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Communication

Comparison of two commercial DNA extraction kits for the analysis of nasopharyngeal bacterial communities

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Abstract: Characterization of microbial communities via next-generation sequencing (NGS) requires an extraction of microbial DNA. Methodological differences in DNA extraction protocols may bias results and complicate inter-study comparisons. Here we compare the effect of two commonly used commercial kits (Norgen and Qiagen) for the extraction of total DNA on estimating nasopharyngeal microbiome diversity. The nasopharynx is a reservoir for pathogens associated with respiratory illnesses and a key player in understanding airway microbial dynamics.

Total DNA from nasal washes corresponding to 30 asthmatic children was extracted using the Qiagen QIAamp DNA and Norgen RNA/DNA Purification kits and analyzed via Illumina MiSeq 16S rRNA V4 amplicon sequencing. The Norgen samples included more sequence reads and OTUs per sample than the Qiagen samples, but OTU counts per sample varied proportionally between groups (r = 0.732). Microbial profiles varied slightly between sample pairs, but alpha- and beta-diversity indices (PCoA and clustering) showed high similarity between Norgen and Qiagen microbiomes. Moreover, no significant differences in community structure (PERMANOVA and adonis tests) and taxa proportions (Kruskal-Wallis test) were observed between kits. Finally, a Procrustes analysis also showed low dissimilarity ($M^2 = 0.173$; $P < 0.001$) between the PCoAs of the two DNA extraction kits.

Contrary to what has been observed in previous studies comparing DNA extraction methods, our 16S NGS analysis of nasopharyngeal washes did not reveal significant differences in community composition or structure between kits. Our findings suggest congruence between column-based

chromatography kits and support the comparison of microbiome profiles across nasopharyngeal metataxonomic studies.

Keywords: 16S rRNA; asthma; DNA extraction; metataxonomics; microbiome; nasopharynx

1. Introduction

The advent of next-generation sequencing (NGS) technology has significantly facilitated characterization of microbial communities (microbiomes) residing in the human body [1–4]. Numerous metagenomic (shotgun sequencing) and metataxonomic (amplicon sequencing; e.g., 16S rRNA) approaches [see 5 for distinction] have been developed and widely applied to describe and compare human microbiomes during health and disease [2,6–9]. Given the plethora of available methods, initial choices in upstream analysis may cause biases in the subsequent estimation of microbial (taxa or OTUs) profiles (downstream analysis), hindering interpretation and comparison of studies and threatening their veracity [10,11]. NGS metagenomic and metataxonomic projects usually start with a microbiome DNA extraction, which also requires choosing a specific protocol. Multiple systematic comparisons of available commercial kits for DNA extraction have shown that variation in their design and components (e.g., reagents, disruption procedure, filtering column) can lead to technical biases (non-biological differences) in microbial composition and structure [12–14]. Most of these studies, however, have been focused on gut, skin, and oral microbiotas [15], and less frequently have included other organs of interest, such as the respiratory tract. One exception is the study by Willner et al. [15], which compared five DNA extraction methods for microbial community profiling of bronchoalveolar lavage samples. In this study the authors revealed that differences between extraction methods were significantly greater than differences between technical replicates, emphasizing the importance of standardizing methodologies for airway microbiome research and the need for further testing. Here we use nasopharyngeal washes to perform a new comparative study of two additional DNA extraction protocols not included in Willner's study for characterizing airway microbiotas.

The nasopharynx is considered an anatomical reservoir from which pathogenic microbes can spread to the lower and upper respiratory airways and cause respiratory infections, or invade the bloodstream to cause sepsis and meningitis [16–19]. Consequently, given its importance, the nasopharynx has been the focus of intense microbiome research over the last few years. Numerous metagenomic and metataxonomic studies have identified commensal and pathogenic members of the nares and investigated how nasal microbiotas change during health and disease [18–30]. Several of these studies used Qiagen and Norgen commercial kits to extract microbial DNA; however, no technical comparative study so far has assessed the effect that these, or any other commercial kits, have on nasopharyngeal profiling. Consequently, how upstream methodological choices bias estimation of nasopharyngeal microbial diversity and structure is unknown. In this study we used high-throughput 16S amplicon data to evaluate the effect that two commercial kits (Qiagen and Norgen) commonly used for the extraction of DNA have on microbial profiling of nasopharyngeal washes from asthmatic children.

