Comparison of Small RNA Sequencing Profiles of Exosomal RNA Extracted from Plasma, Urine and Saliva

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Introduction:

Liquid biopsies have emerged as an important alternative to tissue biopsies as they carry useful information for detection of the disease but is also a non-invasive or minimally invasive approach to collect sample [(1)]. Exploring the extracellular vesicles (EVs) that are present in the form of exosomes and microvesicles are gaining importance in theirs use in disease diagnosis as they carry important cargo that represent the current physiological condition [(2)]. The cargo, which is in the form of proteins, lipids, RNA including protein coding as well as non-coding RNAs such as micro RNA (miRNA), PIWI-interacting RNA (piRNA) and long non-coding RNA (lncRNA) as well as DNA are known to play an important role in cell-to-cell communication [(2, 3, 4)].

To explore the potential of the EV components as a prognostic or diagnostic marker for several pathological conditions including cancers, infectious and autoimmune diseases is thus important [(2)]. Amongst the cargo present in the extracellular vesicles, characterization of RNA is of highest importance as RNA provides information that is more specific to cell-to-cell communication as well as disease transmission. There has been enough evidence of exosomal RNA to be associated with several diseases such as pancreatic and colon cancer, sarcoma, prostate cancer, lung cancer and sarcomas [(5)].

Amongst all the liquid biopsies, blood (serum and plasma) is one of the most reliable and widely used liquid biopsy. However, blood is slightly invasive as compared to other non-invasive samples such as urine and saliva, which are not only non-invasive but are also easy to collect at the clinic or at home based on the convenience of the patient/donor. However, before employing any sample type as an alternative to a well-established sample, it is important to verify the capabilities alternative sample type to perform diagnosis with the same accuracy and reliability. This is important as the selection of a wrong sample type could lead to inaccurate diagnosis. In the current study, we compare the two most easily available liquid biopsies, Urine and Saliva with the most reliable and well-established liquid biopsy, plasma, to qualify them as an alternative to the gold standard.

Methods:

Blood, urine, and saliva samples were collected from six donors on the same day. Blood was collected on Norgen's cf-DNA/cf-RNA Preservative Tubes (Cat. 63950), urine was collected on Norgen's Urine Collection and Preservation Tubes (Cat. 18113) and Saliva was collected on Norgen's Saliva Exosome Collection and Preservation Kit (Cat. 65400). All the samples types form all 6 donors were preserved for 7 days at room temperature. After the end of incubation, blood from all donors was processed as per manufacturer's specifications and the plasma was separated. Blood, urine and salvia were further processed to make them cell-free by centrifuging at 2500 x g for 10 minutes and the supernatants were stored at -80°C until further use.

All the liquid biopsies from 6 donors were thawed and intact exosomes were extracted from 4 mL plasma using Norgen's Plasma/Serum Exosome Purification Midi kit (Cat. 57500), from 10 mL urine using Norgen's Urine Exosome Purification Midi kit (Cat. 57800), and from 1 mL saliva using Norgen's Saliva Exosome Purification kit (Cat. 65300). Exosomes from all sample types were eluted in 400 µL of ExoR Buffer. An aliquot of 50 µL from each sample was sent to The Hospital for Sick Children (SickKids) where the exosomes were characterized and visualized with the NanoSight LM10 instrument. The rest of the exosomes (150 µL) were used to extract exosomal RNA using EXTRAClean Exosomal RNA Isolation Kit (Cat. 72800). The extracted RNA was quantified and analyzed using RNA 6000 Pico assay using Agilent 2100 Bioanalyzer System (Agilent Technologies, USA) as well as Qubit microRNA assay kit.

Small RNA libraries were constructed from all the exosomal RNA samples using Norgen's Small RNA Library Prep Kit for Illumina (Cat. 63600). Libraries were quantified on the High Sensitivity DNA Analysis Chip using the Agilent 2100 Bioanalyzer System (Agilent Technologies, USA). All the libraries were diluted to 4 nm concentration, pooled and sequenced on the Illumina NextSeq 550 platform using the NextSeq 500/550 High Output Kit v2.5 (75 cycles). Fifty-one cycles were used for the sequencing run.

