratory pathogens that are not normally present within the oral microbiota may colonise the mouth and again, move to the lungs, thereby resulting in infection [34]. Interestingly, after cessation of mechanical ventilation, respiratory pathogens that were colonising dental plaque are often lost from the plaque [34]. As well as movement between the mouth and lower airways, microorganisms may also grow as biofilms within the lumen of the ETT [12]. These biofilms may harbour typical oral microorganisms together with respiratory pathogens, with the former potentially facilitating pathogen presence.

The aim of this research was to use metataxonomics involving 16S rRNA gene sequencing as a means to compare the microbiome of the dental plaque, lower airway (NBL) and ETT lumen from mechanically ventilated patients. Metataxonomics is a widely used technique that exploits both conserved and variable regions within the bacterial 16S rRNA gene to enable comprehensive analysis of microbial communities [35, 36]. In this manner, microbial associations between these sites may be drawn, thereby highlighting likely movement of microorganisms within mechanically ventilated patients, which could have importance in VAP occurrence, its management and prevention. Whilst traditional culture methods remain the gold standard in clinical diagnostics, there are limitations to such approaches, primarily relating to the inability of culture media to allow growth of all microorganisms and logistical restraints of using selective media for each species. Within a given microbial community an oversight of completely novel pathogens or those that are currently unculturable could therefore occur using culture-based methods [37].

The microbiome at separate sites was analysed for 12 mechanically ventilated patients. Dental plaque was obtained from all patients along with an NBL and or an ETT, when possible. It was not possible to obtain all samples simultaneously for a single patient, but given that biofilms develop over time, we did not consider this to be detrimental in our comparison of

dental plaque and ETT biofilm communities. It could be argued NBL samples might be more variable with temporal change, which that could be regarded as a potential limitation to the study. Whilst metataxonomics have previously been used to characterise the microbiology in ETTs [38], dental plaque [13] or lavage samples [39], this is the first study that has simultaneously compared all three sites within a given patient.

An important finding was that dental plaque contained bacteria not normally associated with healthy mouths, but recognised as pathogens in respiratory infection. Amongst the top 20 species in dental plaque were *S. aureus*, *P. aeruginosa*, *S. pneumoniae* and *H. influenzae*, which are all respiratory pathogens and at least one of these species was found in the dental plaque of all patients. The typically low prevalence of these species in the dental plaque of healthy individuals has previously been shown. Molecular studies revealed a *S. aureus* prevalence in plaque of 3% [40] and an absence of *S. pneumoniae* in the mouths of 265 individuals [41]. Significantly, in a recent study employing real-time PCR analysis of samples from 120 patients with suspected VAP, these respiratory pathogens were also found to be the most prevalent in ETT aspirates and bronchoalveolar lavages [42]. Culture-based analyses have shown a change in dental plaque during mechanical ventilation leading to respiratory pathogen (including *P. aeruginosa* and *S. aureus*) colonisation [13, 43, 44]. These findings once again implicate dental plaque as a reservoir of VAP pathogens in mechanically ventilated patients. The respiratory pathogens were also amongst the top 20 species present in NBLs and ETT biofilms, and of note was the observation that they were accompanied by bacteria normally associated with the oral environment *e.g.* *Streptococcus anginosus*, *Streptococcus oralis* and *Fusobacterium nucleatum*. *Fusobacterium nucleatum* is a common oral bacterium associated with co-aggregation of bacteria and biofilm stability [45], and its presence in ETTs could therefore conceivably enhance colonisation by respiratory pathogens.

Interestingly, *E. hirae* was in the top 5 species found overall, however it was only found in two patients, one of whom had very high OTU reads in their dental plaque (71730) and NBL (9808). This microorganism is mainly associated with disease in mammals and birds and is considered a rare pathogen in humans [46]. However, there have been recent reports of urinary tract infections and severe bacteraemia caused by this species [47, 48]. These reports combined with an understanding that other members of the *Enterococcus* family (*E. faecalis* and *E. faecium*) behave as opportunistic pathogens [49], makes *E. hirae* colonisation clinically relevant.

Overall, the results revealed a high level of similarity between the bacterial microbiomes of the three sample types, strongly implying common origins for the microorganisms present. In addition, it was evident that when all community profiles were compared, for 4 patients (P03, P24, P28, P27), the microbiome of either the ETT or the NBL had highest similarity to the dental plaque from the same patient. This would suggest that any inter-patient variation between dental plaque communities was on occasion greater than the variation between different sample types from the same patient. These findings again suggest microbial links between the sample types analysed. NMDS showed that the microbiome of dental plaque and the ETT were most similar, with higher variability evident for NBLs. This could reflect the relative ability of different species to persist at these particular sites.