2. Materials and Methods

2.1. Ethics approval and consent to participate

All participants in this study were part of the AsthMaP2 (Asthma Severity Modifying Polymorphisms) Study. AsthMaP2 is an ongoing study of urban children and adolescents designed to find associations among airway microbes, environmental exposures, allergic sensitivities, genetics, and asthma [31]. AsthMaP2 and the study presented here were approved by the Children's National Medical Center Institutional Review Board (Children's National IRB), which requires that consent is obtained and documented prior to conducting study procedures and collection of samples for research. Written consent was obtained from all independent participants or their legal guardians using the Children's National IRB approved informed consent documents.

2.2. Nasopharyngeal samples and molecular analyses

A total of 30 children and adolescents (ages 6 to 18 years) were recruited from the metropolitan Washington, DC, area. All had been physician-diagnosed with asthma for at least one year prior to recruitment. Individuals who reported a medical history of chronic or complex cardiorespiratory disease were ineligible. Their nasopharynges were sampled by instilling 5 ml of isotonic sterile saline buffer into each nare, holding it for 10 seconds, and then blowing into a specimen collection container. Nasal washes were then split in half and extracted using the Qiagen QIAamp DNA Kit (Catalog # 51304) and the Norgen RNA/DNA Purification Kit (Product # 48600, 48700). These two kits are simple and commonly used in the study of airway microbiomes, but never have been compared before. In both kits, DNA purification is based on heating, and chemical and enzymatic reactions followed by spin column chromatography. These processes involve a pre-incubation in 100 µL of lysozyme-TE buffer pH = 8.0 for 15 minutes at 37 ℃, followed by a lysing step via lysis solution and proteinase K. The DNA in the lysate is then captured and purified on a DNA purification column. Potential differences between kits involve the composition of the lysis, washing and elution solutions, proteinase K, and the silica gel membrane inside the spin columns. No specific information for any of these components is provided by the kit manufacturers. All extractions yielded >50 ng of total DNA (as indicated by NanoDrop 2000 UV-Vis Spectrophotometer measuring). No significant differences (paired t-test) in DNA concentration and quality (ratios of absorbance at 260 and 280 nm) were detected between extraction kits. DNA extractions were prepared for sequencing using the Schloss' MiSeq_WetLab_SOP protocol (09.2015) in Kozich et al. [32]. Each DNA sample was amplified for the V4 region (~250 bp) of the 16S rRNA gene and libraries were sequenced using the Illumina MiSeq sequencing platform at University of Michigan Medical School.

2.3. Sequence analyses

Raw FASTQ files were processed in mothur v1.35.1 [33]. Default settings were used to minimize sequencing errors as described in Schloss et al. [34]. Clean sequences were aligned to the SILVA_v123 bacterial reference alignment at www.mothur.org. Chimeras were removed using uchime [35] and non-chimeric sequences were classified using the naïve Bayesian classifier of

Wang et al. [36]. Sequences were clustered into Operational Taxonomic Units (OTUs) at the 0.03 threshold (species level). OTU sequence representatives and taxonomy were imported (BIOM format) into QIIME [37] for subsequent analyses. The mothur OTU table was filtered to a minimum of 2 observations (sequences) per OTU. Samples were subsampled (rarefaction analysis) to the smallest sample size (1,916 sequences) to remove the effect of sample size bias on community composition.

Trees for phylogenetic diversity calculations were constructed using FastTree [38]. Taxonomic alpha-diversity was estimated using the number of observed OTUs and the Chao1, Simpson, Fisher and Shannon indexes. Phylogenetic alpha-diversity (PD) was calculated by the Faith's phylogenetic diversity index [39]. Similarly, both taxonomic (Bray-Curtis and Euclidean) and phylogenetic (unweighted and weighted unifrac) beta-diversity metrics were calculated. Relatedness among samples was assessed using Procrustes (only weighted unifrac distances), PCoA ordination and neighbor-joining (NJ) clustering analyses. Alpha- and beta-diversity metrics were compared between samples grouped by DNA extraction kit (Qiagen *versus* Norgen) using the Kruskal-Wallis test and a non-parametric version of the t-test. Taxonomic and phylogenetic distances were also compared among groups using the non-parametric PERMANOVA and adonis tests from the vegan R's library [40]. Sample pairs were also compared using Fisher's exact test. Significance was determined through 10,000 permutations. Bonferroni or Benjamini-Hochberg FDR multiple test correction methods were applied. All analyses were performed in mothur, QIIME, and RStudio [41]. Sequence data have been uploaded to the GenBank under SRA accession number SRP069020.

