A Comparison of RNA- Versus DNA-Based 16S rRNA Amplicon Sequencing Analysis of the Equine Uterine Microbiome

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An increasing number of studies in humans and large animals indicate an important role of the uterine microbiome in endometrial receptivity. However, the study of this low biomass microbiome is challenging and biased by bacterial DNA contaminations introduced from different sources and high background of host DNA in most of the samples used for 16S rRNA amplicon sequencing. To overcome these challenges, this study investigated if i) RNA as input for 16S amplicon generation is more sensitive compared to DNA (higher copy number of ribosomes per bacterial cell compared to gene copies/genome; ~15,000 vs. 1-15) and ii) RNA as input reveals mainly information about bacteria alive while DNA could also be derived from dead bacteria. Based on a previously established 16S rRNA V3-V4 amplicon PCR, this study compared sequencing results starting from RNA or DNA isolated from uterine cytobrush samples as template for amplicon generation.

Uterine cytobrush samples were collected in estrus (1-3 days before ovulation) from mares (n=14) of the University of Illinois Urbana-Champaign or client-owned mares presented at the Veterinary Hospital (IACUC approvals 21237, 21238). From the same sample, RNA and DNA were extracted. The 16S rRNA V3-V4 region was amplified using universal prokaryotic primers Pro341F and Pro805R, binding the conserved regions flanking the V3-V4 regions. For RNA-based PCR, RNA samples were first reverse transcribed with Pro805R, and first-strand cDNA was used as input for 16S PCR. PCR products with a size of approx. 465 bp were isolated and purified from agarose gels. Illumina barcodes were added and paired-end 300 bp reads generated on an NextSeq 2000 instrument (Functional Genomics Center Zurich). Processing of fastq files and analysis of microbial composition was done with QIIME2. Amplicon sequence variants (ASV) were clustered at 97% sequence identity, using SILVA database (v. 138).

Overall, sufficient PCR product was obtained with 32 cycles for RNA-based compared to 35 cycles DNA-based amplicon PCR. Gel electrophoresis showed higher band intensity of RNA-derived amplicons indicating an at least 10-fold higher sensitivity of RNA-based PCR. Sequencing revealed 1.58 to 3.02 million paired-end reads per sample. After processing and merging the paired reads, about 70% of the reads remained for subsequent analysis. A

preliminary data analysis revealed 26,201 ASVs. The predominant phyla obtained for both RNA and DNA samples were Actinobacteriota, Proteobacteria, Firmicutes, and Bacteroidota confirming results of previous studies in the mare. The alpha diversity was significantly higher in RNA compared to DNA samples (Chao1: $P = 0.0001$, Simpson: $P = 0.003$). In addition, the analysis of beta diversity revealed significant differences of the RNA group compared to the DNA group (Bray-Curtis distances; $P = 0.001$). The analysis of RNA samples detected more operational taxonomic units (OTUs) (>2,500) than DNA samples (>1,100). A considerable number of OTUs was exclusively/mainly detected in RNA or DNA samples. Most OTUs only detected in RNA samples had counts in more than half of the samples, while OTUs only detected in DNA samples had counts only in a few of the 14 samples,

The results of this study indicate that 16S rRNA amplicon sequencing is more sensitive and reveals a higher microbiome diversity when RNA samples are used as starting material. The identification of OTUs only found in DNA samples suggests origin from dead bacteria.

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