British Journal of Medicine & Medical Research
4(10): 1980-1991, 2014

SCIENCEDOMAIN *international*
www.sciencedomain.org

Comparison of DNA Extraction Methods for Human Oral Microbiome Research

Jing Wu[1], I-Hsin Lin[1], Richard B. Hayes[1] and Jiyoung Ahn[1*]

[1]Division of Epidemiology, Department of Population Health, New York University School of Medicine, New York, NY10016, USA.

Authors' contributions

This work was carried out in collaboration between all authors. Author JW conceived and designed the study, conducted laboratory experiments and statistical analysis, and drafted manuscript. Author IHL conducted statistical analysis. Author RBH conducted critical revision of the manuscript for important intellectual content. Author JA obtained funding, conceived and designed the experiment, and drafted the manuscript. All authors read and approved the final manuscript.

Original Research Article

Received 15th June 2013
Accepted 28th July 2013
Published 14th January 2014

ABSTRACT

The oral micro biome is highly diverse and its composition is associated with oral disease and potentially diseases at other sites. Our objective is to evaluate DNA extraction methods potentially suitable for population-based investigations on the oral human microbiome and disease risk. Six commonly used microbial DNA extraction kits, employing either enzymatic methods or mechanical bead beating for cell lysis, were evaluated for the following aspects total DNA yield and quality and 16s rRNA DNA product and representation of microbial diversity. All analyses were carried out using a pooled and homogenized sample from one study subject. 16s rRNA gene sequence data were processed using the QIIME pipeline. One way ANOVA and Kruskal-Wallis tests were used to compare the different DNA extraction methods. We found that enzymatic extraction kits produced higher human genomic DNA, compared with mechanical extraction kits, however, phylogenic diversity in oral microbiome community structure from 16s rRNA gene sequence reads revealed no important differences between kit types. Enzymatic and mechanical bead beating kits provide alternative approaches for DNA extraction of oral microbiome DNA from oral wash samples. Greater total DNA yields are found in enzymatic approaches but microbial diversity can be similarly well characterized by either enzymatic or mechanical bead beating approaches.

**Corresponding author: Email: Jiyoung.Ahn@nyumc.org*;

Keywords: DNA extraction; oral microbiome; 16s rRNA sequencing; qiime pipline.

1. INTRODUCTION

The human mouth hosts a structured microbial community, the oral microbiome [1]. Until recently, studies of the human microbiota have been based on bacterial culture, which is a limited and insensitive approach, as non-culturables (up to 80%) cannot be studied [2]. With high-throughput genetic-based microbiome assays, we are just beginning to understand the role of human microbiome in human health and disease. The NIH Human Micro biome Project (HMP) revealed that individuals have a shared 'core' microbiome component [3,4] and a component exhibiting significant inter-individual variation [5]. Advances are being made relating microbiome variability to host disease susceptibility, including to obesity [6], gastrointestinal cancers [7,8,9] and inflammatory diseases [10,11].

16S rRNA gene sequencing is an accurate and high-throughput technique for microbiome assay. This assay involves DNA extraction, 16S ribosomal RNA gene (DNA) amplicon generation by PCR, followed by amplicon sequencing. While each step of the assay could involve potential error [12], it remains unclear if different DNA extraction methods could affect microbiome sequencing results.

Our purpose is to evaluate DNA extraction methods potentially suitable for large population-based investigations on the oral human microbiome and disease risk. In this study, we compared 6 commonly used DNA extraction methods for oral microbiome, employing either enzymatic or mechanical bead beating methods for cell lysis. We evaluated the following aspects: total DNA yield and quality and 16s rRNA DNA product and representation of microbial diversity.

2. MATERIALS AND METHODS

2.1 Specimen Collection

One subject provided mouth wash specimens for six consecutive days under same condition at the same time (around 11am) of each day. Following a procedure developed for oral wash collection in large epidemiologic cohorts [13], the subject was asked to swish vigorously with 10ml Scope mouthwash at (Proctor and Gamble) for 45 seconds and was directed to expectorate into a sterile 50 ml Corning screw cap tube. The daily specimens were immediately frozen to -80ºC. Upon thawing, the six daily specimens were pooled and mixed well and aliquot as 1.5ml samples into PCR clean level microcentrifuge tubes (Eppdendorf). The 1.5 ml aliquot oral wash specimens were centrifuged (first 6000g and then 10000g) and the pellets were retrieved for DNA extraction. This study was approved by the New York University IRB.