3. Results and Discussion

3.1. Sequences and OTUs

Total DNA from 30 nasal washes corresponding to 30 asthmatic children was extracted using Qiagen and Norgen kits and analyzed via MiSeq sequencing of 16S rRNA V4 amplicons. Norgen samples generated a total of 637,624 sequences ranging from 2,016 to 51,194 sequences per sample (mean = 21,987) after quality control analyses and OTU filtering. While Qiagen samples generated a total of 480,443 sequences ranging from 1,916 to 53,966 sequences per sample (mean = 16,567) after quality control and OTU filtering. The lower read yield of the Qiagen approach might result from DNA loss during column purification (i.e., differences in the affinities of the silica gel membrane inside the spin columns). Sequence yield variation across samples was weakly correlated [Pearson correlation coefficient (r) = 0.236, n.s.] and not significantly different (paired t-test) between DNA extraction methods. The mothur pipeline identified 69–309 OTUs (mean = 165) per sample in the Norgen sample group and 38–282 OTUs (mean = 145) per sample in the Qiagen sample group. Interestingly, observed OTU estimates across samples were strongly correlated (r = 0.732***) and significantly different (paired t-test; $P = 0.011$) between commercial kits. Only 2.8% and 2.85% of the OTUs were unclassified at the genus level in the Norgen and Qiagen groups, respectively.

3.2. Microbial composition

Nasopharyngeal microbiomes in Norgen (N) and Qiagen (Q) kits were dominated by the following eight genera: *Moraxella* (N = 24.9%, Q = 26.8%), *Staphylococcus* (N = 25.1%, Q = 12.5%), *Corynebacterium* (N = 7.6%, Q = 9.5%), *Haemophilus* (N = 7.0%, Q = 9.0%), *Prevotella* (N

= 5.4%, Q = 6.5%), *Streptococcus* (N = 5.2%, Q = 7.2%), *Dolosigranulum* (N = 4.6%, Q = 7.3%) and *Fusobacterium* (N = 4.2%, Q = 4.1%) (see Figure 1). The hard-to-lyse Gram-positive genus *Staphylococcus* showed the largest difference in microbial proportions between DNA extraction methods and those proportions were significantly different (Fisher's exact test; effect size ≤ 5%; $P < 0.05$) in three of the sample pairs compared. Those same three sample pairs showed the largest dissimilarity in the beta-diversity analyses below. Both of our extraction procedures included a 15-minute pre-incubation with the same lysozyme to lyse Gram-positive bacteria, but the lysis buffer used in each protocol was different. Differences in cell wall composition and structure can lead to variations in bacterial susceptibility to different lysis procedures [14]. Hence, we suspect that the Norgen Lysis Solution + lysozyme buffer is probably more effective than the Qiagen ATL + lysozyme buffer at lysing Gram-positive bacteria.

