Blood vs. saliva: analysis of the effect of sample type on variant calling confidence for human Whole Genome Sequencing

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Introduction

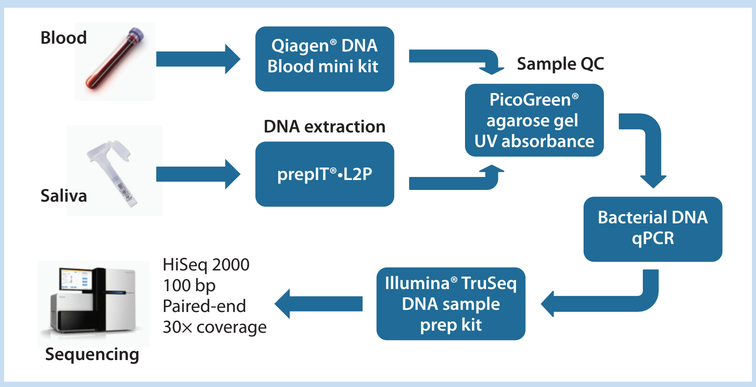
Saliva collected using the Oragene® self-collection kit is a non-invasive alternative to blood as a source of large amounts of high quality genomic DNA. Oragene enables large-scale population studies by improving donor access and compliance, and its utility has been well-documented in over one thousand peer-reviewed publications. However, data on the performance of DNA from saliva in Whole Genome Sequencing is scarce in the existing literature.

In this study, we present a systematic, multi-sample analysis of the effect of sample type (blood vs. saliva) on variant calling confidence and the effect of bacterial DNA in saliva on sequence alignment.

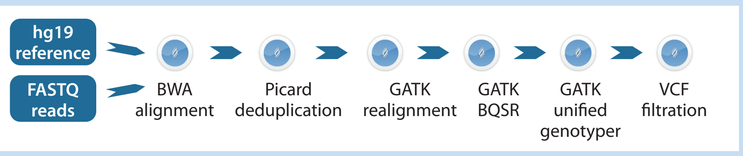
Materials and methods

Sample collection: Blood and saliva samples were collected from each member of two families using K-EDTA tubes and Oragene self-collection kits, respectively. These particular study participants were selected because the bacterial DNA content in the saliva samples (determined by 16S qPCR) ranged from below average to significantly above average. Four and three blood/saliva pairs were obtained from family 1 and 2, respectively.

Sample preparation and sequencing: Standard sample preparation protocols were used to extract and quantify DNA, and to prepare TruSeq libraries for sequencing on the Illumina HiSeq 2000. Samples from Family 2 were prepared and sequenced in duplicate to provide technical replicates. All 20 prepared libraries were sequenced to a target coverage of 30×.



Data analysis: Variants were called from the sequencing reads on the Seven Bridges platform for bioinformatics analysis using a BWA+GATK pipeline conforming to the Broad Institute's best-practices recommendations. Reads were aligned to the hg19/B37 reference and all called variants were filtered using hard filters set according to Broad Institute's recommendations.

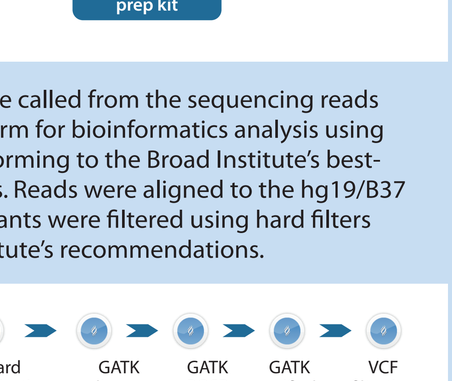


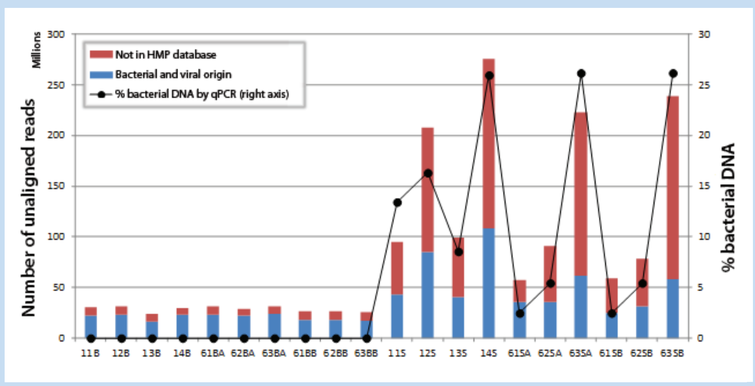
To determine if unaligned reads in blood and saliva samples were of bacterial origin, they were aligned to sequences contained in the Human Microbiome Project (HMP)[1] database using BWA MEM 0.7.4.

