Supplemental Online Content

Hanna N, Heffes-Doon A, Lin X, et al. Detection of messenger RNA COVID-19 vaccines in human breast milk. *JAMA Pediatr*. Published online September 26, 2022. doi:10.1001/jamapediatrics.2022.3581

eMethods.

This supplemental material has been provided by the authors to give readers additional information about their work.

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This cohort study was conducted from February to October 2021 and included 11 healthy lactating mothers who received either the BNT162b2 (Pfizer) or mRNA-1273 (Moderna) COVID vaccine within six months after delivery. Demographic data were collected through in-person interviews (Table 1). NYU Institutional Review Board approval was obtained, and all study volunteers signed written informed consent.

Isolation of extracellular vesicles (EVs) from BM:

Extracellular vesicles (EVs) were isolated from 3 ml of BM by sequential ultra-centrifugation method. After removing cell and bulky debris by serial centrifugation at 2,000- and 17,000 x g for 10 min at 4°C, the supernatant was collected and subjected to 100,000 g centrifugation in a TLA 110 rotor for 18 hrs at 4 °C. The EVs pellet was suspended in PBS, and EVs concentration was determined by ZetaView by Particle Metrix ZetaView Nanoparticle Tracking Analysis (Particle Metrix, Germany).

Detection of Covid-19 vaccine mRNA in BM by qRT-PCR:

Covid-19 mRNA vaccines were assayed by two-step RT-PCR. Total RNA was isolated from 0.6 mL of whole milk by miRNeasy mini kit (cat# 217004, Qiagen, Germantown, MD) according to the manufacturer's instructions. One-third of the eluted RNA was used for reverse transcription reaction. Reverse transcription was carried out using the High-Capacity cDNA Reverse Transcription Kit (cat# 4368814, ThermoFisher, Waltham, MA) with random primers. After diluted 1:5, 2 uL cDNA was used in real-time PCR reaction. Based on the putative sequences of vaccine BNT162b2 (Pfizer) and mRNA1273 (Moderna) that were previously published, two sets of primers targeted two regions of each vaccine mRNA were designed, and the primers and

probes were synthesized by Integrated DNA Technologies (Coralville, IA). Expression of RNAse P and beta Actin transcripts were assayed as an internal control to verify the sample quality throughout the RNA isolation, reverse transcription, and PCR processes. Real-time PCR was performed using Lightcycle 480 Probes Master (cat#04707494001, Roche LifeScience, Indianapolis, IN) on the QuantStudio3 (Applied Biosystems, Foster City, CA). Cycle threshold (Ct) was calculated with Quantstudio Design and Analysis software version 15.1 (Applied Biosystems, Foster City, CA). BNT162b2 (Pfizer) and mRNA-1273 (Moderna) leftover vaccines immediately after clinical use in our hospital (that were designated to be discarded) were used to validate the real-time PCR assay. For assay validation, a known amount of vaccine in the range of 100,000 to 0.1 pg/mL was spiked in whole milk collected from individuals prior to June 2019. Standard curves of real-time RT-PCR assays were performed. COVID mRNA vaccines were 10-fold serially diluted in BM. The ranges on both vaccines were from 1 to 100,000 pg/mL BM, and the detection limit was 1 pg vaccine mRNA/mL BM.