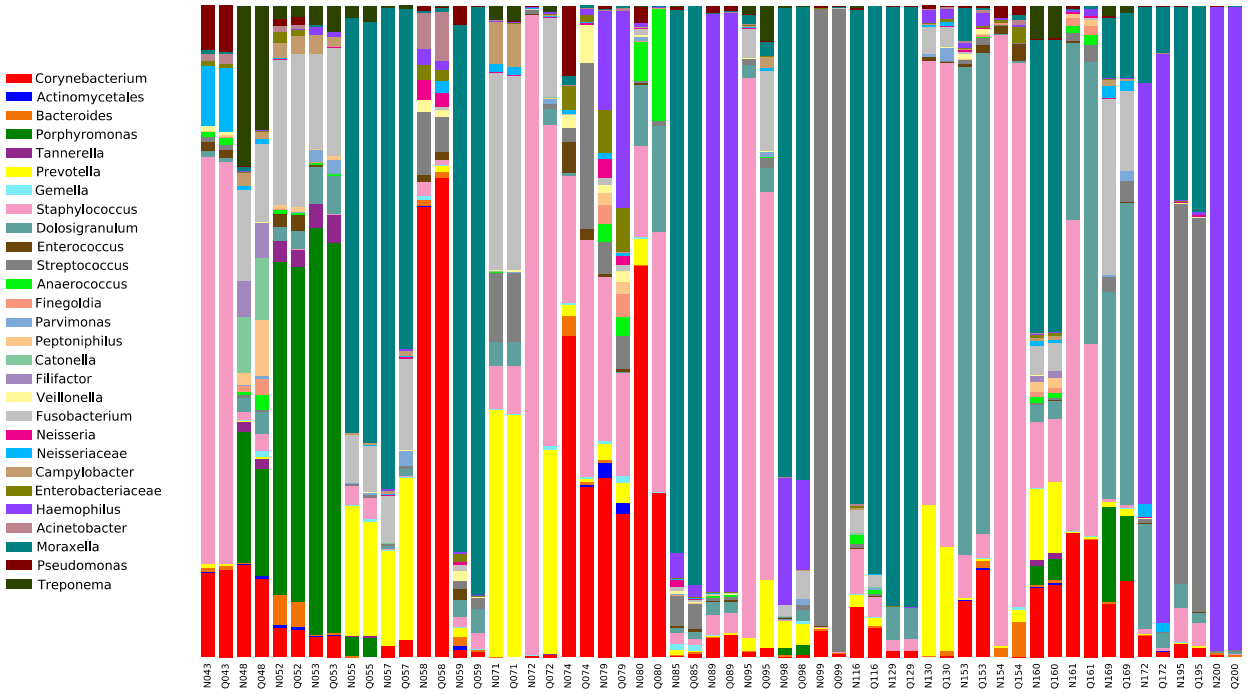

Figure 1. Taxonomic profiles of Norgen (N) and Qiagen (Q) nasopharyngeal microbiome pairs from 30 asthmatic children. Only the 28 most abundant bacterial genera are shown.

All the bacterial genera dominating the nasopharynges of asthmatic children and adolescents in this study (see green spheres in Figure 2) have also been detected in previous 16S metataxonomic studies of nasal microbiotas in infants [18,19,29,42] and adults [43–45] with and without respiratory infections, but in different proportions.

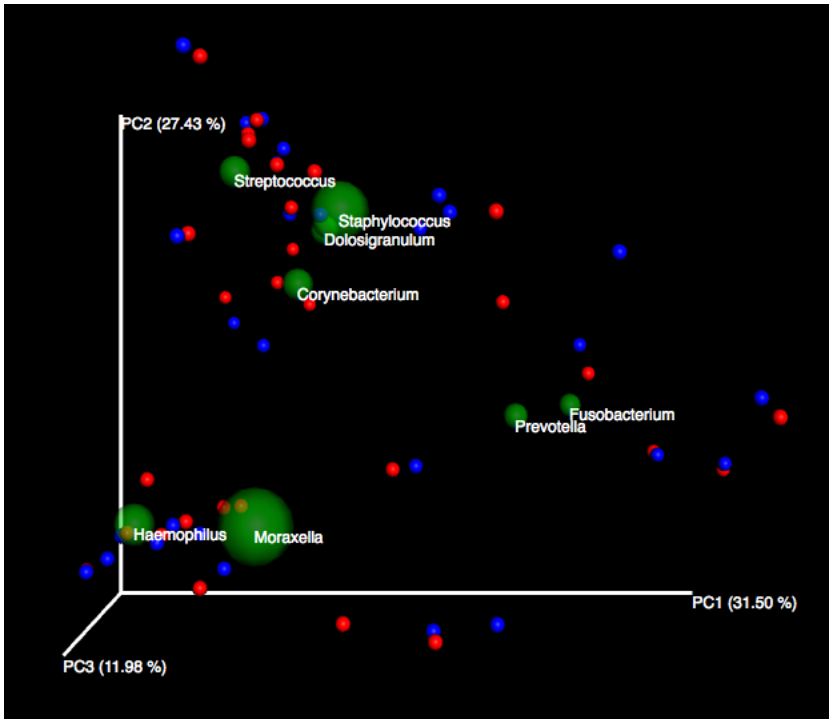

Figure 2. 3D Principal Coordinates Analysis (PCoA) of weighted unifrac distances between Norgen (red) and Qiagen (blue) samples. Green spheres show the more prevalent genera in the different areas of the PCoA plot.

3.3. Microbial diversity

Microbial profiles varied slightly between sample pairs (Figure 1), but alpha-diversity indices (Figure 3) did not significantly varied (paired t-test) between kits.