Results and Discussion:

The Nanoparticle Tracking Analysis (NTA) data from the NanoSight NS300 instrument revealed that plasma showed the least exosome concentration (5.51E+08 particles/mL) as compared to urine (1.35E+09 particles/mL) and saliva (7.82E+08 particles/mL) (Figure 1).

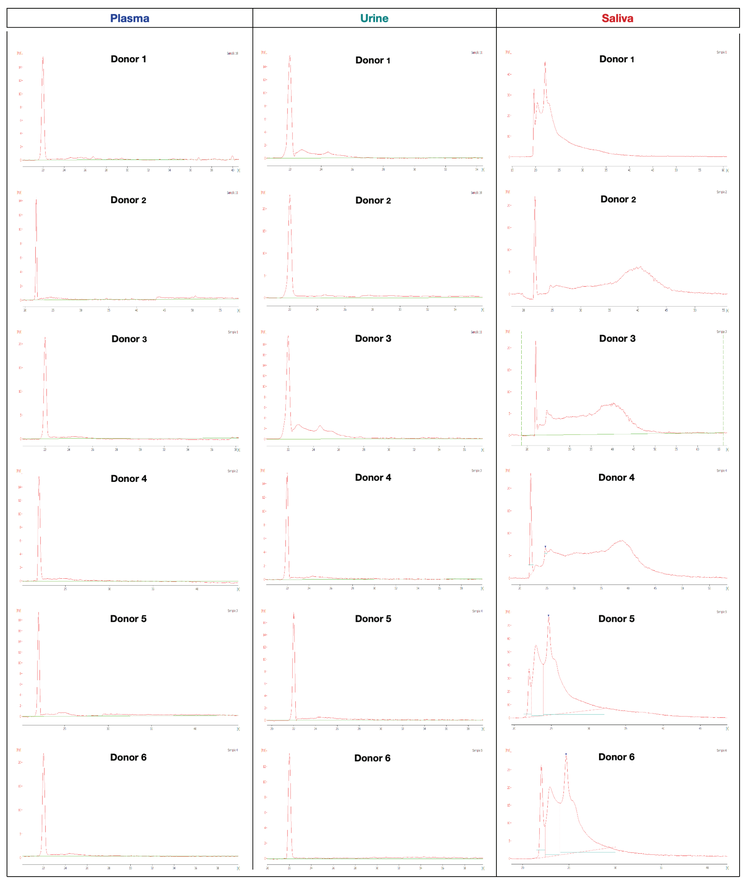

Figure 1. Representative image of quality and quantity analysis of exosomal RNA for each liquid biopsy type using RNA 6000 pico assay on Agilent Bioanalyzer 2100.

It was also discovered that plasma showed the least nanoparticle size (131.70 nm) as compared to urine (176.00 nm) and saliva (195.82 nm) (Figure 2). The difference in the size and the concentration of exosomes extracted from various body fluids can be observed in (Figure 3). In previous study by Vagner et, al., (6) it was observed that larger size EVs carry heavier cargo. A similar pattern was observed in the current study where plasma carried the smallest RNA size fragments, followed by urine and saliva, which had intermediate and larger EV size (Figure 4). The larger RNA fragment size also contributed to higher RNA yield, which could be corroborated from Bioanalyzer data obtained by analyzing RNA yield from EVs isolation from plasma (1.5 ng), urine (3.33 ng) and saliva (76.06 ng).

Small RNA sequencing analysis revealed that plasma, urine and saliva produced similar percentage of reads that passed the quality filter and were further used for alignment and analysis (Figure 5). Out of the reads that were used for alignment, 10.48% reads of the total input reads were mapped to genome in case of plasma RNA, which was similar to urine (8.86%) and saliva (14.77%) (Figure 6). However, on further analyzing the reads mapped to genome, it was observed that RNA extracted from urine exosomes showed the highest miRNA sequences (40.17%) as compared to plasma (24.91%) and saliva (1.69%) (Figure 7). It was surprising to observe that saliva exosomal RNA which had exhibited the highest RNA yield showed the least number of reads that were aligned to miRNA sequences. This is indeed intriguing and thus the reads from all samples were further analyzed for to discover exogenous sequencing as it could be highly possible that the saliva samples are rich in bacterial miRNA/RNA species. Although the urine and plasma showed high miRNA reads and saliva did not, it is essential to compare the miRNA profiles of these samples in order to qualify urine or saliva as an alternative to plasma samples.