Given the clear associations between dental plaque communities and bacterial biofilms in the ETT and lower airways (NBLs), this study highlights the need to improve oral hygiene in mechanically ventilated patients to limit the bioburden that can subsequently aspirate to the lungs and ETT [50]. The reported decline in oral hygiene in mechanically ventilated patients would likely lead to an exacerbation of this microbial translocation and increase the risk of VAP. Appropriate oral care interventions can improve oral hygiene in these patients and this study highlights the importance of such practices.

Conclusion

The microbiomes of dental plaque, NBL and ETT were similar in terms of diversity, it was noted that the similarities between samples of the same patient were higher than for sample type. Oral species were most frequently found in all sample types and a high abundance of respiratory pathogens was evident in dental plaque, ETT and NBL. This work highlights the importance of the oral microbiome in the intensive care setting where patients are at high risk of developing life-threatening infections such as VAP.

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Conflict of Interest and funding

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All other authors declare no conflicts of interest.

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Table 1. Demographics of the 12 patients participating in this study

Patient	Sex	Age (yrs)	Diagnosis	Days between hospital and critical care admission	Days intubated at time of recruitment	Antibiotic therapy*
P01	M	53	Sepsis	2	2	Y
P03	M	70	Pneumonia/Pneumocystis Pneumonia (PCP)	4	2	Y
P05	M	43	Substance overdose/aspiration pneumonia	0	2	N
P08	F	26	Ventricular fibrillation arrest postpartum	0	9	Y
P10	F	68	Head injury	0	0	N
P14	M	29	Head injury	0	0	N
P20	F	23	Alcoholic liver disease and pneumonia	10	0	Y
P21	M	32	Cardiac arrest	0	4	N
P24	F	44	Sepsis	3	1	Y
P25	F	49	Cardiogenic shock	0	3	Y
P27	M	39	Type 1 respiratory failure	1	5	Y
P28	M	52	Septic shock	13	0	Y

*, at time of recruitment

Table 2. Sampling details and oral health indicators from recruited patients

Patient	No. of samples (days of mechanical ventilation when collected)			Oral health status		
	Plaque	NBL	ETT	DMFT	Plaque Index	Gingival index
P01	1 (6)	0	1 (10)	8	1.8	1.7
P03	1 (7)	2 (3, 6)	0	13	2.2	1.6
P05	1 (4)	1 (3)	1 (5)	7	1.5	1.4
P08	1 (12)	0	2 (13)	8	2.1	2.2
P10	1 (4)	1 (3)	1 (11)	17	1.6	1.7
P14	1 (3)	1 (1)	2 (2, 4)	6	2.7	2.8
P20	1 (3)	2 (1)	1 (8)	4	1.9	1.8
P21	1 (6)	1 (6)	1 (12)	10	1.4	2.0
P24	1 (4)	0	1 (6)	4	3.0	3.0
P25	1 (6)	0	1 (12)	9	2.3	1.8
P27	1 (8)	2 (7)	0	9	2.0	1.6
P28	1 (2)	0	1 (3)	9	2.3	2.0
Mean				8.6	2.1	2.0
SD				3.7	0.47	0.49

NBL, non-directed bronchial lavage; ETT endotracheal tube; DMFT, number of Decayed/Missing/Filled Teeth; Plaque and gingival indices were as defined by Silness and Löe [23] where scores range between 0 to 3, with 0 being equal to health and a score of 3 indicating gross plaque deposits or marked gingival inflammation.

Fig. 1a. Chao analysis of similarities in the diversity of the microbiomes of dental plaque, non-directed bronchial lavages (NBLs) and endotracheal tubes (ETTs).

Fig. 1b. Shannon analysis of similarities in the diversity of the microbiomes of dental plaque, non-directed bronchial lavages (NBLs) and endotracheal tubes (ETTs).

Fig. 2. NMDS analysis illustrating position of microbial communities of dental plaque, non-directed bronchial lavages (NBLs) and endotracheal tubes (ETTs) in a multidimensional space.

Fig. 3. Dendrogram demonstrating four major sample clusters each containing communities from dental plaque, non-directed bronchial lavages and endotracheal tubes.

Patient number corresponds to those in Table 1; suffix indicates samples type (P=plaque; T=endotracheal tube; N=non-directed bronchial lavage); when two samples of a given type were obtained this is shown by lettering 'a' and 'b'.

Fig. 4. Most abundant species detected in dental plaque.

Fig. 5. Most abundant species detected in endotracheal tube (ETT) biofilms.

Fig. 6. Most abundant species detected non-directed bronchial lavages (NBLs).