2.2 Cell Lysis and DNA Isolation

Six DNA extraction methods Table 1. Were used to isolate DNA from the pellets. Qiagen (Valencia, CA) kit (Cat # 69504) was used for enzymatic digestion for 30min Method 1. [14]. Or overnight (18 hours) (Method 2). Mo Bio (Carlsbad, CA) kits were used for mechanical bead-beating with different bead sizes (Table 1, Methods 3-6). Each method was carried out in triplicate, thus, 18 aliquot samples were extracted.

2.2.1 Qiagen kit protocol

Cell pellets were pre-processed in 200ul DNA/bacteria decontaminated lysozyme solution (20mg/ml) [14] at 37ºC for 30min, then RNase A and Proteinase K were added following the manufacturer's instruction. The mixed solution was vortexed and incubated at 56ºC for 30 minutes (Method 1) or overnight (Method 2). After incubation, the DNA-containing solution was passed through a silica column for extraction and purification.

2.2.2 Mo bio microbial bead beating kits protocol

Four different types of Mo Bio microbial DNA extraction kits were used. These kits were specially designed to extract microbial DNA from bacterial culture/Method 3 (Cat #12255), food samples/Method 4: Cat # 2100), and environmental soil samples/Method 5 (Cat #12855) and Method 6 (Cat # 12888) with different type and size of beads Table 1. For DNA extraction, the specimen pellets were re-suspended in the corresponding bead solutions (Methods 3-6), and inserted in the Powerlyzer (Mo Bio) instrument for 45s at 4000rpm, to mechanically break the cell walls of the bacteria in the sample. The DNA-containing solution was treated with different solutions to remove PCR inhibitor, and finally was passed through a silica column for extraction and purification.

Table 1. DNA isolation methods compared in this study

Kits	Cell lysis	Cost per sample	Time per sample	Remove PCR inhibitor
1 / Qiagen	Lysozyme/Proteinase K/ RNAnase /chemical	<$3.5	90 min	No
2 / Qiagen Overnight	Lysozyme/Proteinase K/ RNAnase A/chemical	<$3.5	18 hrs	No
3 / Powerlyzer Microbial	Chemical /Mechanic (0.1 mm glass beads)	<$2.5	45 min	No
4 / Food Microbial	Chemical /Mechanic (0.15 mm garnet beads)	<$3.0	60 min	Yes
5 / Powerlyzer soil	Chemical /Mechanic (0.1 mm glass beads)	<$5.0	60 min	Yes
6 / Power Soil	Chemical /Mechanic (0.7 mm garnet beads)	<$5.0	60 min	Yes

2.3 Total DNA Quantification

After DNA extraction, total DNA concentration and amount were determined using the Synergy™ H1M microplate reader (Biotech, VM). To evaluate the DNA purity, we used the OD 260/280 ratio values as calculated by the instrument.

2.4 Proportion of Microbial DNA to Human DNA Measured by qPCR

16S rRNA gene DNA was amplified using primers: 347F- 5'GGAGGCAGCAGTAAGGAAT-3' and 803R-5'CTACCGGGGTATCTAATCC-3' by real time PCR (qPCR) from equal amounts (5ng) of total DNA derived by the six different extraction methods. In the same plate, human housekeeping gene GAPDH (Glyceraldehyde-3-phosphate dehydrogenase) was also

amplified, using previously established primer sequence [15]. qPCR was performed in triplicate in 10ul reaction volume, using power SYBR-Green Master Mix (Applied Biosystems, Foster City, CA). The reaction condition is 3 min at 94ºC for initial denaturing, followed by 40 cycles of 94ºC for 15s, 52ºC 45s and 72ºC for 1min in the ABI 7900HT fast real time PCR system (Applied Biosystems, CA). The yield of bacterial 16S rRNA DNA was normalized to GAPDH DNA amount using the comparative ΔCt method [15].

2.5 454 Pyrosequencing of 16S rRNA Genes

16S rRNA amplicon libraries were generated using primers incorporating FLX Titanium adapters and a sample barcode sequence, allowing unidirectional sequencing covering variable regions V3 to V4 (Primers: 347F- 5'GGAGGCAGCAGTAAGGAAT-3' and 803R-5'CTACCGGGGTATCTAATCC-3'). The forward primer for each sample had a ten nucleotides unique specific multiplex identifier (MID). Five ng genomic DNA was used as the template in 25 ul PCR reaction buffer for 16s rRNA amplicon preparation. Cycling conditions were one cycle of 94ºC for 3min, followed by 25 cycles of 94ºC for 15s, 52ºC 45s and 72ºC for 1min followed by a final extension of 72ºC for 8min. After amplification, 1ul of the PCR product was resolved on a 1% agarose gel and visualized by ethidium bromide staining to confirm successful amplification. The generated amplicons were then purified using Agencourt AMPure XP kit (Backman Coulter Company, CA). Purified amplicons were quantified by flurometry using the Quant-iT PicoGreen dsDNA Assay Kit (Invitrogen, Carlsbad, CA). Equimolar amounts (10^7molecules/ul) of purified amplicons with specific MID were pooled for sequencing. Pyrosequencing (Roche 454 GS FLX Titanium) was carried out according to the manufacture's instruction.