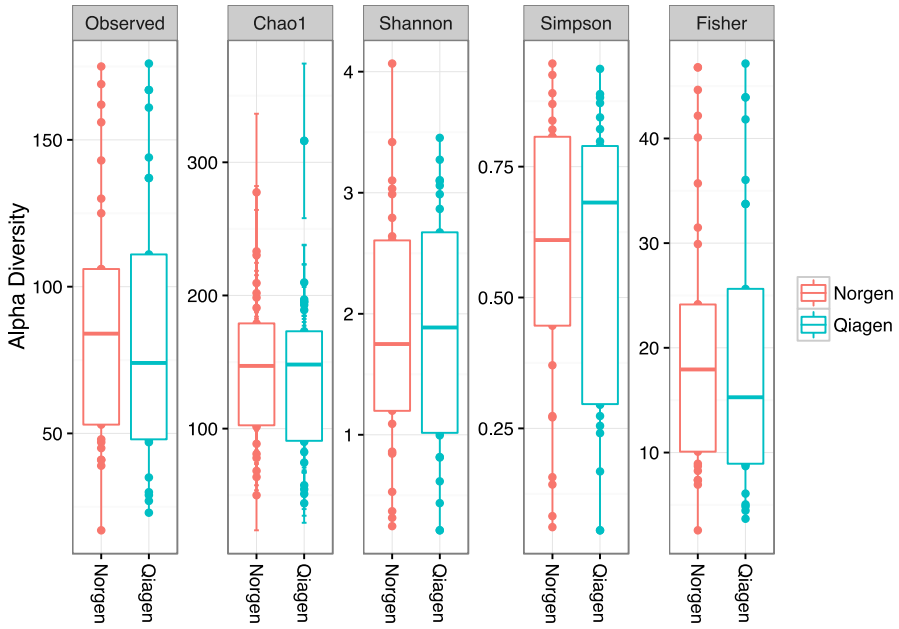

Figure 3. Box plots of alpha-diversity indices comparing Norgen and Qiagen samples.

Beta-diversity ordination analysis of PCoA showed low dissimilarities between Norgen and Qiagen microbiomes for all distances tested (Figure 2). Similarly, no significant differences in community composition (PERMANOVA and adonis tests) were observed between both groups. At the genus and OTU level, microbial abundances did not significantly vary between kits (Kruskal-Wallis test). Procrustes analysis comparing PCoA plots of Norgen and Qiagen microbiomes showed low dissimilarity ($M^2 = 0.173$; $P < 0.001$) between them (Figure 4), with most pairs connected by short bars. This implies that the same beta diversity conclusions could be drawn from either data set.

Finally, clustering analyses based on four different distances also showed that most microbiome sample pairs clustered together according to patient (see Figure 5), with 3 out of 30 microbiome pairs falling in distant clusters. These same three pairs also showed the highest dissimilarity in the Procrustes analysis (Figure 4). However, their differences were not significant (weighted unifrac test) after FDR correction.

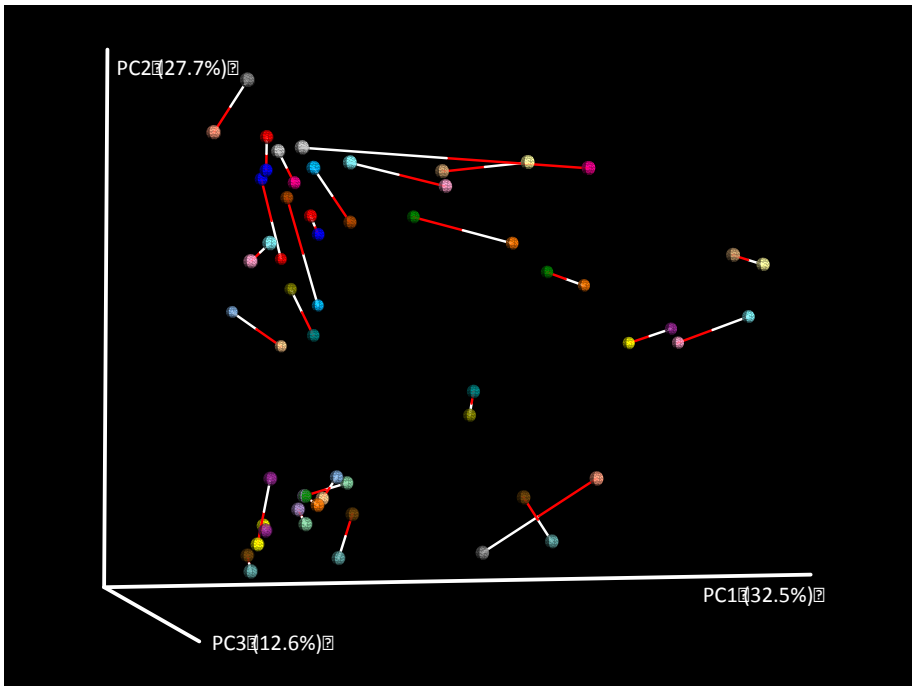

Figure 4. Procrustes analysis of Norgen and Qiagen samples. Intra-patient sample pairs are connected by a line, the white end indicating its Norgen origin and the red end indicating its Qiagen origin.