Results

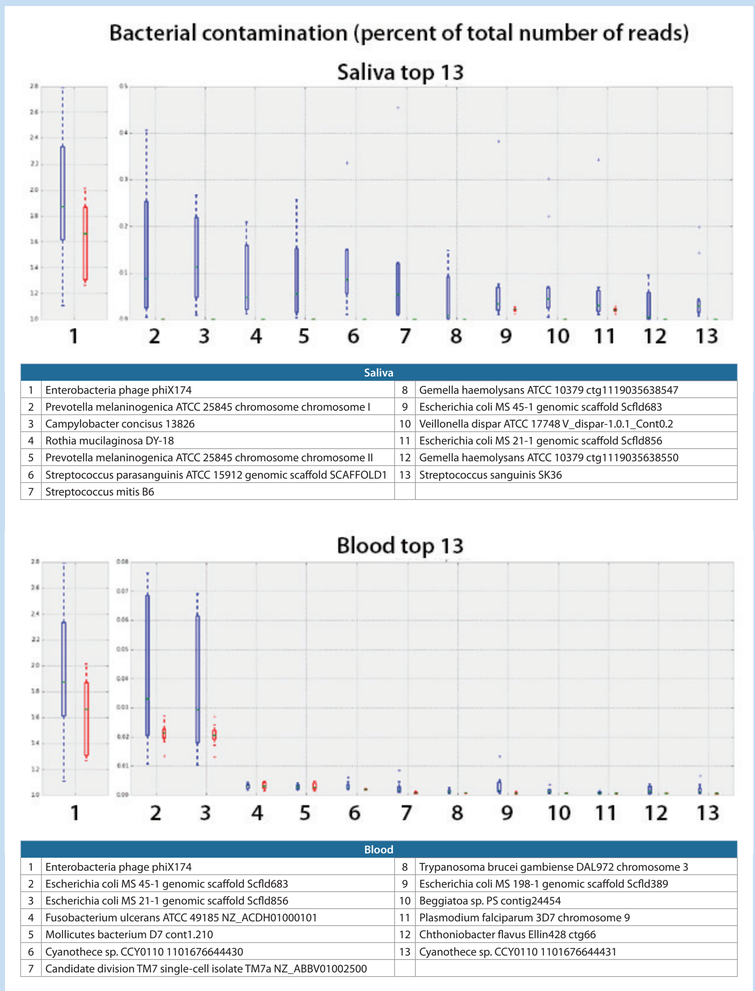
Bacterial DNA content in the samples correlates very closely with the number of bases (reads) that align to the hg19 reference, having a Pearson correlation coefficient of 0.9731. This indicates that the bacterial DNA content of a sample has a linear effect on sequencing coverage.

Reads that did not map to the hg19 reference were aligned to the HMP database. An average of 37% of the unaligned reads in saliva are of bacterial or viral origin while blood reads were higher at 72%, on average. Assembly of the reads that did not align to either hg19 or HMP revealed contigs that resemble bacteria and some unknown organisms previously identified in gut and soil samples. The presence of sequences from such organisms suggests that the HMP database is incomplete.





To quantify the amount of different bacteria in each sample the number of reads aligning to each bacterial genome was expressed as a percentage of the total number of reads in the sample. The figure below shows the number of reads originating from the top 13 viruses and bacteria found in saliva and blood.

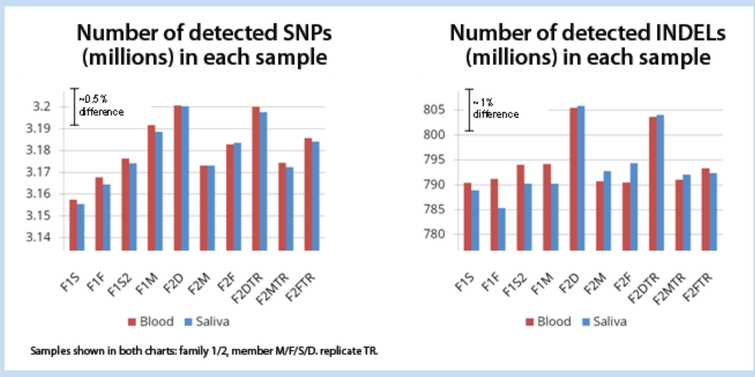


Saliva			
1	Enterobacteria phage phiX174	8	Gemella haemolysans ATCC 10379 ctg1119035638547
2	Prevotella melaninogenica ATCC 25845 chromosome chromosome I	9	Escherichia coli MS 45-1 genomic scaffold Scfld683
3	Campylobacter concisus 13826	10	Veillonella dispar ATCC 17748 V_dispar-1.0.1_Cont0.2
4	Rothia mucilaginosa DY-18	11	Escherichia coli MS 21-1 genomic scaffold Scfld856
5	Prevotella melaninogenica ATCC 25845 chromosome chromosome II	12	Gemella haemolysans ATCC 10379 ctg1119035638550
6	Streptococcus parasanguinis ATCC 15912 genomic scaffold SCAFFOLD1	13	Streptococcus sanguinis SK36
7	Streptococcus mitis B6		