2.6 Processing of 16s RNA Sequence Data Analysis

16S rRNA amplicon sequences were processed and analyzed using the QIIME pipeline [16]. Multiplexed libraries were deconvoluted based on the barcodes assigned to each sample. Poor quality sequence was filtered based on sequence length outside the bounds of 200 and 600bp, missing or mean quality score < 25, or mismatched barcode and primer sequences. Chimeric sequence was removed by ChimeraSlayer [17]. The pre-processed sequences were assigned to the Operational Taxonomic Unit (OTU) based on 97% sequence similarity. A representative sequence from each OTU was selected based on UCLUST [18] and aligned based on PyNAST [19]. Each OTU was taxonomically aligned against fully sequenced microbial genomes, the IMG/GG GreenGenes reference package, [20] using the RDP Classifier [21]. Each sample was calculated Shannon's diversity index and Rarefaction Curves for α-diversity. We used Unifrac distance matrices to characterize the inter-group diversity (Beta-diversity) with respect to the different DNA extraction methods. The weighted UniFrac distance metrics [22] were performed to assess the difference in overall microbial community composition. The principal coordinate analysis (PCoA) was used to transform the UniFrac distance matrices into principal coordinates in order to provide visualization of the sample distribution patterns.

2.7 Statistical Method

One way ANOVA was performed to compare the evaluated indices among the tested kits. Only the relative abundance of the taxa distributions was compared by non-parametric Kruskal-Wallis test. Statistical analysis was performed using SPSS (version 17.0). $P<0.05$ was treated as significant difference.

3. RESULTS

3.1 Total Genomic DNA Yield

Total genomic DNA yields from 1.5 ml mouth wash samples are shown in Fig. 1. According to the six different DNA extraction methods. Overall, the two Qiagen DNA extraction methods (Methods 1 and 2) produced higher genomic DNA amount than the Mo Bio kits (Methods 3-6) ($P<0.01$). Qiagen enzymatic digestion overnight with lysozyme in Method 2 (18 hours) did not significantly increase total DNA yields in Method 1 (30 minutes) ($P=0.08$). Among the mechanical bead beating kits, the food microbial kit (Method 4) produced the highest ($p<0.01$) and the Powerlyzer Microbial kit (Method 3) generated the lowest yields ($P<0.01$). For all kits, a second elution yielded only minor additions to total DNA yield (shown in red, Fig. 1). The Powerlyzer microbial kit also yielded a lower OD260/280 ratio ($\leq$1.66), whereas the other kits yielded ratios close to or above 1.8, indicating good quality DNA. Comparison of the replicates for each kit showed excellent reproducibility for total DNA yield.

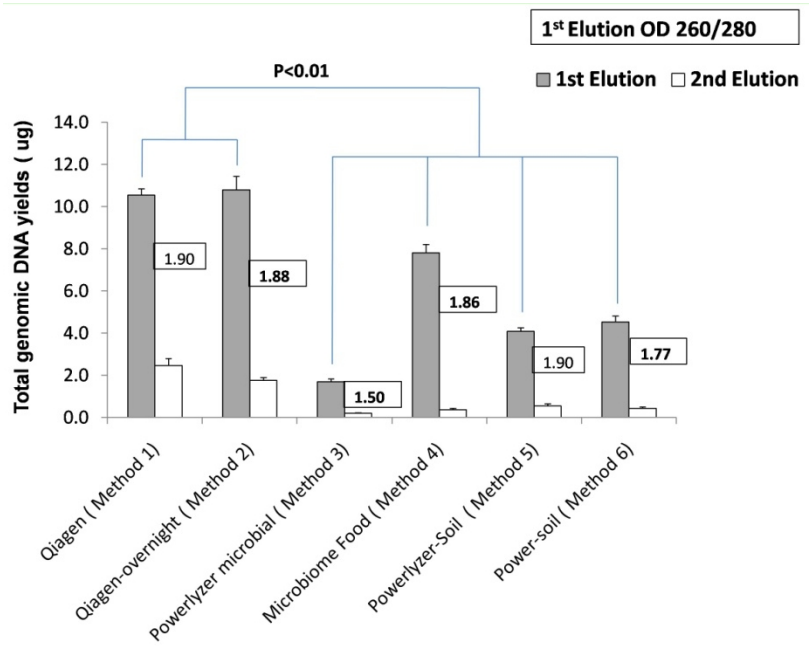

Fig. 1. DNA Yield Amount by Each Kit

DNA were extracted from three identical aliquot oral wash specimens. ANOVA method was conducted to compare the DNA amount differences among the methods. Overall, there was good reproducibility of the DNA recovery of each sample. Qiagen kits had significantly higher yield of DNA ($p<0.01$). The fewest DNA yield amount was from Powerlyzer microbial kit ($p=0.03$). Food microbial kit produced highest DNA among the all Mo Bio microbial kits ($p<0.04$). The DNA amounts existing in second elution buffer were much less than that in first elution buffer. OD260/280 above 1.8 showing the obtained DNA were clean

