Brazilian Journal of
OTORHINOLARYNGOLOGY

ORIGINAL ARTICLE

High microbiome variability in pediatric tracheostomy cannulas in patients with similar clinical characteristics

Leonardo Palma Kuhl [a,b,*], **Paulo José Cauduro Marostica** [b,c], **Alexandre José Macedo** [d], **Gabriel Kuhl** [a], **Marina Siebert** [e,f,g], **Denise Manica** [a,b], **Leo Sekine** [h], **Cláudia Schweiger** [a,b]

[a] *Hospital de Clínicas de Porto Alegre (HCPA), Serviço de Otorrinolaringologia e Cirurgia Cérvico-Facial, Porto Alegre, RS, Brazil*
[b] *Universidade Federal do Rio Grande do Sul (UFRGS), Programa de Pós-Graduação em Saúde da Criança e do Adolescente, Porto Alegre, RS, Brazil*
[c] *Hospital de Clínicas de Porto Alegre, Serviço de Pneumologia Pediátrica, Porto Alegre, RS, Brazil*
[d] *Universidade Federal do Rio Grande do Sul (UFRGS), Faculdade de Farmácia e Centro de Biotecnologia, Porto Alegre, RS, Brazil*
[e] *Universidade Federal do Rio Grande do Sul (UFRGS), Programa de Pós-Graduação em Gastroenterologia e Hepatologia, Porto Alegre, RS, Brazil*
[f] *Hospital de Clínicas de Porto Alegre (HCPA), Laboratório de Pesquisa Básica e Avançada em Neurociência (BRAIN), Porto Alegre, RS, Brazil*
[g] *Hospital de Clínicas de Porto Alegre (HCPA), Centro de Pesquisa Experimental, Porto Alegre, RS, Brazil*
[h] *Universidade Federal do Rio Grande do Sul (UFRGS), Faculdade de Medicina, Programa de Pós-Graduação em Ciências Médicas, Porto Alegre, RS, Brazil*

HIGHLIGHTS

- Metagenomics can be used for microbiota identification on pediatric tracheostomy cannula.
- Microbiome of pediatric tracheostomy cannulas are highly variable.
- Microbiome did not correlate to any particular clinical characteristic.

Abstract
Objectives: To evaluate the bacterial microbiome found in tracheostomy cannulas of a group of children diagnosed with glossoptosis secondary to Robin Sequence (RS), and its clinical implications.
Methods: Pediatric patients were enrolled in the study at the time of the cannula change in the hospital. During this procedure, the removed cannula was collected and stored for amplicon sequencing of 16s rRNA. DNA extraction was performed using DNeasy PowerBiofilm Kit (QIAGEN® – Cat n° 24000-50) while sequencing was performed with the S5 (Ion S5™ System, Thermo Fisher Scientific), following Brazilian Microbiome Project (BMP) protocol.

* Corresponding author.
  E-mail: leonardokuhl@hotmail.com (L.P. Kuhl).
  Peer Review under the responsibility of Associação Brasileira de Otorrinolaringologia e Cirurgia Cérvico-Facial.

Results: All 12 patients included in the study were using tracheostomy uncuffed cannulas of the same brand, had tracheostomy performed for over 1-year and had used the removed cannula for approximately 3-months. Most abundant genera found were *Aggregatibacter*, *Pseudomonas*, *Haemophilus*, *Neisseria*, *Staphylococcus*, *Fusobacterium*, *Moraxella*, *Streptococcus*, *Alloiococcus*, and *Capnocytophaga*. Individual microbiome of each individual was highly variable, not correlating to any particular clinical characteristic.
Conclusion: The microbiome of tracheostomy cannulas is highly variable, even among patients with similar clinical characteristics, making it challenging to determine a standard for normality.

Introduction

Tracheostomy is a surgical procedure that creates a shortcut for the air to reach the lungs.

It establishes a communication (or tunnel) between the cervical skin and the trachea, which is maintained pervious by a cannula.

Indications for tracheostomy in children nowadays are mainly related to chronic obstruction of upper airways (e.g., laryngeal stenosis or vocal fold paralysis), management of mechanical ventilation, and treatment of bronchopulmonary aspiration.[1–3] Another obstructive airway pathology with potential necessity for tracheostomy is glossoptosis. Glossoptosis is defined as the posterior collapse of the base of the tongue resulting in varying degrees of respiratory obstruction. It may occur as a consequence of mandibular hypoplasia (Robin Sequence), but it also occurs in patients with neurological disorders related to hypotonia.[4–6]

A few days after a tracheostomy is performed, its pathway maturates, resulting in a new microbiological environment. Air goes directly to the lungs without be filtered by the upper airways, and mobilization of tracheal secretions is altered, as well as patients physiological defense systems.[7–10] The cannula itself induces a constant inflammatory (foreign body) reaction, capable of accumulating organic debris and facilitating bacterial colonization and biofilm formation, sometimes resulting in exacerbation of clinical conditions.[11,12]