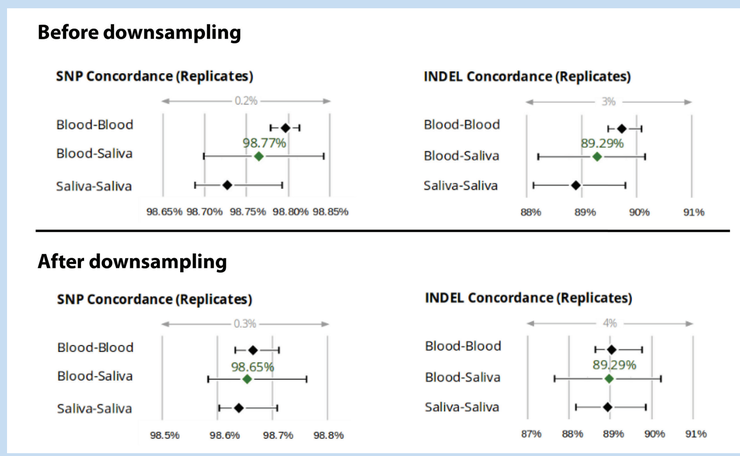
Blood			
1	Enterobacteria phage phiX174	8	Trypanosoma brucei gambiense DAL972 chromosome 3
2	Escherichia coli MS 45-1 genomic scaffold Scfld683	9	Escherichia coli MS 198-1 genomic scaffold Scfld389
3	Escherichia coli MS 21-1 genomic scaffold Scfld856	10	Beggiatoa sp. PS contig24454
4	Fusobacterium ulcerans ATCC 49185 NZ_ACDH01000101	11	Plasmodium falciparum 3D7 chromosome 9
5	Mollicutes bacterium D7 cont1.210	12	Chthoniobacter flavus Ellin428 ctg66
6	Cyanothece sp. CCY0110 1101676644430	13	Cyanothece sp. CCY0110 1101676644431
7	Candidate division TM7 single-cell isolate TM7a NZ_ABBV01002500		

The most significant contributor of reads (2.0% and 2.8% in blood and saliva respectively) was the Enterobacteria phage PhiX174. During preparation for sequencing, this virus is added to each sample as part of Illumina's preparation protocol to improve calibration and quality control.[2] The remaining bacterial/viral sequences are present in much lower amounts (<0.5% for saliva and <0.08% for blood), and most of the species found in saliva are known inhabitants of the mouth. The presence of bacterial sequences in blood (for example, those from E. coli) are likely due to contamination during sample or library preparation and similar contamination is also present in saliva samples.

No significant difference in the total number of variants (SNPs and INDELs) called from blood and saliva samples was observed. The average differences in SNP and INDEL counts were 0.06% and 0.30%, respectively.



Concordance of SNPs and indels between blood replicates, saliva replicates and between blood-saliva pairs is generally very high, however a small, systematic difference between blood and saliva can be observed. In order to determine if the concordance difference was due to coverage differences, the blood samples were downsampled to a coverage equal to that of the saliva samples. Once differences in coverage were accounted for, the average SNP and indel concordances for replicates are within 0.05% and 0.25%, of each other respectively.



In order to check if there are any regions of the human genome which were enriched with bacterial reads, human reference-aligned reads were also aligned to the HMP reference. All reads not aligning to both hg19 and HMP were discarded and a moving average coverage was calculated per base with a 100 bp window. A region was classified as enriched if a 20× average coverage was observed. These regions were inspected for the following things to identify potential bacterial contamination:

- Unusually high mismatch ratio (# mismatches in reads/total bases in region)
- Existence of HMP-enriched regions detected only in saliva samples
- High ratio of alignments with map quality zero
- Unusually low concordance between blood and saliva

Manual inspection of regions falling into one or more of the above categories revealed that none of them showed contamination with bacterial reads. Although this is not conclusive proof that there is no bacterial read contamination, it nonetheless provides confidence that bacterial reads do not accumulate enough to affect the overall mutation calling quality.

Conclusions

The amount of bacterial DNA in a saliva sample and the number of reads that do not align to the human reference are closely correlated. However, the coverage loss due to bacterial DNA is relatively small, with coverage dropping approximately 3% for every 5% bacterial DNA in the sample.

The majority (72%) of the unaligned reads in blood aligned to the HMP database indicating that the source of these sequences is indeed bacterial or viral. In saliva, this metric was lower (37%), however many of the remaining unmapped reads showed similarity to other bacterial/viral species not found in the HMP, suggesting that other likely environmentally derived, species are present in the oral cavity.

In spite of the reduced coverage due to the presence of bacterial DNA in the saliva samples, there was no significant difference in the number of SNPs and indels called. The differences in concordance between replicates and saliva/blood pairs was virtually eliminated when blood data was downsampled to a coverage equal to that of saliva, suggesting that coverage differences are, by far, the most significant reason for differences in concordance between sample types.

Finally, a close inspection of HMP-enriched regions of the genome revealed that it is likely that bacterial reads do not accumulate enough to affect mutation calling.

References

1. NIH Human Microbiome Project. http://hmpdacc.org/HMREFG
2. Using a PhiX Control for HiSeq Sequencing Runs. Illumina Inc. March 2013. http://res.illumina.com/documents/products/technotes/technote_phixcontrolv3.pdf

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