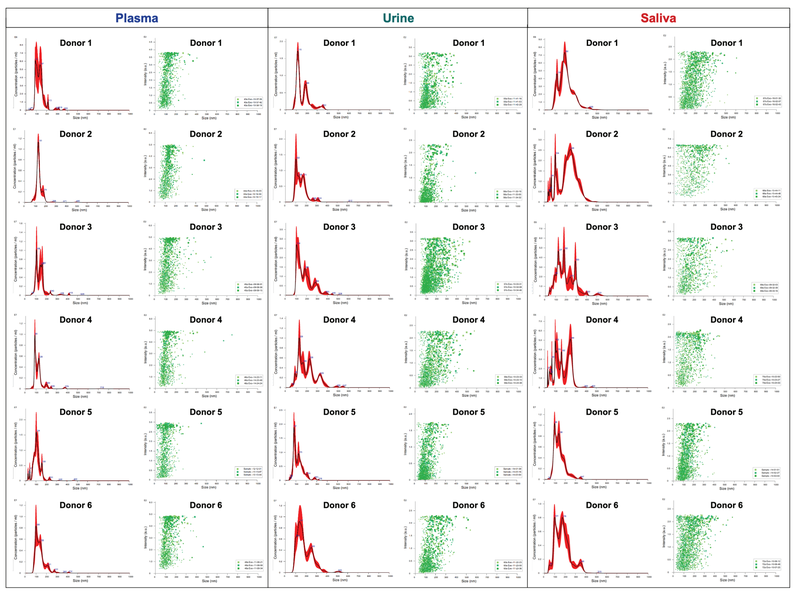

Figure 2. Nanoparticles size distribution analysis: Nanoparticle tracking analysis (NTA) has been performed using NanoSight NS300. Averaged particle size distribution for three measurements indicating a submicron size range between 25 nm and 300 nm with the majority being < 300 nm for all the time points for all liquid biopsy types under study.

Figure 3. Exosome concentration in Plasma, Urine and Saliva across all 6 donors under study.

Figure 4. Exosome average particle size distribution in Plasma, Urine and Saliva samples across all 6 donors under study

The comparison of the top 10 expressed miRNA across all the sample types revealed that 4 out of 10 miRNAs were similar between plasma and urine while only 2 out of 10 were similar between plasma and saliva (Figure 8). To further determine the difference between the miRNA profiles of these fluids, a heat map of the top 50 miRNA was derived using their miRNA read counts and it was clearly observed that neither of the body fluids, urine or saliva resemble to the miRNA profile of plasma (Figure 9). However, several miRNA were expressed at the same level in urine and saliva as compared to plasma. Although the miRNA profile of each sample type is quite distinct, the actual usability of these bodily fluids could be further confirmed by performing similar study on a disease cohort.

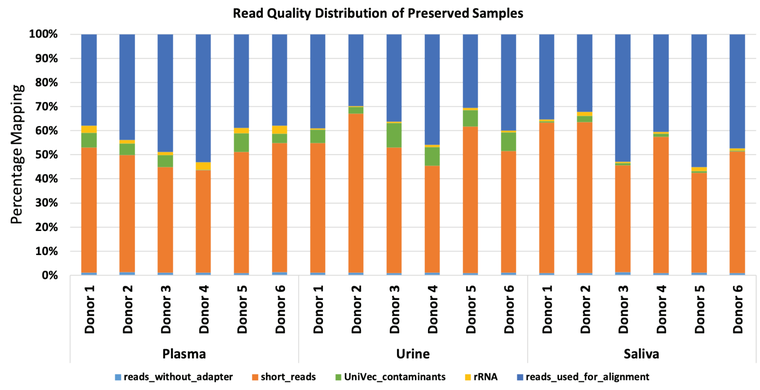

Figure 5. Average read quality distribution relative to raw reads of small RNA sequencing of exosomal RNA extracted from various bodily fluids. Calculations were relative to raw reads.