Precise determination of bacteria present at the tracheostomy tube can be challenging. Culture based methods may favor specific bacteria growth over others and does not contemplate the multitude of microbials present in that environment.[7,13–15]

Recently, high performance next generation sequencing equipment is becoming more accessible and, added to computational evolution and expansion of bioinformatic sector, genetic sequencing costs have been lower.[16] Metagenomic is a culture independent method for phylogenetic identification of bacterial communities. Referred as shotgun sequencing, whole microbial genome sequencing is performed, resulting in taxonomic and possibly function gene identification.[17–20] Another culture independent genetic sequencing method for microbiome determination is amplicon sequencing. Targeting specific known genes, shorter sequences can be used for taxonomic identification, therefore lowering costs while keeping efficacy at the study of bacterial communities.[21–24]

This study aims to evaluate pediatric tracheotomy bacterial microbiome using amplicon sequencing of 16S rRNA gene in a specific population of children with Robin Sequence and to describe the findings amidst recent literature.

Methods

Subjects and study design

This study received a certificate of ethic appreciation from ''Plataforma Brasil'' numbered 93356618.0.000.5327. The study was also approved by the Ethics and Research Committee (CEP) of Hospital de Clínicas de Porto Alegre. All patients had consent and approval forms signed by parents or legal caretakers prior to inclusion. We targeted a group of patients who performed tracheostomy due to glossoptosis associated with mandibular hypoplasia (Robin Sequence), with a history or plan for osteogenic distraction.

Data and samples collection

Prior to the tracheostomy cannula exchange, which is performed at our hospital by the Otolaryngology team, caretakers were questioned on patient current clinical condition (fever, use of antibiotics, etc.) and electronic medical chart was reviewed.

At the moment of cannula exchange, patients had their cannula collected with the use of sterile techniques. The cannula was bisected longitudinally as to include both distal (in contact to trachea) and proximal (in contact to skin) portions and placed into 2 different 15 mL conical tubes (Fig. 1). These tubes were stored in a $-80\,°C$ freezer within the first two hours of collection for future DNA extraction and sequencing.[25–27]

At the moment of the cannula exchange, cervical skin was also examined for the presence or absence of peristomal granuloma.

DNA extraction and amplicon sequencing

All samples were processed following the protocol of the Experimental Research Center of Hospital de Clínicas de Porto Alegre. DNA extractions were conducted using DNeasy

Figure 1 Incisions performed on tracheostomy cannula.

PowerBiofilm Kit (QIAGEN® – Cat nº 24000-50). For every patient the content of one 15 mL conical tube was used (half of tracheostomy tube) (Fig. 1). Once removed of the −80 °C freezer, samples were kept at room temperature for 10 min to facilitate handling. Using sterile material, tracheostomy tube was sectioned into small fragments on a Petri dish and DNA extraction was performed following DNeasy Power-Biofilm Kit protocol.

Once extracted, samples quality was tested using DNA spectrophotometer NanoDrop 1000 (Thermo Fischer Scientific®).

Approximately 50 ng of DNA for each sample were submitted to amplification of the V4 region of rRNA 16S gene by PCR using the following pair of primers: 515F (5′-GTGCCAGCMGCCGCGGTAA-3′) and 806R (5′-GGACTACHVGGGTWTCTAAT-3′). In order to process multiple samples at the same reaction, primer-fusion method was used so each sample received a unique nucleotide sequence (barcode) attached to its amplified products. Sequencing was conducted using S5 (Ion S5™ System, Thermo Fisher Scientific). The resulting raw data were compacted and exported as a single FastQ file.

Bioinformatics

Raw data generated from DNA sample sequencing was processed according to the Brazilian Microbiome Project (BMP) protocol,[23] using scripts in QIIME and RDP for 16S rRNA gene phylogenetic representation.

Sequences were filtered based on size and quality score with removal of chimeras. RDP_GOLD identifier was used for Operational Taxonomic Unit (OTU) picking based on 97% similarity, and phylogenetic identity of represented OTU was achieved using Green Genes database. Sequences which failed to achieve 97% similarity were defined as unclassified. Quality control was performed with FASTQC.

Data processing and STF extension files were also created using QIIME (v1.9.1). Analysis was performed using STAMP (v2.1.3).

Statistical analysis

Principal Components Analysis (PCA) plots were used to identify clusters while separating samples for different variables such as feeding route, use of antibiotics in the last 3-months and presence of granuloma.

Results

Twelve patients with Robin Sequence and tracheostomy were included in the study. Patient characteristics are summarized in Table 1. All tracheostomy tubes collected were of the same brand (PORTEX® Blue Line® Tracheostomy Tube) and uncuffed. No complications occurred.

