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An assessment of Oxford Nanopore sequencing for human gut metagenome profiling: A pilot study of head and neck cancer patients

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ABSTRACT

Gut metagenome profiling using the Oxford Nanopore Technologies (ONT) sequencer was assessed in a pilot-sized study of 10 subjects. The taxonomic abundance of gut microbiota derived from ONT was comparable with Illumina Technology (IT) for the high-abundance species. IT better detected low-abundance species through amplification, when material was limited.

1. Introduction

Head and neck cancer (HNC) constitutes approximately 4% of all new cancer reported in United States with almost 50,000 new cases in 2017 (Siegel et al., 2017). The known risk factors for HNC are usage of tobacco or alcohol, infection status of human papillomavirus (HPV) or Epstein-Barr virus (EBV), and poor oral hygiene (Pezzuto et al., 2015). Recently, Hayes et al. (Hayes et al., 2018) reported the association of oral microbiome with the risk for incident head and neck squamous cell cancer, which is the most frequent malignant tumor in HNC. They reported an association between abundant *Corynebacterium* or *Kingella* with decreased cancer risk. Many other studies also found associations of oral microbiome with HNCs (Galvao-Moreira and da Cruz, 2016; Lim et al., 2018; Shin et al., 2017; Wolf et al., 2017).

Recently United States Food and Drug Administration (FDA) have approved immuno-therapy for use in treatment of cancer patients with 10 tumor types (Sharma et al., 2017) include HNC. HPV therapeutic vaccines are in development, and may potentially be used to treat HNC in the future (Coleman et al., 2016; Wang et al., 2018). New studies showed that the gut microbiome plays an important role in host immune system functions are associated with dysbiosis, affecting oncogenesis as well as anticancer therapy (Sharma et al., 2017; Zitvogel et al., 2015; Zitvogel et al., 2018). Some commensal bacteria have reduced the efficacy of immunotherapy; for example, the gut microbiome modulates immunotherapy responses in melanoma patients (Gopalakrishnan et al., 2018a, 2018b; Matson et al., 2018). The knowledge surrounding the association between the gut microbiome and HNCs and vaccination therapy is still limited and needs further investigation.

The whole shotgun metagenome sequencing approach has multiple advantages in taxonomic resolution and gene function, as compared to the 16S amplicon sequencing approach (Ranjan et al., 2016). Traditionally, shotgun metagenome sequencing employs short-read sequencing technologies to generate deep sequencing data that can capture and identify taxonomic diversity and gene functions (Quince et al., 2017). Recently, Oxford Nanopore technologies (ONT), release a mini-sequencer, MinION, that has advantages over short-read sequencing, including long-read output and portable rapid real-time sequencing with no fixed or maintenance costs. With these capabilities, we believe that the technology can be further adopted for clinical metagenomic

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0167-7012/

Table 1
Demographic and clinical details of HNC participants (n = 10).

Characteristics		Patient
Gender	Female	2
	Male	8
Age (Mean ± SD)		61.42 ± 7.26
On antibiotics	No	6
	Yes	3
	Unknown	1
Smoking	Never	2
	Former	4
	Current	2
Alcohol	Never	4
	Former	3
	Current	3
Location of cancer	Oral	5
	Oropharynx	3
	Others	2
Disease stage	II	1
	III	2
	IV	7
Treatment	Chemo, radiation, surgery	5
	Chemo, radiation	5
P16 status[a]	Negative	5
	Positive	2
	Unknown	3

[a] P16 staining is a surrogate marker for HPV status.

research.

In this pilot study, we assessed the feasibility of ONT sequencing to confidently assign species-level taxonomy for the gut microbiome. Whole shotgun metagenome sequencing of fecal samples obtained from 10 HNC patients was performed using ONT, and the results were compared with those obtained using Illumina Technology (IT).

2. Methods

Written informed consent was obtained from the participants before collecting samples (IRB-approved: 217267, University of Arkansas for Medical Sciences). Demographic and clinical details are shown in Table 1. Fecal samples were collected in the OMNIgene.GUT tubes (DNA Genotek, Canada). DNA extraction of the samples was accomplished using ZymoBIOMICS DNA Kits (Zymo Research, USA). The extracted DNA for individual samples was aliquoted for ONT and IT sequencing to reduce technical variations.