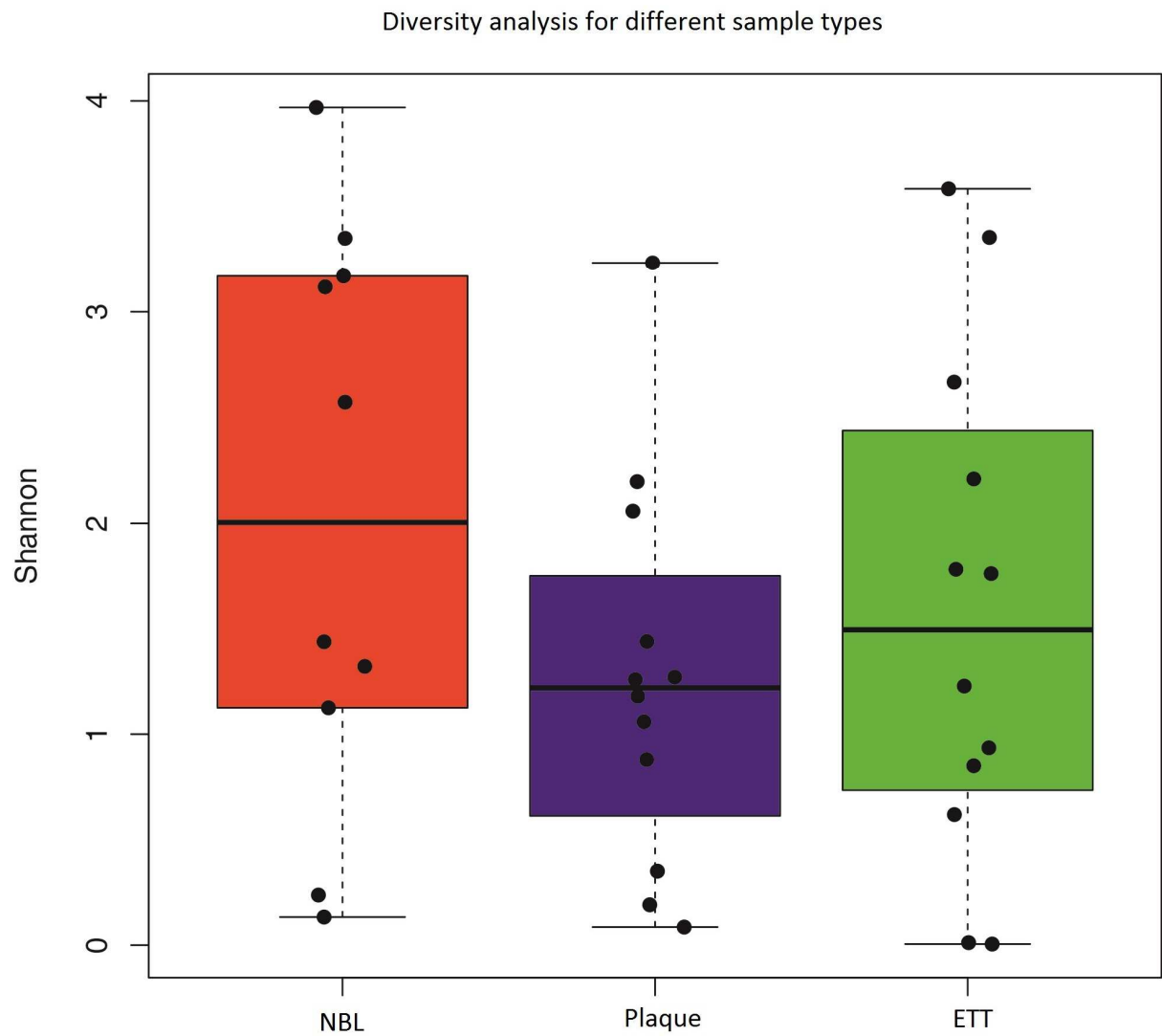


Fig. 1a. Chao analysis of similarities in the diversity of the microbiomes of dental plaque, non-directed bronchial lavages (NBLs) and endotracheal tubes (ETTs).