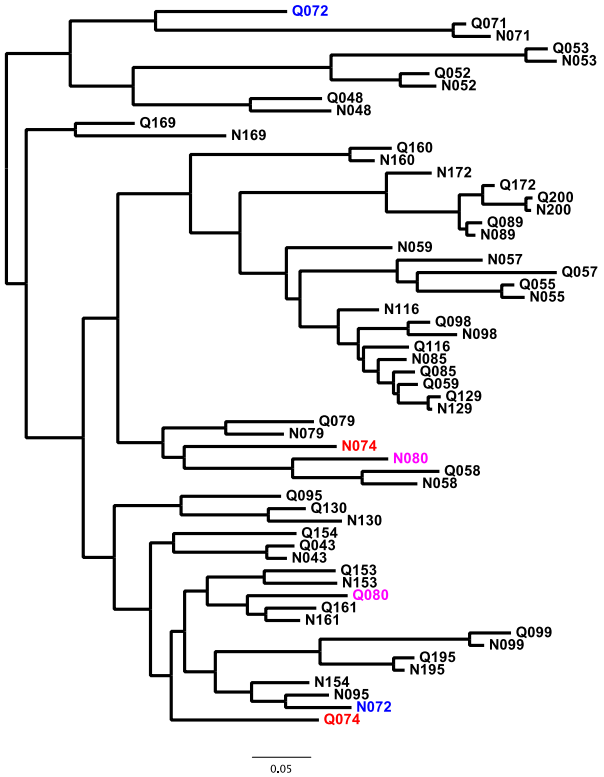

Figure 5. Neighbor-joining tree of weighted unifrac distances between Norgen (N) and Qiagen (Q) samples. Distant sample pairs are colored.

Previous studies comparing DNA extraction methods in mock communities (i.e., a mixture of microbes created *in vitro* to simulate the composition of a microbiome sample), oral and bronchoalveolar lavages [11,15,46,47] revealed that DNA yield and bacterial species representation varied with DNA extraction methods. However, kit-based extractions showed less technical variation than non-commercial methods – presumably due to the use of premade buffers and purification columns which likely reduce technical error. Those studies then suggested that DNA preparation methods have a profound effect on microbial diversity estimation, and implied that samples prepared with different protocols may not be suitable for comparative metagenomics or metataxonomics. Similarly, several 16S metataxonomics studies of the gut microbiota have also revealed significantly large community differences between extraction methods [12,48]. Although a recent systematic comparison [49] of five DNA extraction methods based on 16S V4 amplicon data (like in our study) showed that the largest portion of variation (34%) in gut bacterial profiles was attributed to differences between subjects, with a smaller proportion of variation (9%) associated with DNA extraction method and intra-subject variation. This study did not detect significant differences (paired t-test) in alpha-diversity between extraction methods, while beta-diversity estimates (PERMANOVA and adonis test) varied significantly between most of the estimators. As for the oral cavity, other study comparing two DNA extraction methods in the analysis of salivary bacterial communities [14] also showed a high degree of congruence in alpha-diversity between extraction methods; the same study revealed significant differences in the structure of the microbiotas. As in all studies above, our analyses of the nasopharyngeal microbiota using two commercial DNA extraction

kits also revealed significant differences (t-test; $P < 0.05$) in sequence depth and OTU count. Similarly to Lazarevic et al. and Mackenzie et al. [14,49], we did not see significant differences in alpha-diversity between groups. Our intra-patient analysis showed significant differences (Fisher's exact test) in bacterial proportions in three sample pairs (see Fig. 5) for some of the genera (e.g., *Staphylococcus*). However, contrary to what has been reported in previous studies, our 16S metataxonomic analysis of nasal washes did not reveal significant differences in community structure between groups of samples extracted via Norgen and Qiagen kits.

4. Conclusion

Our 16S NGS analysis of nasopharyngeal washes from 30 asthmatic children suggests that nasopharyngeal microbial profiles are congruent between column-based chromatography kits and supports their comparison across studies. Cheaper and easier-to-use commercial DNA extraction kits are constantly being developed. Such kits allow researchers to quickly and efficiently extract DNA, with minimal clean-up steps before amplification. We hope the methods examined and results generated in this study contribute to the ongoing debate regarding DNA extraction standardization and reproducibility in human subjects.

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Conflict of Interest

All authors declare no conflicts of interest in this study.

References

1. Ding T, Schloss PD (2014) Dynamics and associations of microbial community types across the human body. *Nature* 509: 357–360.
2. Human Microbiome Project C (2012) Structure, function and diversity of the healthy human microbiome. *Nature* 486: 207–214.
3. Human Microbiome Project C (2012) A framework for human microbiome research. *Nature* 486: 215–221.
4. Integrative HMPRNC (2014) The Integrative Human Microbiome Project: dynamic analysis of microbiome-host omics profiles during periods of human health and disease. *Cell Host Microbe* 16: 276–289.