Only one patient was using antibiotics during sampling, which had been started two days before because of increasing of tracheostomy secretion, without fever or any other symptoms. Five other patients used antibiotics previously while in use of the collected tracheostomy tube, all of them finished treatment for at least one month before cannula removal.

Metagenomics

DNA concentration after extraction varied between 69.65 ng/mL to 312.45 ng/mL (median = 176.84 ng/mL). After filtering and quality control, amplicon sequencing resulted in a total of 647,256 reads, ranging from 16,850 to 137,586 per sample (median = 53,938). The microbiome of each tracheostomy tube can be viewed on Fig. 2.

A total of 68 OTUs was identified at genus taxonomic level. Considering all sequences identified, most abundant OTUs included: *Aggregatibacter* (17%), *Pseudomonas* (15.9%), *Haemophilus* (12.4%), *Neisseria* (8.8%), *Staphylococcus* (8.3%), *Fusobacterium* (6.5%), *Moraxella* (5.8%), *Streptococcus* (4.6%), *Alloiococcus* (3%) and *Capnocytophaga* (2.9%). Other OTUs with lower abundance included: *Corynebacterium* (2.5%), *Prevotella* (2.5%), *Porphyromonas* (1.8%), *Eikenella* (1.2%), *Treponema* (0.9%), *Elizabethkingia* (0.9%), *Stenotrophomonas* (0.8%), *Mycoplasma* (0.8%), *Achromobacter* (0.5%) and *Acinetobacter* (0.5%) (Table 2). The 48 remaining OTUs accounted for less than 3% of all identified reads. Relative proportion of OTUs found per sample can be observed in Table 3.

Bacteria and clinical data of the patients

Principal Component Analysis (PCA) plots were performed searching for clusters accounting for variables such as food intake, use of antibiotics or presence of granuloma; however, no cluster patterns were found (see Supplemental Material).

Discussion

After analysis of tracheostomy cannula microbiome of 12 children with glossoptosis secondary to mandibular hypoplasia and other common features (i.e., use of uncuffed tube, use of the same tracheostomy tube brand, similar length of time in use of the analyzed cannula, and tracheostomy surgical procedure performed for over a year) we can state that there is a very high microbiome variability among patients, making the establishment of procedure routines very challenging.

257

Table 1 Clinical characteristics of the 12 patients.

Sample	Age (years)	Time from tracheostomy (years)	Feeding route	Syndrome	Comorbidities	Recurrent LRI	Time from last cannula change (days)	Cannula size	Peristomal granuloma	ATB (day of sampling)	ATB (last 3-months)
001	8.2	8.1	Oral	Facio-auricular-vertebral	Yes	No	98	5	No	No	No
002	5.4	5.2	G-tube	None	Yes	No	98	4.5	Yes	No	Yes
003	7.7	7.5	Oral/G-tube	Richieri-Costa-Pereira	Yes	No	98	4.5	No	No	No
004	2.1	1.9	NG-tube	None	Yes	No	98	3.5	No	No	Yes
005	9.2	9	Oral	Stickler	No	No	91	4.5	No	No	No
006	2.2	2.1	Oral	None	Yes	No	91	3.5	Yes	No	No
007	4	1.4	NG-tube	None	Yes	Yes	138	4	No	No	Yes
008	7.2	3.9	Oral	None	Yes	No	80	5	No	No	No
009	11.7	8.3	Oral	Picnodisostosis	No	No	98	4	No	No	Yes
010	5.4	5.2	G-tube	Apert	Yes	No	119	4.5	No	No	Yes
011	9.1	9	Oral	Treacher-Collins	Yes	No	98	4	No	No	No
012	2.2	2.1	NG-tube	None	Yes	No	91	3.5	Yes	Yes	No

LRI, Lower Respiratory Infection; ATB, Antibiotics; G-tube, Gastrostomy; NG-tube, Nasogastric Tube.

Table 3 Genera proportion per sample.

001		002		003		004	
Genus	%	Genus	%	Genus	%	Genus	%
Aggregatibacter	29.48	*Aggregatibacter*	50.67	*Moraxella*	34.42	*Haemophilus*	81.27
Pseudomonas	26.33	*Fusobacterium*	18.69	*Treponema*	8.30	*Streptococcus*	6.98
Fusobacterium	9.21	*Capnocytophaga*	11.62	*Fusobacterium*	7.49	*Moraxella*	2.95
Streptococcus	6.07	*Eikenella*	7.33	*Alloiococcus*	7.15	*Corynebacterium*	2.66
Capnocytophaga	4.36	*Pseudomonas*	6.69	*Neisseria*	6.75	*Aggregatibacter*	1.58
Mycoplasma	4.32	*Neisseria*	2.44	*Aggregatibacter*	6.42	*Fusobacterium*	1.54
Haemophilus	4.05	*Prevotella*	0.72	*Porphyromonas*	5.55	*Pseudomonas*	1.28
Neisseria	2.87	*Porphyromonas*	0.59	*Streptococcus*	4.11	*Achromobacter*	0.57
Prevotella	2.81	*Haemophilus*	0.40	*Capnocytophaga*	4.08	*Prevotella*	0.43
Treponema	2.43	*Leptotrichia*	0.31	*Mycoplasma*	2.19	*Neisseria*	0.27
Others	8.07	Others	0.54	Others	13.54	Others	0.47