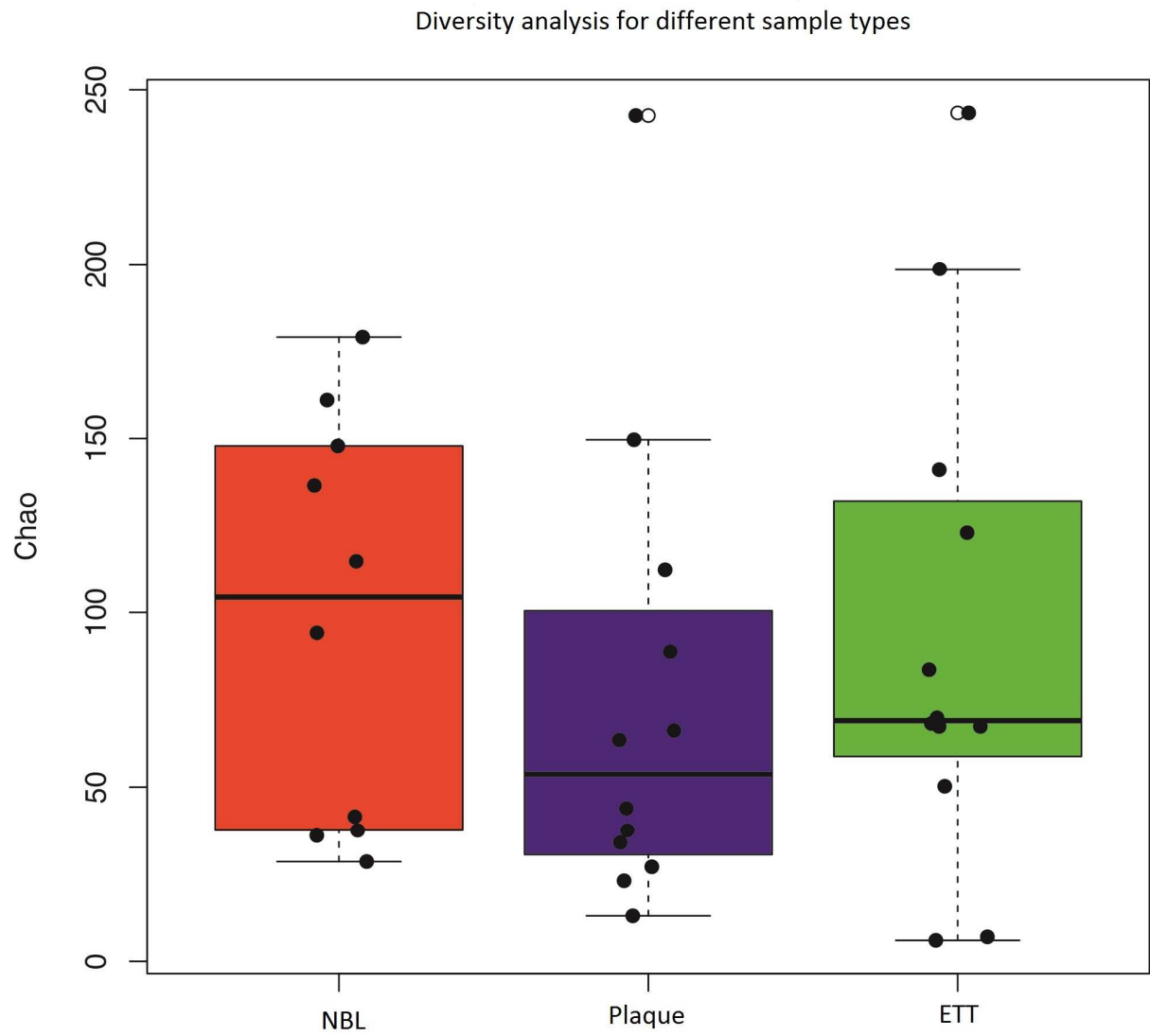


Fig. 1b. Shannon analysis of similarities in the diversity of the microbiomes of dental plaque, non-directed bronchial lavages (NBLs) and endotracheal tubes (ETTs).