5. Marchesi JR, Ravel J (2015) The vocabulary of microbiome research: a proposal. *Microbiome* 3: 31.
6. Kuczynski J, Lauber CL, Walters WA, et al. (2012) Experimental and analytical tools for studying the human microbiome. *Nat Rev Genet* 13: 47–58.
7. Althani AA, Marei HE, Hamdi WS, et al. (2015) Human Microbiome and its Association With Health and Diseases. *J Cell Physiol* 231: 1688–1694.
8. Martin R, Miquel S, Langella P, et al. (2014) The role of metagenomics in understanding the human microbiome in health and disease. *Virulence* 5: 413–423.
9. Cox MJ, Cookson WO, Moffatt MF (2013) Sequencing the human microbiome in health and disease. *Hum Mol Genet* 22: R88–94.
10. Brooks JP, Edwards DJ, Harwich MD, Jr., et al. (2015) The truth about metagenomics: quantifying and counteracting bias in 16S rRNA studies. *BMC Microbiol* 15: 66.
11. Abusleme L, Hong BY, Dupuy AK, et al. (2014) Influence of DNA extraction on oral microbial profiles obtained via 16S rRNA gene sequencing. *J Oral Microbiol* 6.
12. Wu GD, Lewis JD, Hoffmann C, et al. (2010) Sampling and pyrosequencing methods for characterizing bacterial communities in the human gut using 16S sequence tags. *BMC Microbiol* 10: 206.
13. Momozawa Y, Deffontaine V, Louis E, et al. (2011) Characterization of bacteria in biopsies of colon and stools by high throughput sequencing of the V2 region of bacterial 16S rRNA gene in human. *PLOS ONE* 6: e16952.
14. Lazarevic V, Gaia N, Girard M, et al. (2013) Comparison of DNA extraction methods in analysis of salivary bacterial communities. *PLOS ONE* 8: e67699.
15. Willner D, Daly J, Whiley D, et al. (2012) Comparison of DNA extraction methods for microbial community profiling with an application to pediatric bronchoalveolar lavage samples. *PLOS ONE* 7: e34605.
16. Bogaert D, De Groot R, Hermans PW (2004) Streptococcus pneumoniae colonisation: the key to pneumococcal disease. *Lancet Infect Dis* 4: 144–154.
17. Garcia-Rodriguez JA, Fresnadillo Martinez MJ (2002) Dynamics of nasopharyngeal colonization by potential respiratory pathogens. *J Antimicrob Chemother* 50 Suppl S2: 59–73.
18. Biesbroek G, Tsivtsivadze E, Sanders EA, et al. (2014) Early respiratory microbiota composition determines bacterial succession patterns and respiratory health in children. *Am J Respir Crit Care Med* 190: 1283–1292.
19. Teo SM, Mok D, Pham K, et al. (2015) The infant nasopharyngeal microbiome impacts severity of lower respiratory infection and risk of asthma development. *Cell Host Microbe* 17: 704–715.
20. Feazel LM, Santorico SA, Robertson CE, et al. (2015) Effects of Vaccination with 10-Valent Pneumococcal Non-Typeable Haemophilus influenza Protein D Conjugate Vaccine (PHiD-CV) on the Nasopharyngeal Microbiome of Kenyan Toddlers. *PLOS ONE* 10: e0128064.
21. Prevaes SM, de Winter-de Groot KM, Janssens HM, et al. (2015) Development of the Nasopharyngeal Microbiota in Infants with Cystic Fibrosis. *Am J Respir Crit Care Med*.
22. Cremers AJ, Zomer AL, Gritzfeld JF, et al. (2014) The adult nasopharyngeal microbiome as a determinant of pneumococcal acquisition. *Microbiome* 2: 44.
23. Allen EK, Koeppel AF, Hendley JO, et al. (2014) Characterization of the nasopharyngeal microbiota in health and during rhinovirus challenge. *Microbiome* 2: 22.