005		006		007		008	
Genus	%	Genus	%	Genus	%	Genus	%
Staphylococcus	95.23	*Aggregatibacter*	63.12	*Neisseria*	45.55	*Neisseria*	25.77
Corynebacterium	3.09	*Streptococcus*	16.15	*Haemophilus*	24.89	*Fusobacterium*	21.78
Streptococcus	1.54	*Haemophilus*	8.68	*Porphyromonas*	9.82	*Aggregatibacter*	18.99
Anaerococcus	0.05	*Peptococcus*	1.87	*Acinetobacter*	5.89	*Capnocytophaga*	7.95
Finegoldia	0.03	*Selenomonas*	1.78	*Streptococcus*	3.61	*Haemophilus*	7.89
Alloiococcus	0.01	*Prevotella*	1.66	*Prevotella*	1.90	*Alloiococcus*	3.77

L.P. Kuhl, P.J. Marostica, A.J. Macedo et al.

Table 3 (*Continued*)

005		006		007		008	
Genus	%	Genus	%	Genus	%	Genus	%
Facklamia	0.01	*Fusobacterium*	1.55	*Moraxella*	1.78	*Serratia*	2.76
Actiomyces	0.01	*Capnocytophaga*	1.14	*Fusobacterium*	1.42	*Eikenella*	2.34
Abiotrophia	0.01	*Neisseria*	1.10	*Eikenella*	0.59	*Staphylococcus*	1.72
Peptoniphilus	0.01	*Veillonella*	1.08	*Aggregatibacter*	0.55	*Porphyromonas*	1.28
Others	0.01	Others	1.87	Others	4	Others	5.75

| 009		010		011		012	
Genus	%	Genus	%	Genus	%	Genus	%
Pseudomonas	72.83	*Pseudomonas*	36.42	*Pseudomonas*	31.18	*Neisseria*	18.44
Haemophilus	14.25	*Aggregatibacter*	22.11	*Moraxella*	30.10	*Pseudomonas*	15.33
Corynebacterium	7.25	*Alloiococcus*	13.71	*Elizabethkingia*	11.08	*Prevotella*	12.01
Streptococcus	2.47	*Corynebacterium*	8.20	*Alloiococcus*	10.14	*Fusobacterium*	11.36
Staphylococcus	1.64	*Achromobacter*	5.05	*Stenotrophomonas*	8.64	*Aggregatibacter*	11.29
Fusobacterium	1.19	*Capnocytophaga*	4.23	*Corynebacterium*	4.51	*Streptococcus*	11.04
Anaerococcus	0.10	*Fusobacterium*	3.60	*Streptococcus*	1.38	*Haemophilus*	6.55
Porphyromonas	0.09	*Neisseria*	2.92	*Achromobacter*	1.06	*Mycoplasma*	2.99
Finegoldia	0.05	*Porphyromonas*	1.81	*Haemophilus*	0.92	*Porphyromonas*	2.07
Peptoniphilus	0.05	*Streptococcus*	0.85	*Lampropedia*	0.36	*Eikenella*	1.89
Others	0.02	Others	1.1	Others	0.63	Others	7.03
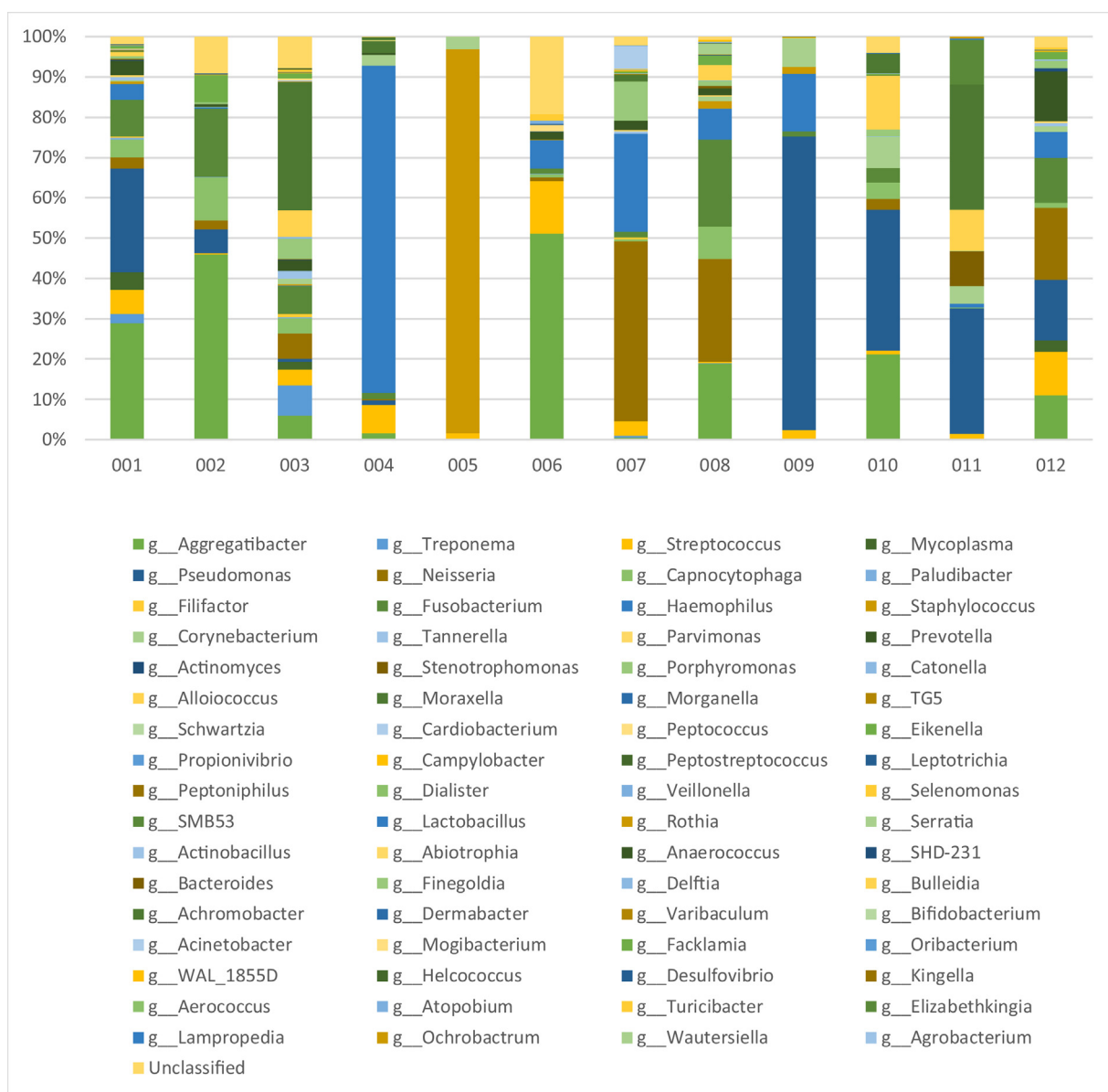
Figure 2 Microbiome per sample.