3.2 Proportion of Microbial DNA to Human DNA

Across the kits and kit replicates for the single pooled subject samples, the ratio of 16s rRNA gene to human GAPDH gene is greatest for the Powerlyzer Microbial DNA kit (Method 3) (Fig. 2. $p<0.05$). There are no differences among other methods.

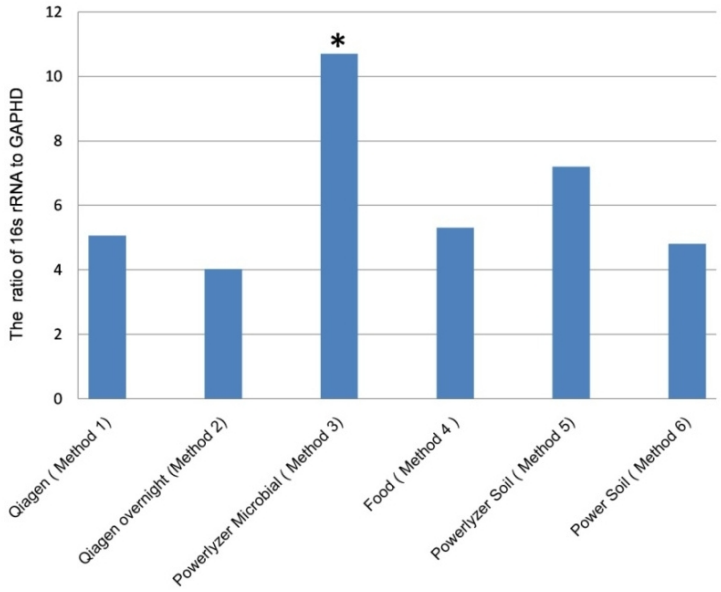

Fig. 2. Proportion of Microbial DNA to Human DNA

The amount of 16s rRNA gene from same amount of DNA extracted by six methods were compared with same sample's human housekeeping gene. ANOVA shows 16S Rrna gene expression from Powerlyzer Microbial DNA kit (Method 3) was significantly higher than other methods 16S rRNA gene (p=0.01). Other methods' 16S rRNA expressions are not significantly different

3.3 16S Sequencing Assay Based Microbial Diversity

Analyzable sequence reads subsequent to Qiime pipeline filtering are shown in Table 2. All extraction kits yielded similar number of sequence reads (>14,000 per sample), similar average size (400-500 bp) and generally identified >8 bacterial phyla, and >50 bacterial genera for each method.

We have examined a global signature of the entire microbial community according to the six different DNA extraction methods. Rarefaction curve (Fig. 3a) indicated no significant differences in richness, assessed by number of OTU (i.e., observed species) in the oral microbiome community structure according to the six different DNA extraction methods ($P\geq0.29$). The Shannon Index was also similar across the six methods ($p\geq0.31$). In order to examine whether each method cluster separately, we performed principle component analysis (PCA) (Fig. 3b). We found that there is no significant cluster based on the methods, which is consistent with rarefaction analysis results (Fig. 3a). In taxonomic analysis, according to the six DNA extraction methods, Firmicutes and Bacteroidetes were the major phyla (average of 59% and 34%, respectively), Streptococcus and Prevotella were the major genera identified across the six extraction methods. There were no significant difference of

the major (more than 1% composition) phylum ($p>0.05$, Fig. 4a.) and genera ($p>0.05$, Fig. 4b.) between the six methods.