24. Biesbroek G, Bosch AA, Wang X, et al. (2014) The impact of breastfeeding on nasopharyngeal microbial communities in infants. *Am J Respir Crit Care Med* 190: 298–308.
25. Sakwinska O, Bastic Schmid V, Berger B, et al. (2014) Nasopharyngeal microbiota in healthy children and pneumonia patients. *J Clin Microbiol* 52: 1590–1594.
26. Bassis CM, Tang AL, Young VB, et al. (2014) The nasal cavity microbiota of healthy adults. *Microbiome* 2: 27.
27. Perez-Losada M, Castro-Nallar E, Bendall ML, et al. (2015) Dual Transcriptomic Profiling of Host and Microbiota during Health and Disease in Pediatric Asthma. *PLOS ONE* 10: e0131819.
28. Castro-Nallar E, Shen Y, Freishtat RJ, et al. (2015) Integrating metagenomics and host gene expression to characterize asthma-associated microbial communities. *BMC Medical Genomics* 8: 50.
29. Bogaert D, Keijser B, Huse S, et al. (2011) Variability and diversity of nasopharyngeal microbiota in children: a metagenomic analysis. *PLOS ONE* 6: e17035.
30. Pérez-Losada M, Crandall KA, Freishtat RJ (2016) Two sampling methods yield distinct microbial signatures in the nasopharynx of asthmatic children. *Microbiome* [in press].
31. Benton AS, Wang Z, Lerner J, et al. (2010) Overcoming heterogeneity in pediatric asthma: tobacco smoke and asthma characteristics within phenotypic clusters in an African American cohort. *J Asthma* 47: 728–734.
32. Kozich JJ, Westcott SL, Baxter NT, et al. (2013) Development of a dual-index sequencing strategy and curation pipeline for analyzing amplicon sequence data on the MiSeq Illumina sequencing platform. *Appl Environ Microbiol* 79: 5112–5120.
33. Schloss PD, Westcott SL, Ryabin T, et al. (2009) Introducing mothur: Open-Source, Platform-Independent, Community-Supported Software for Describing and Comparing Microbial Communities. *Appl Environ Microbiol* 75: 7537–7541.
34. Schloss PD, Gevers D, Westcott SL (2011) Reducing the effects of PCR amplification and sequencing artifacts on 16S rRNA-based studies. *PLOS ONE* 6: e27310.
35. Edgar RC, Haas BJ, Clemente JC, et al. (2011) UCHIME improves sensitivity and speed of chimera detection. *Bioinformatics* 27: 2194–2200.
36. Wang Q, Garrity GM, Tiedje JM, et al. (2007) Naive Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. *Appl Environ Microbiol* 73: 5261–5267.
37. Caporaso JG, Kuczynski J, Stombaugh J, et al. (2010) QIIME allows analysis of high-throughput community sequencing data. *Nat Methods* 7: 335–336.
38. Price MN, Dehal PS, Arkin AP (2010) FastTree 2-Approximately Maximum-Likelihood Trees for Large Alignments. *PLOS ONE* 5.
39. Faith DP (1992) Conservation evaluation and phylogenetic diversity. *Biol Conserv* 61: 1–10.
40. Dixon P (2003) VEGAN, a package of R functions for community ecology. *J Veg Sci* 14: 927–930.
41. RStudioTeam (2015) RStudio: Integrated Development for R. RStudio, Inc., Boston, MA URL http://www.rstudio.com/.
42. Biesbroek G, Wang X, Keijser BJ, et al. (2014) Seven-valent pneumococcal conjugate vaccine and nasopharyngeal microbiota in healthy children. *Emerg Infect Dis* 20: 201–210.
43. Yan M, Pamp SJ, Fukuyama J, et al. (2013) Nasal microenvironments and interspecific interactions influence nasal microbiota complexity and S. aureus carriage. *Cell Host Microbe* 14: 631–640.

44. Cremers AJH, Zomer AL, Gritzfeld JF, et al. (2014) The adult nasopharyngeal microbiome as a determinant of pneumococcal acquisition. *Microbiome* 2.
45. Bassis CM, Erb-Downward JR, Dickson RP, et al. (2015) Analysis of the upper respiratory tract microbiotas as the source of the lung and gastric microbiotas in healthy individuals. *MBio* 6: e00037.
46. Morgan JL, Darling AE, Eisen JA (2010) Metagenomic sequencing of an in vitro-simulated microbial community. *PLOS ONE* 5: e10209.
47. Yuan S, Cohen DB, Ravel J, et al. (2012) Evaluation of methods for the extraction and purification of DNA from the human microbiome. *PLOS ONE* 7: e33865.
48. P OC, Aguirre de Carcer D, Jones M, et al. (2011) The effects from DNA extraction methods on the evaluation of microbial diversity associated with human colonic tissue. *Microb Ecol* 61: 353–362.
49. Mackenzie BW, Waite DW, Taylor MW (2015) Evaluating variation in human gut microbiota profiles due to DNA extraction method and inter-subject differences. *Front Microbiol* 6: 130.

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