Aggregatibacter was the OTU with the highest number of reads in metagenomics (17% of all samples reads), present in 10 of the 12 analyzed tracheostomies tubes, representing over 10% of microbiome genera proportion in 6 samples. Belonging to the *Pasteurellaceae* family (also common to the *Haemophilus* genus) its most noticeable representative is *Aggregatibacter actiomycetemcomitans*, a gram-negative facultative anaerobe bacterium frequently identified in oral cavity and associated with periodontal disease. *Aggregatibacter* has great biofilm formation capabilities, which can have mutualistic characteristics with *Fusobacterium nucleatum* and *Veillonella spp.*[28-31] Unlike our results *Aggregatibacter* was not found among most observed OTUs on other tracheostomy microbiome studies.[32-34] Dickson et al. (2015) and Charlson et al. (2011) described that, although different environments in the same patient such as oral cavity, lower and upper airway show different bacterial communities, they are not isolated from each other. Closer environments sustain microbiomes with higher resemblance than distance environments, suggesting that these regions are not completely separate, but linked by transition areas.[35,36] It is possible the abundance of *Aggregatibacter* and other oral cavity typical OTUs (as *Neisseria, Fusobacterium, Streptococcus, Prevotella* and *Porphyromonas*) can be correlated with patients' disease-associated high probability of dysphagia and aspiration.[5] In fact, among the 12 patients, 6 were in use of G-tube or nasogastric tube, and the others with oral feeding probably showed a high risk of silent aspiration, according to the studies of Gasparin et al. (2017) and Monasterio et al. (2004).[37,38]

Bacteria such as *P. aeruginosa, Staphylococcus aureus, F. nucleatum* and *A. actinomycetemcomitans* are known for development of biofilm and can create polymicrobial complexes among themselves or associated with other bac-

Table 2 Prevalence of total reads per genus on metagenomics.