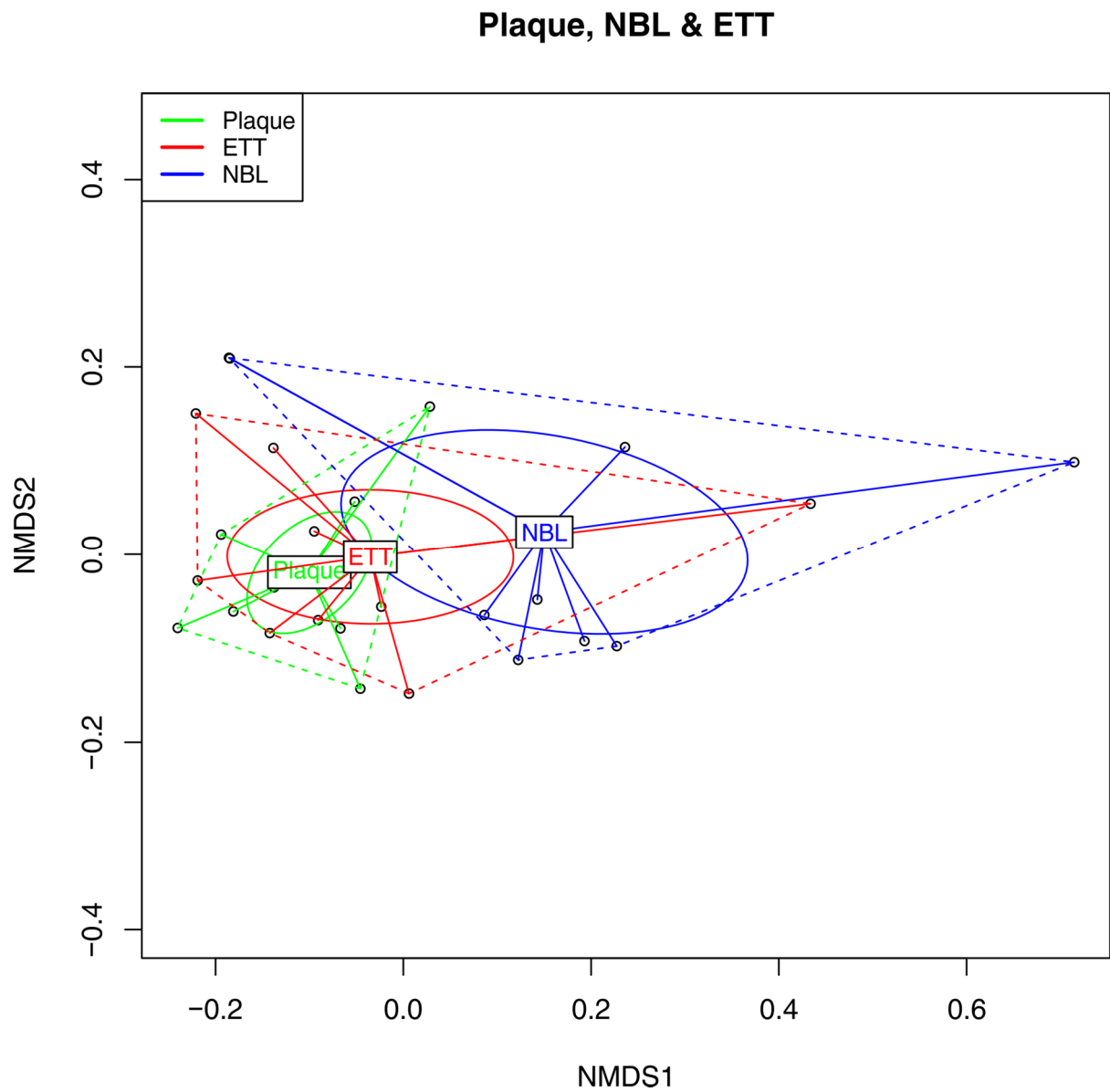


Fig. 2. NMDS analysis illustrating position of microbial communities of dental plaque, non-directed bronchial lavages (NBLs) and endotracheal tubes (ETTs) in a multidimensional space.