ONT sequencing library preparation was performed following the 1D Genomic DNA sequencing (SQK-LSK108) protocol (ONT, USA). An R9.4/FLO-MIN106 flow cell on a MinION Mk1B sequencer was used for sequencing of the library. The raw fast5 files were base-called using Albacore version 2.1.3 to generate fastq files. The IT library preparation was prepared with the KAPA HyperPlus kit (Roche, USA) following the manufacturer's protocol (Biosystems, 2015). We employed NexSeq 500 (Illumina, USA) for sequencing the library.

The raw fastq files derived from ONT and IT sequencing were pre-processed before taxonomic profiling. The mean quality score of 7 and read length longer than 200 bp were used to filter the ONT fastq using our in-house script (Jenjaroenpun et al., 2018), and trimming adaptors using porechop version 0.2.3. For Illumina fastq, adapter trimming and quality filtering were performed by Trimmomatic software version 0.36 (Bolger et al., 2014) with default parameters. The filtered fastqs obtained from the both sequencing technologies were used as the inputs for taxonomic classification and quantification using Centrigue software (Kim et al., 2016) version 1.0.4 with default parameters to generate species tables, based on the reference database of non-redundant prokaryote species provided with the software. Due to differences in sequencing depth between IT and ONT, rare fraction analyis was performed before further analysis using the *phyloseq* package (McMurdie and Holmes, 2013). The tables were then visulized on a taxonomic hierarchy using the *pavian* package and were compared diversity using the *phyloseq* package (McMurdie and Holmes, 2013).

3. Results and discussion

The amounts of sequencing data generated and read lengths from the shotgun metagenome by IT and ONT are summarized in the Fig. 1A. The sequencing chemistry of IT includes amplification steps; therfore, the number of reads and amount of sequencing data were much higer with IT than with ONT sequencing, which does not amplify the DNA molecules in the sample. With the limited amount of input DNA (see Supplementary Table S1) which was less than the amount (~1500 ng) recommended by the SQK-LSK108 protocol, high sequencing depth could not be achieved. Nevertheless, ONT produced much longer mean read length with the longest read length longer than 30 kb on average. Using the reference genome based approach, Centrifuge software generated a species table containing ~2300 known prokaryal species (at least one mapped read detected in at least one sample, see Supplementary Table S2 for detail). The microbiome obtained from IT and ONT were compared as a bar plot of relative abundance (Fig. 1B). We found 57 species considered highly abundant (> 0.5% relative abundance) in the cohort as illustrated in the Fig. 1C. The high-abundance species, such as *Bacteroides sp., Eubacerium rectale, Escherichia coli, Lachclostridium sp., Alipstipes finegoldii, Flavonifractor plautii* and *Akkermansia muciniphila*, were previously reported by others (Karlsson et al., 2013; Li et al., 2014; Lloyd-Price et al., 2017). The within-sample similarity of gut microbiome profiles derived from ONT and IT was observed in Sankey diagrams for combined samples (Fig. 2A and B) and other patients (Supplementary Figure). The alpha diveristy based on the Simpson Index of diversity of individual samples is shown in Fig. 2C. The diversity of gut microbiome profile derived from ONT and IT were comparable. IT gave a higher richness than ONT, because of the higher sequencing depth (see the species richness based on Chao1 method in Supplementary Fig. S2). This subsequently increased the sampling probability of low-abundance species. This was true for all samples except for HNC_07, which had too low a sequencing depth (see Table S1 in details). This strongly indicated a strength of IT is that can amplify the signal of low-abundance species. The beta diversity of each sample and sequencing technologies were calculated and plotted as principal coordinate analysis (PCoA) as shown in Fig. 2D. The plot clearly shows the intrinsic virability of the diversity between samples was higher than within the sample, supporting the reproducibility of microbiome profiling using ONT and IT for high-abundance species. The functional content, important information that can be obtianed from shotgun metagenome (Karlsson et al., 2014), was not compared in this study. Because of the low squencing depth of ONT data in this study, we could not achieve a sufficient consensus accuracy of assembled contigs.