Genus	%
Aggregatibacter	17%
Pseudomonas	15.9%
Heamophilus	12.4%
Neisseria	8.8%
Staphyloccus	8.3%
Fusobacterium	6.5%
Moraxella	5.8%
Streptococcus	4.6%
Alloiococcus	3.0%
Capnocytophaga	2.9%
Corynebacterium	2.5%
Prevotella	2.5%
Porphyromonas	1.8%
Eikenella	1.2%
Treponema	0.9%
Elizabethkingia	0.9%
Stenotrophomonas	0.8%
Mycoplasma	0.8%
Achromobacter	0.5%
Acinetobacter	0.5%
Others	2.4%
Total	100%

teria as *Haemophilus influenzae* and *Veillonella spp*.[39–41] We found high abundances of these genus among our patients, which is believed to be expected as tracheostomy tube serves as an anchor point for these structures.

Regarding distribution, patients seemed to present with either a balanced proportion of different bacteria or a predominance of one or two genus. In four samples two genera corresponded to more than 75% of all reads (i.e., sample 005 with *Staphylococcus* representing 95.23% of its reads). Although these patterns could be highlighted, either group had no distinguished characteristics that could explain this finding.

Only one patient (sample 012) was receiving antibiotics during sampling, indicated for suspected lower respiratory exacerbation. The patient had increased tracheostomy secretion without fever or other symptoms. Amplicon sequencing of this sample evidenced no particular pathological bacterial genus, suggesting it was a viral or non-infectious condition. Perez-Losada shown that a microbiota altered by an acute infection tends to balance itself after four weeks. All the five patients who had received previous antibiotics had their treatment concluded more than a month before sampling. It is not clear if the use of antibiotics played a role in their microbiome at the time of the study, as their microbiome showed no particular characteristics.[34]

Wang et al. (2018) studied the microbiome of 4 children with tracheostomy, of whom 2 had suprastomal granuloma tissue identified by airway endoscopy.[32] Both patients with granuloma tissue were in use of antibiotics and grew *H. influenzae* in respiratory bronchoalveolar lavage culture. Microbiome evaluation of these patients' tracheostomy tubes through shotgun metagenomics revealed that besides *H. influenzae*, *F. nucleatum*, *M. catahrralis* and *Streptococ-*

cus pneumoniae had higher abundances, potentially being associated with the presence of granuloma. Although airway endoscopy was not performed during our sampling, periostomal granulation tissue was observed on three patients: 002, 006 and 012. Amplicon sequencing observed 19 OTUs common to all three samples, highlighting only the ones with higher abundancy we observe: *Aggregatibacter* (50.7% vs. 63.1% vs. 11.3%), *Fusobacterium* (18.7% vs. 1.5% vs. 11.4%), *Neisseria* (2.4% vs. 1.1% vs. 18.4%), *Prevotella* (0.7% vs. 1.6% vs. 12.0%) and *Haemophilus* (0.4% vs. 8.9% vs. 6.5%). Granulation tissue is potentially influenced by conditions of the tracheostomy tube (oxygenation, secretion, surface), but our results show that its link to specific microbes is still not clear, as these groups of bacteria may be associated either to cause or effect. Other culture-based studies in stents have described relation of granulation tissue with *Staphylococcus aureus* and *P. aeruginosa*.[42,43] According to our results, *Staphylococcus* and *Pseudomonas* populations on the three patients with identified granulomas was not characteristic, with *Staphylococcus* varying from none to 0.16% and *Pseudomonas* from 0.07% to 15.3%. It is possible that studies tend to find these bacteria easier for their capabilities on growing in culture methods and biofilm formation.

Feigelman et al. (2017) describes higher prevalence of *Prevotella*, *Streptococcus*, *Veillonella*, *Haemophilus* and *Neisseria* in bronchoalveolar lavages of healthy individuals without tracheostomy.[44] Our samples showed a mixture of these genera intertwined with potentially pathological bacteria. As patient's microbiome had variable presentations, they did not correlate to clinical characteristics studied (feeding route, use of antibiotics or presence of granuloma). Recent literature suggests that infections are more related to a shift in community balance than the introduction of a new bacteria, and a static microbiome analysis may not be sufficient to understand correlations between network patterns and its causes or consequences.[43,44]

More studies are needed in order to understand bacterial community networks and how they interact among themselves, with the environment and what the clinical repercussions are.

Conclusion

Our study describes the use of 16s RNA metagenomics for bacterial microbiome identification present on tracheostomy cannulas of a specific group of children.

Although some bacterial genera demonstrated greater abundance (e.g., *Aggregatibacter*, *Pseudomonas*, *Haemophilus*, *Neissera*, *Staphylococcus*, *Fusobacterium* and *Moraxella*), composition of patients' individual microbiome showed high variability, even with similar clinical characteristics.

Despite the descriptive nature of this study, we hope to create foundations for more complex questions regarding microbiology interactions of these patients.

Financing

Conflicts of interest

The authors declare no conflicts of interest.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.bjorl.2022.05.001.