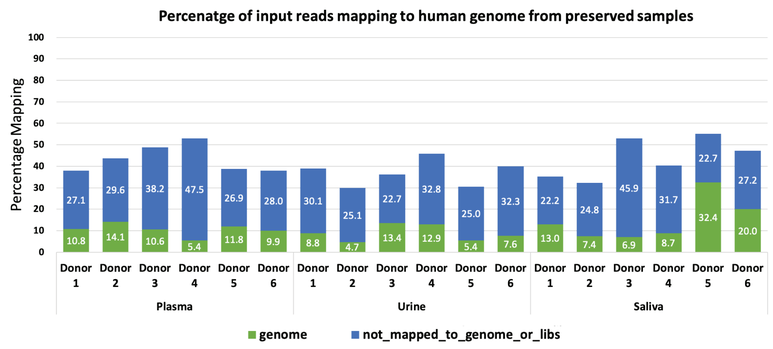

Figure 6. Genome mapping distribution relative to reads used for alignment obtained from small RNA sequencing of exosomal RNA extracted from various bodily fluids.

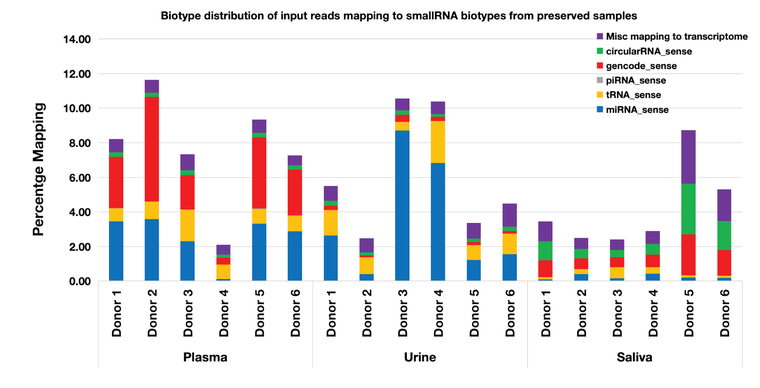

Figure 7. Average small RNA biotype distribution relative to the genome mapped reads obtained from sequencing of exosomal RNA extracted from various bodily fluids.

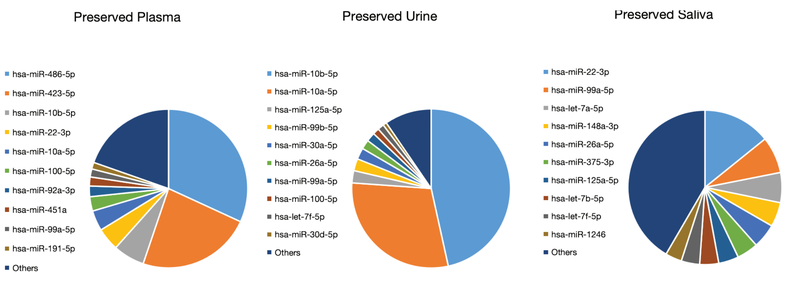

Figure 8. Average small RNA biotype distribution relative to the genome mapped reads obtained from sequencing of exosomal RNA extracted from various liquid biopsy types.

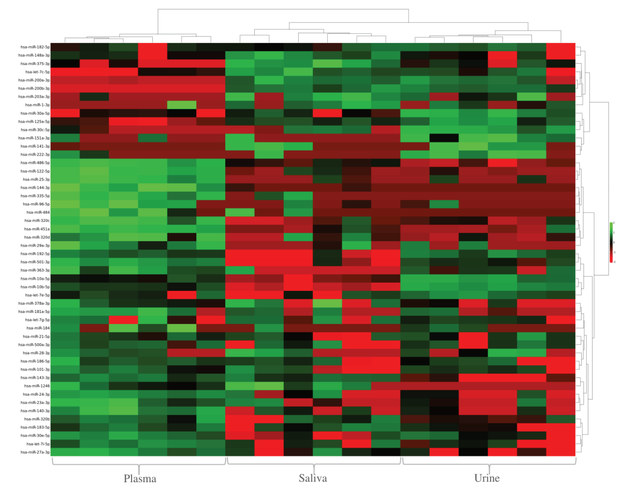

Figure 9. The heatmap visually represents the difference in expression between the top 50 miRNA observed between the 3 sample sets of Plasma, Urine, and Saliva. Each row represents a miRNA and each column represents a sample.

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