Table 2. 16s rRNA Sequencing Informatics

Method	Sequence Reads	Average Size	Phyla Number	Genera Number
Qiagen / Method 1	16707	416	10	57
Qiagen / Method 1	18090	416	10	67
Qiagen Overnight / Method 2	18846	422	10	49
Qiagen Overnight / Method 2	20037	416	9	70
Powerlyzer Microbial / Method 3	17693	416	8	72
Powerlyzer Microbial / Method 3	19947	413	8	62
Food Microbial / Method 4	18630	420	10	50
Food Microbial / Method 4	20306	415	10	67
Powerlyzer Soil / Method 5	17511	413	10	66
Powerlyzer Soil / Method 5	18443	421	11	52
Power Soil / Method 6	18436	418	9	57
Power Soil / Method 6	14364	423	11	52
Average	18250	417	10	60

Sequence reads and obtained phyla and genera numbers of each sample from various DNA extraction kits. All extraction kits had good reproducibility (n=2), and DNA extraction kits did not strong affect each sample's phyla number ($p\geq0.99$) and genera number (and $p\geq0.136$)

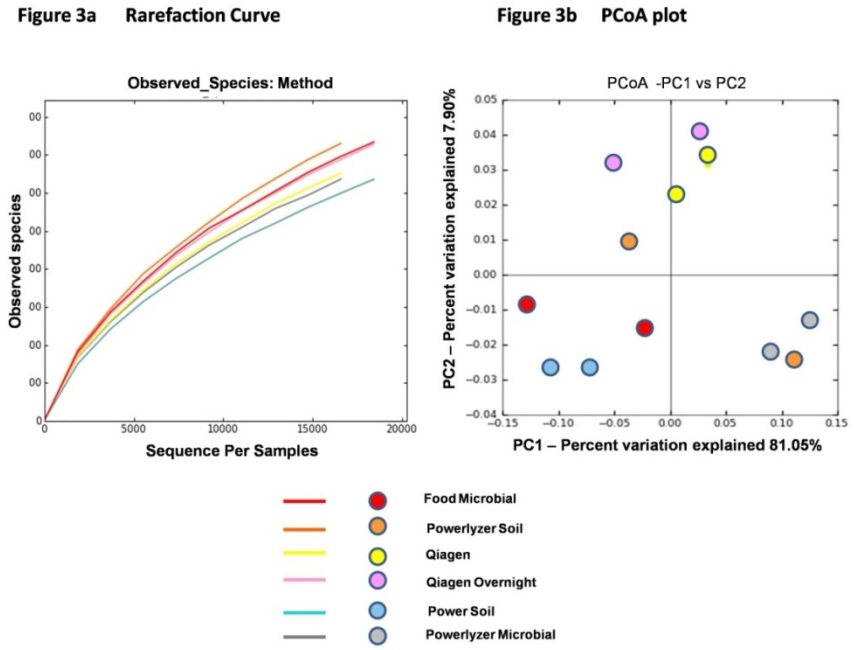

Fig. 3. 16s rRNA Pyrosequencing Results

Fig. 3a: Rarefaction curve showing number of operational taxonomic unit richness (OTUs, observed species) by each DNA extraction method. Fig. 3b. PCoA plots based on weighted UniFrac according to the 6 different DNA extraction methods. Figs. 3a and 3b suggest that DNA extraction methods did not have a significant influence on overall bacterial community composition