References

1. de Trey L, Niedermann E, Ghelfi D, Gerber A, Gysin C. Pediatric tracheotomy: a 30-year experience. J Pediatr Surg. 2013;48:1470–5.
2. Watters KF. Tracheostomy in infants and children. Respir Care. 2017;62:799–825.
3. Yaremchuk K. Regular tracheostomy tube changes to prevent formation of granulation tissue. Laryngoscope. 2003;113:1–10.
4. Sorin A, McCarthy JG, Bernstein JM. Predicting decannulation outcomes after distraction osteogenesis for syndromic micrognathia. Laryngoscope. 2004;114:1815–21.
5. Schweiger C, Manica D, Kuhl G. Glossoptosis. Semin Pediatr Surg. 2016;25:123–7.
6. Manica D, Schweiger C, Sekine L, Fagondes SC, Kuhl G, Collares MV, et al. The role of flexible fiberoptic laryngoscopy in Robin Sequence: a systematic review. J Craniomaxillofac Surg. 2017;45:210–5.
7. Cline JM, Woods CR, Ervin SE, Rubin BK, Kirse DJ. Surveillance tracheal aspirate cultures do not reliably predict bacteria cultured at the time of an acute respiratory infection in children with tracheostomy tubes. Chest. 2012;141:625–31.
8. Brook I. Role of anaerobic bacteria in infections following tracheostomy, intubation, or the use of ventilatory tubes in children. Ann Otol Rhinol Laryngol. 2004;113:830–4.
9. Pozzi M, Galbiati S, Locatelli F, Granziera M, Colombo D, Pellegrino P, et al. Prevention of respiratory infections in tracheostomized patients of a pediatric long-term rehabilitation setting. Am J Infect Control. 2015;43:394–6.
10. Rodney J, Ojano-Dirain CP, Antonelli PJ, Silva RC. Effect of repeated tracheostomy tube reprocessing on biofilm formation. Laryngoscope. 2016;126:996–9.
11. Backman S, Björling G, Johansson UB, Lysdahl M, Markström A, Schedin U, et al. Material wear of polymeric tracheostomy tubes: a six-month study. Laryngoscope. 2009;119:657–64.
12. Silva RC, Ojano-Dirain CP, Antonelli PJ. Effectiveness of pediatric tracheostomy tube cleaning. Arch Otolaryngol Head Neck Surg. 2012;138:251–6.
13. Schuurmans MM, Palheiros Marques M, Freitag L, Zinkernagel AS, Achermann Y. Biofilm formation in a permanent tracheal stent implanted for twenty-five years. Respiration. 2015;90:327–8.
14. Sanders CD, Guimbellot JS, Muhlebach MS, Lin FC, Gilligan P, Esther CR Jr. Tracheostomy in children: epidemiology and clinical outcomes. Pediatr Pulmonol. 2018;53:1269–75.
15. Morar P, Singh V, Makura Z, Jones AS, Baines PB, Selby A, et al. Oropharyngeal carriage and lower airway colonisation/infection in 45 tracheotomised children. Thorax. 2002;57:1015–20.
16. National Human Genome Research Institute. The cost of sequencing a human genome. https://www.genome.gov/about-genomics/fact-sheets/Sequencing-Human-Genome-cost. Accessed February 14, 2020.
17. Quince C, Walker AW, Simpson JT, Loman NJ, Segata N. Corrigendum: shotgun metagenomics, from sampling to analysis. Nat Biotechnol. 2017;35:1211.
18. Martín R, Miquel S, Langella P, Bermúdez-Humarán LG. The role of metagenomics in understanding the human microbiome in health and disease. Virulence. 2014;5:413–23.
19. Pienkowska K, Wiehlmann L, Tümmler B. Airway microbial metagenomics. Microbes Infect. 2018;20:536–42.
20. Weinstock GM. Genomic approaches to studying the human microbiota. Nature. 2012;489:250–6.
21. Hamady M, Knight R. Microbial community profiling for human microbiome projects: tools, techniques, and challenges. Genome Res. 2009;19:1141–52.
22. Garrido-Cardenas JA, Garcia-Maroto F, Alvarez-Bermejo JA, Manzano-Agugliaro F. DNA sequencing sensors: an overview. Sensors (Basel). 2017;17:588.
23. Pylro VS, Roesch LF, Morais DK, Clark IM, Hirsch PR, Tótola MR. Data analysis for 16S microbial profiling from different benchtop sequencing platforms. J Microbiol Methods. 2014;107:30–7.
24. Bukin YS, Galachyants YP, Morozov IV, Bukin SV, Zakharenko AS, Zemskaya TI. The effect of 16S rRNA region choice on bacterial community metabarcoding results. Sci Data. 2019;6:190007.
25. Cuthbertson L, Rogers GB, Walker AW, Oliver A, Hafiz T, Hoffman LR, et al. Time between collection and storage significantly influences bacterial sequence composition in sputum samples from cystic fibrosis respiratory infections. J Clin Microbiol. 2014;52:3011–6.
26. Lauber CL, Zhou N, Gordon JI, Knight R, Fierer N. Effect of storage conditions on the assessment of bacterial community structure in soil and human-associated samples. FEMS Microbiol Lett. 2010;307:80–6.
27. Wiehlmann L, Pienkowska K, Hedtfeld S, Dorda M, Tümmler B. Impact of sample processing on human airways microbial metagenomes. J Biotechnol. 2017;250:51–5.
28. Periasamy S, Kolenbrander PE. Aggregatibacter actinomycetemcomitans builds mutualistic biofilm communities with Fusobacterium nucleatum and Veillonella species in saliva. Infect Immun. 2009;77:3542–51.
29. Nørskov-Lauritsen N. Classification, identification, and clinical significance of Haemophilus and Aggregatibacter species with host specificity for humans. Clin Microbiol Rev. 2014;27:214–40.
30. Gholizadeh P, Pormohammad A, Eslami H, Shokouhi B, Fakhrzadeh V, Kafil HS. Oral pathogenesis of Aggregatibacter actinomycetemcomitans. Microb Pathog. 2017;113:303–11.
31. Belibasakis GN, Maula T, Bao K, Lindholm M, Bostanci N, Oscarsson J, et al. Virulence and pathogenicity properties of Aggregatibacter actinomycetemcomitans. Pathogens. 2019;8:222.
32. Wang JC, Bergeron M, Andersen H, Tikhtman R, Haslam D, Hunter T, et al. Feasibility of shotgun metagenomics to assess microbial ecology of pediatric tracheostomy tubes. Laryngoscope. 2019;129:317–23.
33. Pérez-Losada M, Graham RJ, Coquillette M, Jafarey A, Castro-Nallar E, Aira M, et al. The temporal dynamics of the tracheal microbiome in tracheostomised patients with and without lower respiratory infections. PLoS One. 2017;12:e0182520.
34. Pérez-Losada M, Graham RJ, Coquillette M, Jafarey A, Castro-Nallar E, Aira M, et al. Tracheal microbiota in patients with a tracheostomy before, during and after an acute respiratory infection. Pediatr Infect Dis J. 2018;37:e269–71.
35. Dickson RP, Erb-Downward JR, Freeman CM, McCloskey L, Beck JM, Huffnagle GB, et al. Spatial variation in the healthy human lung microbiome and the adapted island model of lung biogeography. Ann Am Thorac Soc. 2015;12:821–30.
36. Charlson ES, Bittinger K, Haas AR, Fitzgerald AS, Frank I, Yadav A, et al. Topographical continuity of bacterial populations in the healthy human respiratory tract. Am J Respir Crit Care Med. 2011;184:957–63.
37. Gasparin M, Schweiger C, Manica D, Maciel AC, Kuhl G, Levy DS, et al. Accuracy of clinical swallowing evaluation for diagnosis