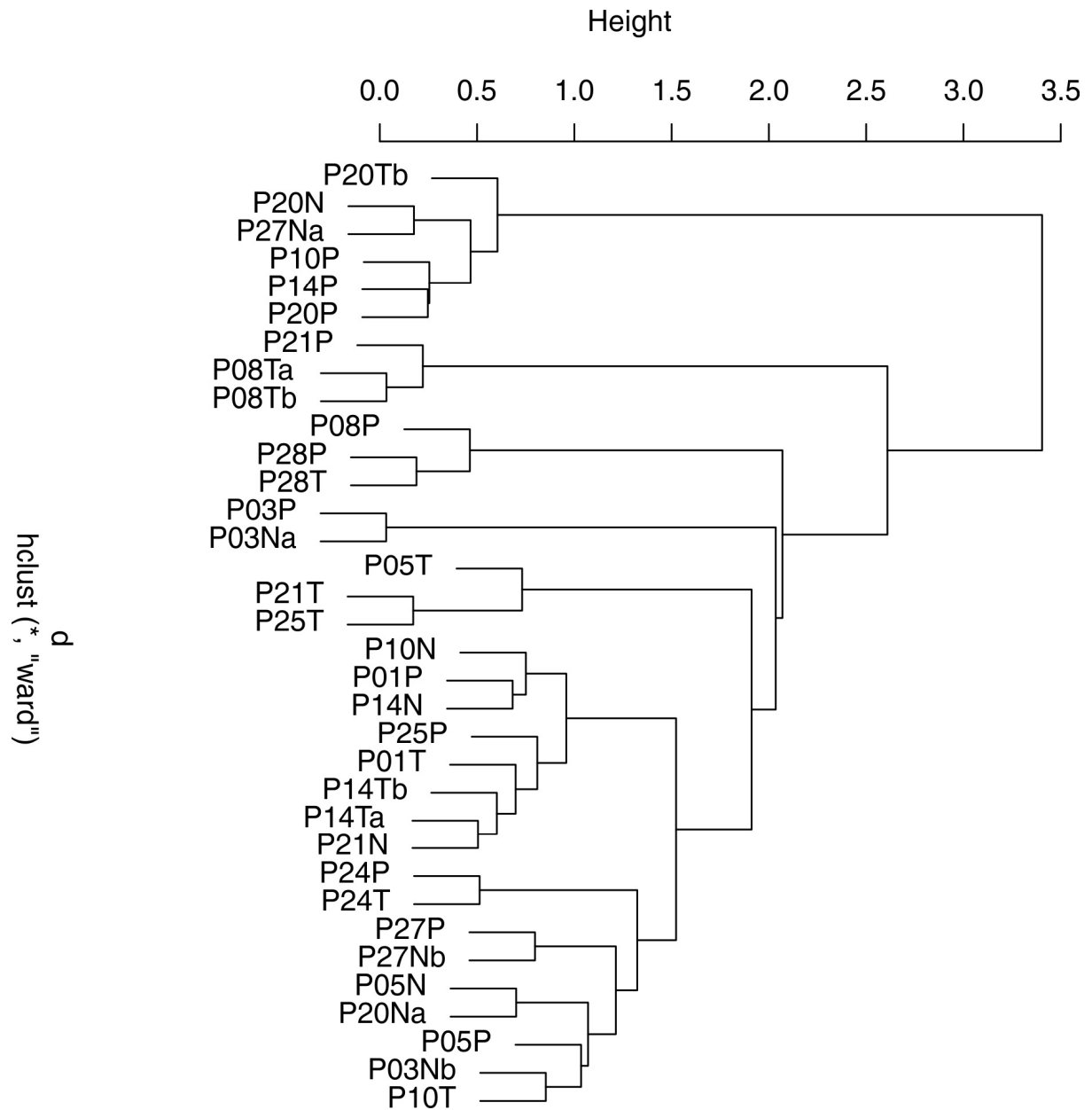


Fig. 3. Dendrogram demonstrating four major sample clusters each containing communities from dental plaque, non-directed bronchial lavages and endotracheal tubes.

Patient number corresponds to those in Table 1; suffix indicates samples type (P=plaque; T=endotracheal tube; N=non-directed bronchial lavage); when two samples of a given type were obtained this is shown by lettering 'a' and 'b'.