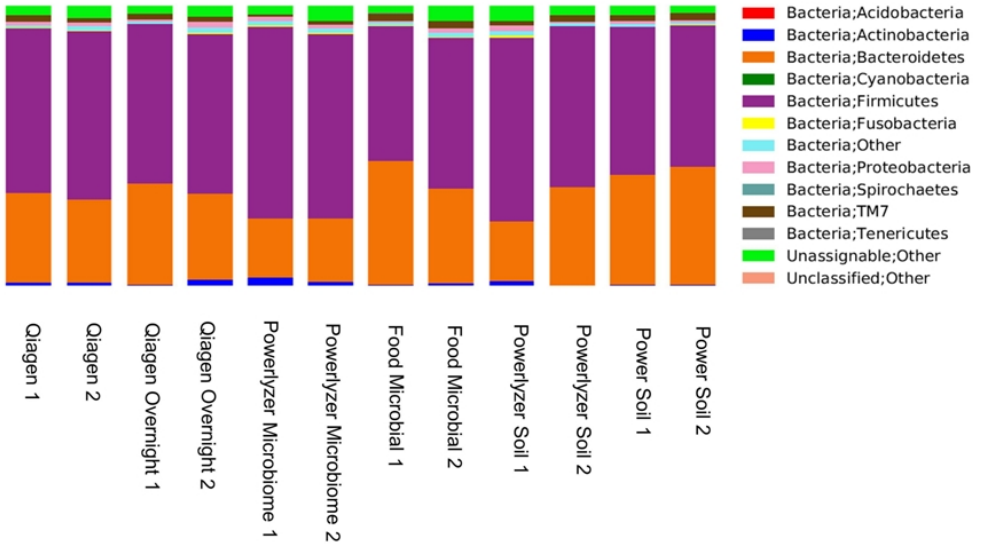

Fig. 4a. Relative Abundances of Phyla

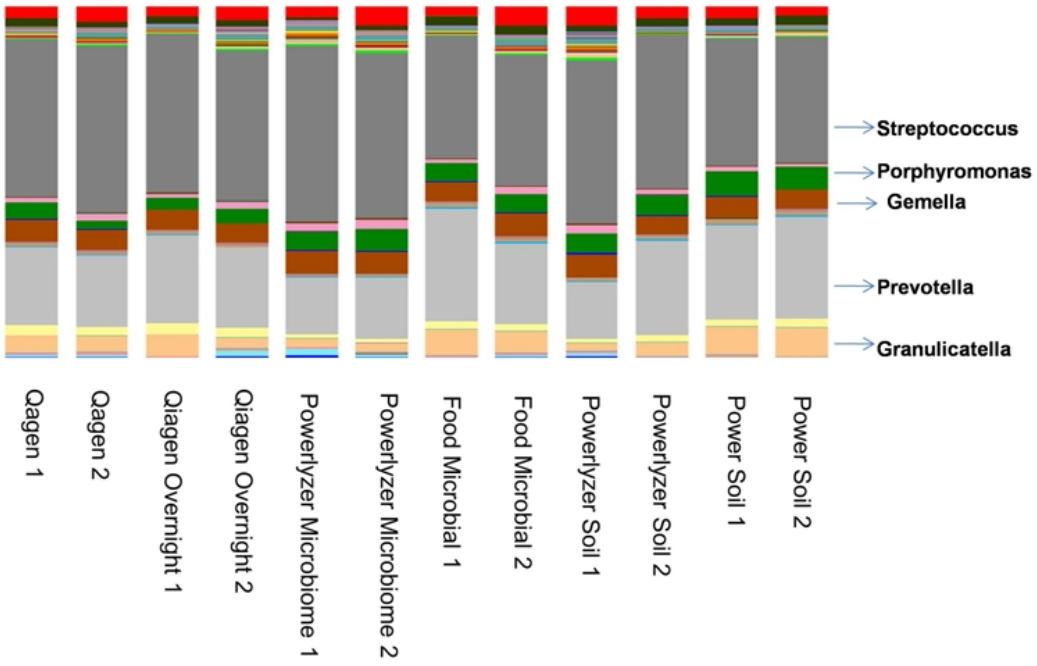

Fig. 4b. Relative Abundances of Genera

Fig. 4. Taxonomic Composition IN Each Method

Fig. 4. is the composition of Phylum (Fig. 4a) and Genera (Fig. 4b) obtained from each method. Kruskal Willis test results indicate there were no significant difference of the majority (more than 1% composition) phylum ($p>0.05$) and genera ($p>0.05$)

5. DISCUSSION

In this study, we compared six different methods for microbial DNA extraction of oral mouth wash specimens for microbiome analysis. We found that total DNA recovery tended to be greater for the enzymatic (Qiagen) than for the mechanical bead beating approach (Mo Bio). There are several reasons why the enzymatic approach yielded greater amounts of total DNA. The Qiagen procedure involves transfer of the all digested specimen directly to the silica column while the Mo Bio extraction methods cannot avoid the DNA contained solution remaining between beads. Mo Bio kits also involve multiple transfer steps to remove PCR *inhibitor, non-DNA organic and inorganic material including humic substances, cell debris,* and proteins, leading to possible DNA losses. In particular, the Mo Bio Food and Soil kits use only a portion of the sample, directly resulting in lower yields. Other considerations are the differential adherence of garnet beads (used for Power food kits) and small glass beads (used for Powerlyzer soil kit). All kits except the Powerlyzer microbial kit (Method 3) showed good DNA purity, based on OD 260/280 above 1.8. The poorer OD260/280 result for the Powerlyzer microbial kit may be related to lower DNA concentration, and cause lower absorbance at 260nm, and therefore affecting calculation of the ratio value.

Although total DNA yields varied between kit types, the relative yield of microbial to human DNA from Powerlyzer microbial kit (method 3) tended to be higher than all other five types of kits. It has been reported that achieved microbial composition is mainly affected by the efficiency of cell lysis instead of DNA recovery [23,24,25]. The Mo Bio mechanical bead beating kits are recommended for the ability of mechanical bead beating to physically break down the bacterial cell wall. This may be particularly important in analysis of the total microbiome because gram positive cell walls are thicker and may be more resistant to enzymatic degradation than the cell walls of gram negative bacteria, potentially leading to artifactual differentials in bacterial type identification from non-mechanical methods (http://www.mobio.com/images/custom/file/pdf/12255_apnote_web.pdf). Normalized to human GAPDH gene, as an indicator of human DNA extraction efficiency, our experiments showed that both the enzymatic and mechanical bead beating methods tended to yield substantial and roughly equivalent amounts of bacterial 16S rRNA gene product except Powerlyzer microbial kits (Method 3). For the Powerlyzer microbial DNA kit (Method 3), which yielded the lowest total DNA amount, compared to other approaches, but achieved the greatest microbial to human DNA yield. Higher bacterial DNA extraction efficiencies by this kit could be attributed to mechanical disruption of microbial cell walls with thick layers of peptidoglycan by bead-beating [26], which was also found in other studies [24,27,28], particularly in relation to the smaller size beads in the Powerlyzer microbial kit [26], as small beads can destroy the thick gram positive cell wall more efficiently than larger beads.