of dysphagia in children with laryngomalacia or glossoptosis. Pediatr Pulmonol. 2017;52:41–7.

38. Monasterio FO, Molina F, Berlanga F, López ME, Ahumada H, Takenaga RH, et al. Swallowing disorders in Pierre Robin sequence: its correction by distraction. J Craniofac Surg. 2004;15:934–41.

39. Wang JC, Cordero J, Sun Y, Aranke M, Wolcott R, Colmer-Hamood JA, et al. Planktonic growth of Pseudomonas aeruginosa around a dual-species biofilm supports the growth of Fusobacterium nucleatum within that biofilm. Int J Otolaryngol. 2017;2017:3037191.

40. Marino PJ, Wise MP, Smith A, Marchesi JR, Riggio MP, Lewis M, et al. Community analysis of dental plaque and endotracheal tube biofilms from mechanically ventilated patients. J Crit Care. 2017;39:149–55.

41. Lima BP, Shi W, Lux R. Identification and characterization of a novel Fusobacterium nucleatum adhesin involved in physical interaction and biofilm formation with Streptococcus gordonii. Microbiologyopen. 2017;6:e00444.

42. Nouraei SA, Petrou MA, Randhawa PS, Singh A, Howard DJ, Sandhu GS. Bacterial colonization of airway stents: a promoter of granulation tissue formation following laryngotracheal reconstruction. Arch Otolaryngol Head Neck Surg. 2006;132: 1086–90.

43. Lim YW, Evangelista JS 3rd, Schmieder R, Bailey B, Haynes M, Furlan M, et al. Clinical insights from metagenomic analysis of sputum samples from patients with cystic fibrosis. J Clin Microbiol. 2014;52:425–37.

44. Quinn RA, Whiteson K, Lim YW, Zhao J, Conrad D, LiPuma JJ, et al. Ecological networking of cystic fibrosis lung infections. NPJ Biofilms Microbiomes. 2016;2:4.