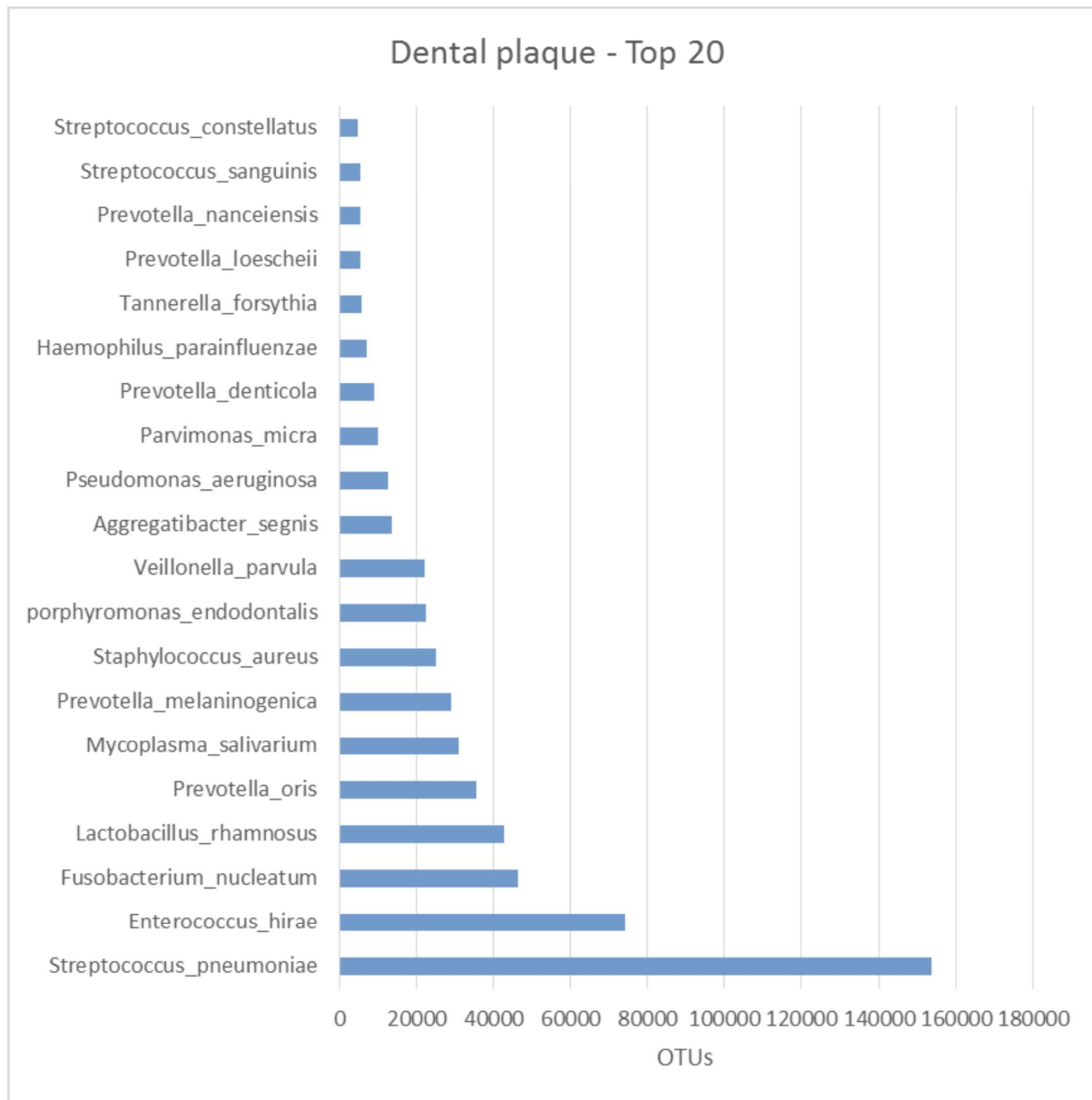


Fig. 4. Most abundant species detected in dental plaque.