Although substantial differentials were observed in total DNA yield and minor differentials were noted in microbial to human DNA extraction ratios, we found that microbiome structure and composition of oral wash specimens did not differ significantly by DNA extraction method. The Shannon Index was similar across the six methods, indicating that microbial diversity was unrelated to extraction method, as also supported by UniFrac and Principle Component Analysis. We also tested some common oral bacteria species by real time PCR method, and do not find differences of these species' expression among six methods (data not shown). Findings suggest that all six DNA extraction methods generate comparable results for microbiome analysis.

Our study is the first to evaluate DNA extraction kits for relative utility in oral microbiome analysis. Because we used a pooled and homogenized sample from one study subject,

observed differentials are not likely due to sampling variability. We also used an adequate sample amount and reduced number of PCR cycles to improve the specificity of the PCR reaction. Therefore, observed differentials are likely due to effectiveness of cell lysis and other aspects of the extraction procedure. A limitation of our approach is that we cannot compare absolute extraction efficiency as can be carried out using a mock microbial community, however; the oral wash specimen mimics the complexity of sampling in human studies. Additional considerations for selecting a DNA extraction kit in epidemiologic investigations of the oral microbiome include cost and technical feasibility, as indicated in Table 1. Qiagen kits require more user-made buffer, requiring preparation time and potentially introducing a source of contamination, while Mo Bio kits for microbial DNA isolation involve multiple transfers between tubes, which may also introduce contamination[29], and for large numbers of samples require additional equipment such as the Mo Bio Powerlyzer or vortex adapter [29].

In summary, enzymatic and mechanical bead beating kits provide alternative approaches for DNA extraction of oral microbiome DNA from oral wash samples. Greater total DNA yields are found in enzymatic approaches but microbial relative abundances can be similarly well characterized by either enzymatic or mechanical bead beating approaches.

CONSENT

All authors declare that “written informed consent” was obtained from the participant for publication of this result.

ETHICAL APPROVAL

All authors hereby declare that all experiments have been examined and approved by the New York University IRB and have therefore been performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki.

ACKNOWLEDGEMENTS

Grant support: National Cancer Institute (R03 CA159414), 2012 Pancreatic Cancer Action Network-AACR Career Development Award supported by the Daniel and Janet Mordecai Foundation (12-20-25-AHN).

COMPETING INTERESTS

Authors have declared that no competing interests exist.

REFERENCES

1. Ahn J, Chen C, Hayes RB. Oral Microbiome and Oral and Gastrointestinal Cancer risk. Cancer Causes Control. 2012;23:399-404.
2. Dewhirst FE, Chen T, Izard J, Paster BJ, Tanner AC, et al. The human oral Microbiome. J Bacteriol. 2010;192:5002-5017.
3. Turnbaugh PJ, Ley RE, Hamady M, Fraser-Liggett CM, Knight R, et al. The Human Microbiome Project. Nature. 2007;449:804-810.
4. Huse SM, Ye Y, Zhou Y, Fodor AA. A Core Human Microbiome as Viewed Through 16S rRNA Sequence clusters. 2012;7:34242.