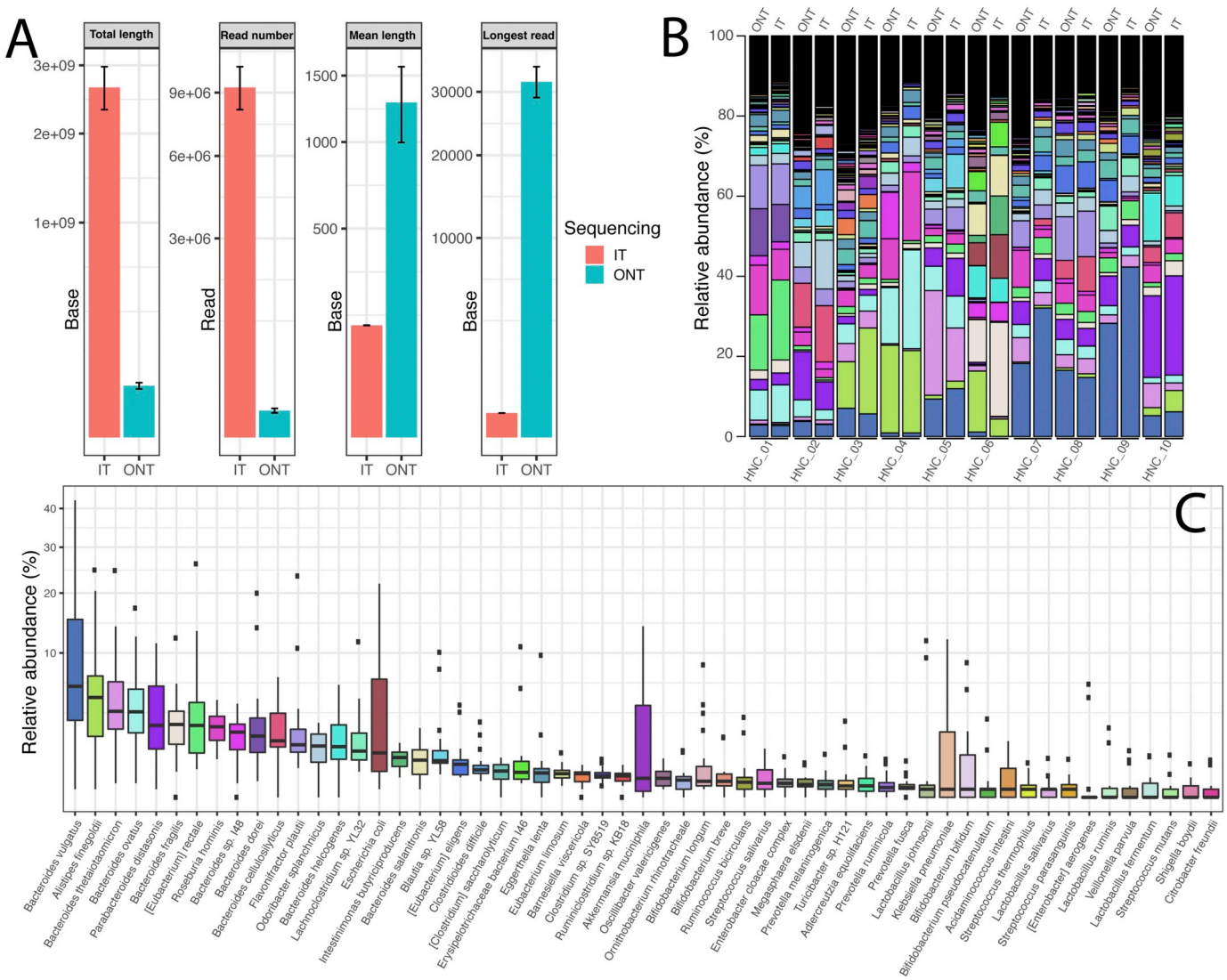

Fig. 1. The sequencing output summary and relative abundance profile of the gut microbiome of HNC patients using IT and ONT. A) Box plots of mean values of output length, number of reads, average read length, and longest read length of IT (red) and ONT (cyan) data. The bars represent standard errors of mean. B) Stacked bar plots showing relative species abundance of individual samples using IT and ONT. The different colors represent different species as shown in panel C. C) Box plots of relative abundance of high-abundance species (> 0.5%) of the cohort with combined IT and ONT data (n = 10; see the plot for individual technology in Supplementary Fig. S1). The boxplot was sorted based on average abundance level of individual taxa across samples from left to right. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

In conclusion, the ONT may be used for taxonomic profile filing of gut microbiome clinically, yielding results comparable with the deep sequencing shotgun metagenomics approach with IT for high-abundance taxa present in the sample. This will be a useful tool for rapid screening of microbiota.