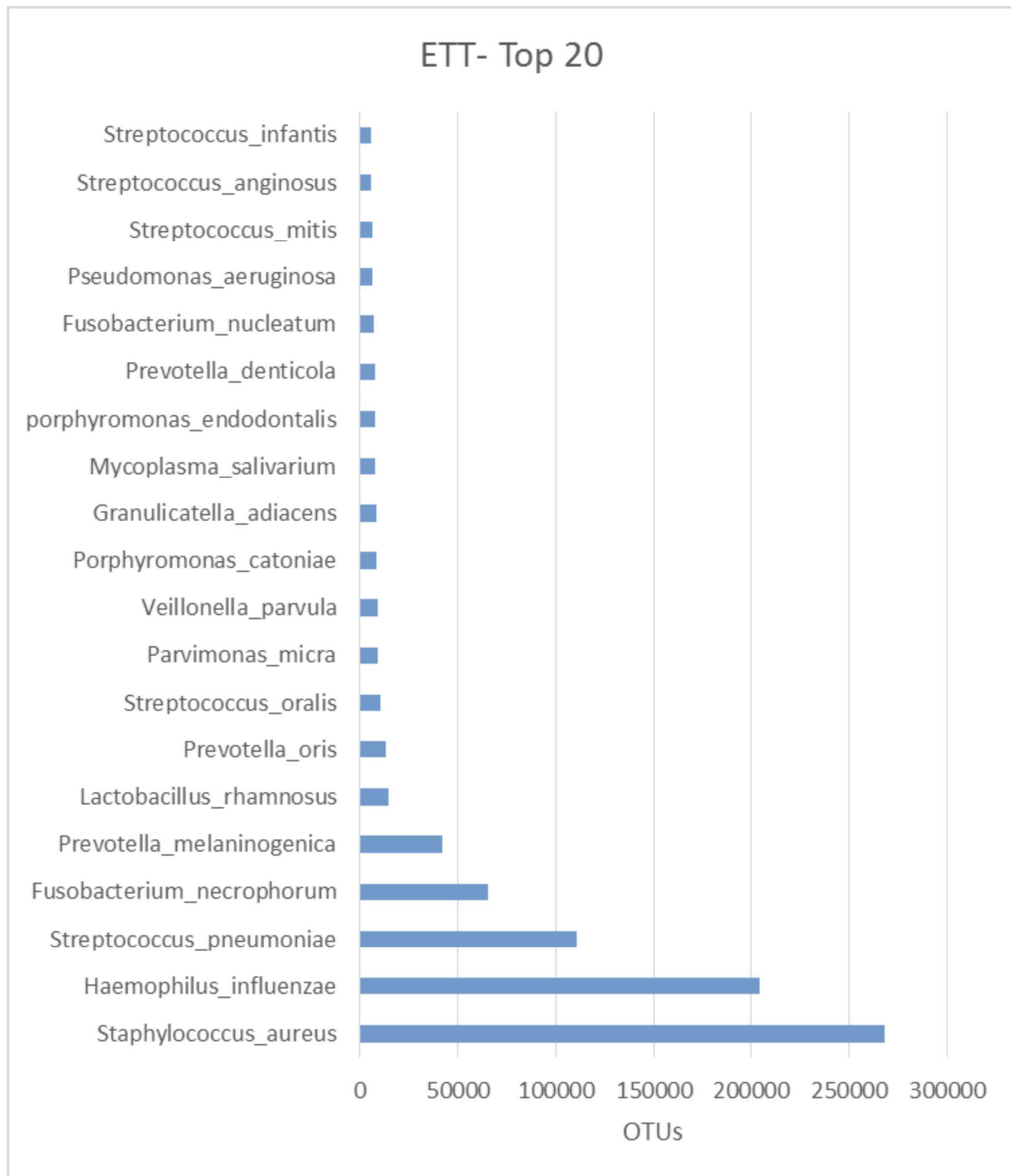


Fig. 5. Most abundant species detected in endotracheal tube (ETT) biofilms.

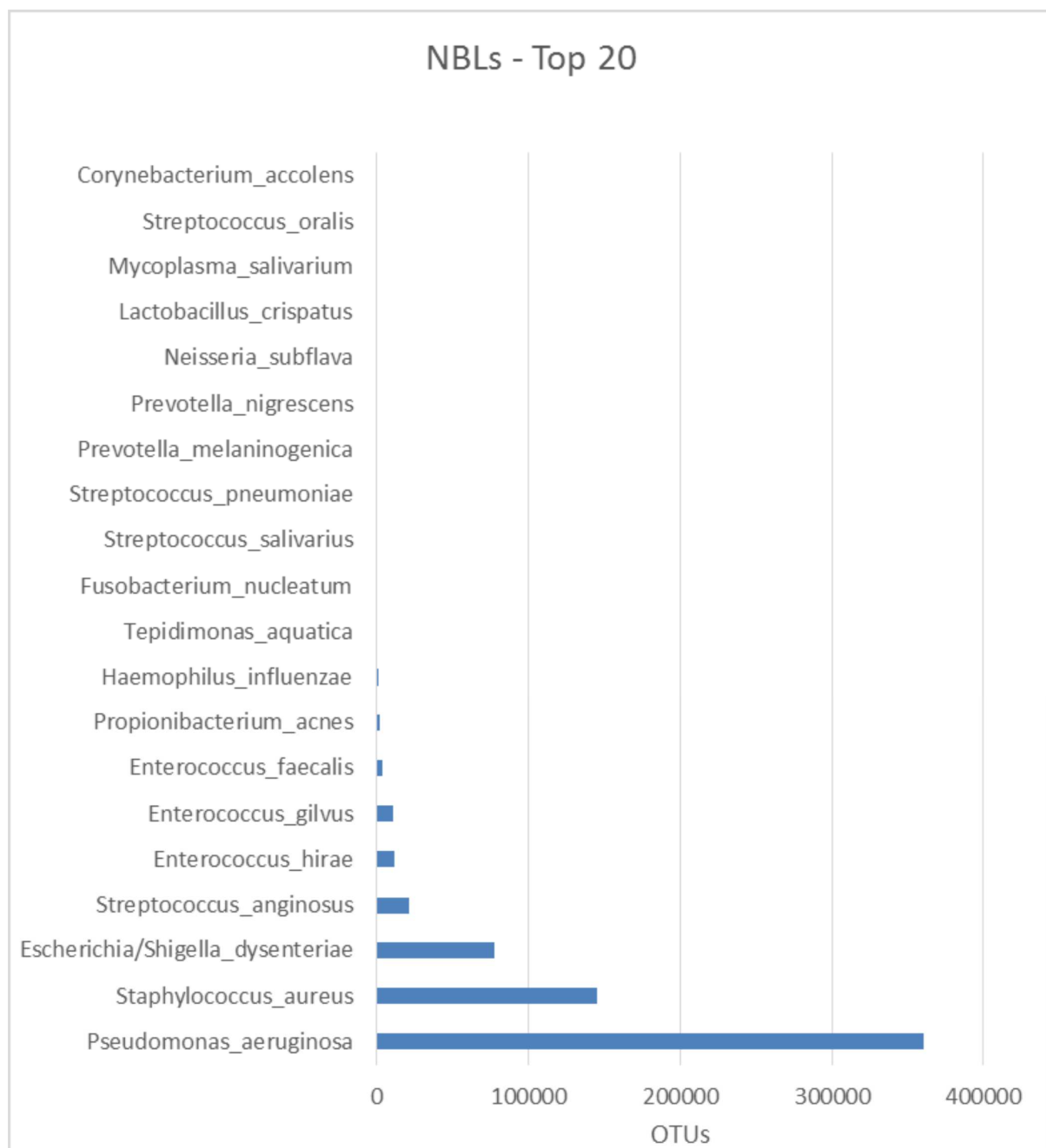


Fig. 6. Most abundant species detected non-directed bronchial lavages (NBLs).