5. Human Microbiome Project: Structure, function and diversity of the healthy human microbiome. Nature. 2012;486:207-214.
6. Turnbaugh PJ, Ley RE, Mahowald MA, Magrini V, Mardis ER, et al. An Obesity-associated Gut Microbiome with Increased Capacity for Energy Harvest. Nature. 2006;444:1027-1031.
7. Castellarin M, Warren RL, Freeman JD, Dreolini L, Krzywinski M, et al. Fusobacterium nucleatum infection is prevalent in human colorectal carcinoma. Genome Res. 2012;22:299-306.
8. Kostic AD, Gevers D, Pedamallu CS, Michaud M, Duke F, et al. Genomic Analysis Identifies Association of Fusobacterium with Colorectal Carcinoma. Genome Res. (2012);22:292-298.
9. Yang L, Ganly I, Morris L, Palmer F, Deng H, et al. Relevance of Microbiome to Cigarette Smoking and oral cancer. J Dent Res abstract; 2012.
10. Morgan XC, Tickle TL, Sokol H, Gevers D, Devaney KL, et al. Dysfunction of the Intestinal Microbiome in Inflammatory Bowel Disease and Treatment. Genome Biol. 2012;13:79.
11. Paju S, Pussinen PJ, Suominen-Taipale L, Hyvonen M, Knuuttila M, et al. Detection of Multiple Pathogenic Species in Saliva is Associated with Periodontal Infection in adults. J Clin Microbiol. 2009;47:235-238.
12. Von Wintzingerode F, Gobel UB, Stackebrandt E Determination of Microbial Diversity in Environmental Samples: pitfalls of PCR-based rRNA analysis. FEMS Microbiol Rev. 1997;21:213-229.
13. Garcia-Closas M, Egan KM, Abruzzo J, Newcomb PA, Titus-Ernstoff L, et al. Collection of genomic DNA from adults in epidemiological studies by buccal cytobrush and mouthwash. Cancer Epidemiol Biomarkers Prev. 2001;10:687-696.
14. Gao Z, Tseng CH, Pei Z, Blaser MJ. Molecular analysis of human forearm superficial skin bacterial biota. Proc Natl Acad Sci U S A. 2007; 104:2927-2932.
15. Vandesompele J, De Preter K, Pattyn F, Poppe B, Van Roy N, et al. Accurate Normalization of Real-time Quantitative RT-PCR data by Geometric Averaging of Multiple Internal control genes. Genome Biol 3; 2002.
16. Caporaso JG, Lauber CL, Walters WA, Berg-Lyons D, Lozupone CA, et al. Global patterns of 16S rRNA diversity at a depth of millions of sequences per sample. Proc Natl Acad Sci USA. 108;1:4516-4522.
17. Haas BJ, Gevers D, Earl AM, Feldgarden M, Ward DV, et al. Chimeric 16S rRNA sequence formation and detection in Sanger and 454-pyrosequenced PCR amplicons. Genome Res. 21:494-504.
18. Edgar,RC Search and Clustering Orders of Magnitude Faster than BLAST. Bioinformatics. 2010;26:2460-2461.
19. Caporaso JG, Bittinger K, Bushman FD, DeSantis TZ, Andersen GL, et al. PyNAST: A Flexible tool for Aligning Sequences to a Template Alignment. Bioinformatics. 26;266-267.
20. DeSantis TZ, Hugenholtz P, Larsen N, Rojas M, Brodie EL, et al. Greengenes, a chimera-checked 16S rRNA Gene Database and Workbench Compatible with ARB. Appl Environ Microbiol. 2006;72:5069-5072.
21. Cole JR, Chai B, Farris RJ, Wang Q, Kulam SA, et al. The Ribosomal Database Project (RDP-II): sequences and tools for high-throughput rRNA analysis. Nucleic Acids Res. 2005;33:294-296.
22. Lozupone C, Knight R. UniFrac: a new phylogenetic method for comparing microbial communities. Applied and Environmental Microbiology. 2005;71:8228-8235.

23. Anderson KL, Lebepe-Mazur S. Comparison of rapid methods for the extraction of bacterial DNA from colonic and caecal lumen contents of the pig. J Appl Microbiol 2003;94:988-993.
24. Li F, Hullar MA, Lampe JW. Optimization of Terminal Restriction Fragment Polymorphism (TRFLP) Analysis of Human gut Microbiota. J Microbiol Methods. (2007);68:303-311.
25. Salonen A, Nikkila J, Jalanka-Tuovinen J, Immonen O, Rajilic-Stojanovic M, et al. Comparative Analysis of fecal DNA Extraction Methods with Phylogenetic Microarray: Effective Recovery of Bacterial and Archaeal DNA using Mechanical cell lysis. J Microbiol Methods. 2010;81:127-134.
26. Yuan S, Cohen DB, Ravel J, Abdo Z, Forney LJ. Evaluation of methods for the extraction and purification of DNA from the human microbiome. 2012;7:33865.
27. Ariefdjohan MW, Savaiano DA, Nakatsu CH Comparison of DNA extraction kits for PCR-DGGE analysis of human intestinal microbial communities from fecal specimens. Nutr J. 9;23.
28. Nylund L, Heilig HG, Salminen S, De Vos WM, Satokari R. Semi-automated extraction of microbial DNA from feces for qPCR and phylogenetic microarray analysis. J Microbiol Methods. 83;231-235.
29. Willner D, Daly J, Whiley D, Grimwood K, Wainwright CE, et al. Comparison of DNA extraction methods for microbial community profiling with an application to pediatric bronchoalveolar lavage samples. 7;34605.

Peer-review history:
The peer review history for this paper can be accessed here:
http://www.sciencedomain.org/review-history.php?iid=403&id=12&aid=3320