Availability of data

The metagenome data sets of the ten HNC patient samples in this study have been submitted to the GenBank, and are publicly available under the accession number SRR7947168-SRR7947187.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

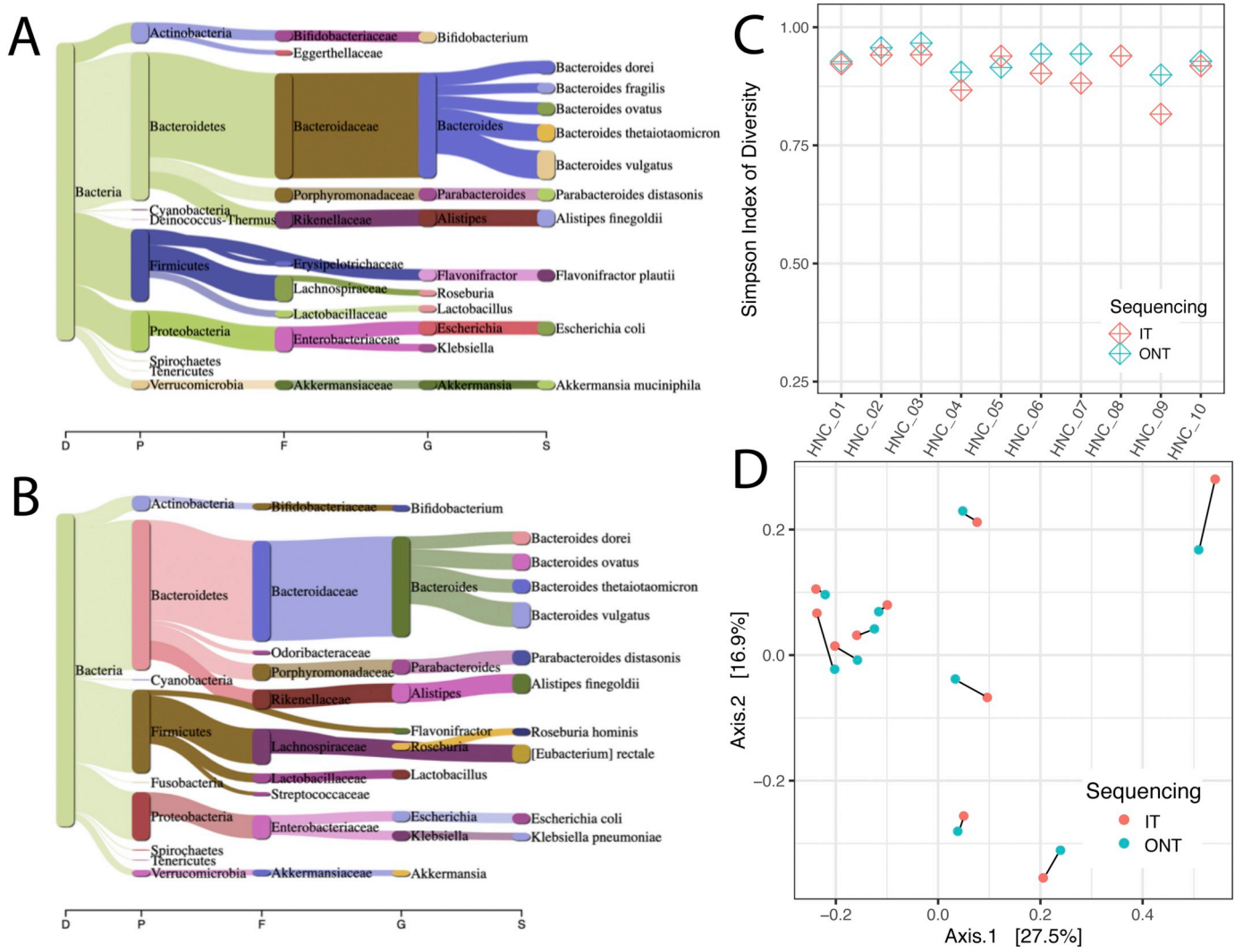

Fig. 2. The profiles of gut microbiome elucidated using IT and ONT are comparable in HNC patients. A) A Sankey diagram shows species abundance in a taxonomic tree of the sample combined using the ONT data. B) The same using the IT data. The Sankey diagram plot of individual subject for both techniques on individual samples are provided in Supplementary Fig. S3 C) Alpha diversity of individual sample based on Simpson index of diversity for IT (red) and ONT (cyan) (see the species richness of individual samples shown using the Chao1 in Supplementary Fig. S2). D) PCoA plot comparing the beta diversity based on Bray-Curtis distant matrices. The black lines link the IT (red) and ONT (cyan) data of the same samples. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.mimet.2019.105739.

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