University of Louisville Journal of Respiratory Infections

METHODOLOGY

Performance of a SARS-CoV-2 RT-PCR Assay with Non-Traditional Specimen Types

*Subathra Marimuthu[1]; Holly Aliesky[1]; Heather Ness[2]; Daniya Sheikh[1]; Satya Rashmit Durugu[1]; Dawn Balcom[1]; Leslie Wolf[1]**

[1]University of Louisville Division of Infectious Diseases, Department of Medicine, School of Medicine, Louisville, KY; [2]University of Louisville School of Public Health, Louisville, KY

*lawolf07@louisville.edu

Recommended Citation: Marimuthu S, Aliesky H, Ness H, Sheikh D, Durugu SR, Balcom D, Wolf L. Performance of a SARS-CoV-2 RT-PCR Assay with Non-Traditional Specimen Types. Univ Louisville J Respir Infect. 2023 Feb 15;7(1):a3. doi: 10.55504/2473-2869.1260.

Abstract

During the first two years of the coronavirus disease 2019 (COVID-19) pandemic, nasopharyngeal (NP) specimens were the gold standard for clinical diagnostic testing. As information about the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) causing the pandemic continued to be shared, it was clear that the virus could be detected in other specimen types during an active infection. The University of Louisville Infectious Diseases Laboratory accepted non-traditional specimen types, most without a paired, positive NP result, for research purposes only to support local epidemiology efforts. A real-time reverse transcription-polymerase chain reaction (RT-PCR) assay originally validated for NP specimens was used for non-traditional specimen types using a variety of specimen preparation methods. Limit of detection (LOD) studies allowed for direct comparison between NP, sputum, and breast milk specimen types. The primary aim of the study was to determine whether SARS-CoV-2 RNA could be detected in different human specimen types. The results showed that the non-traditional specimens were not inherently inhibitory since SARS-CoV-2 RNA was detected in 36 (14.5%) out of 249 non-traditional specimens, and the limit of detection for SARS-CoV-2 in breast milk and sputum was the same as for NP specimens. SARS-CoV-2 was not detected in 15 breast milk specimens from mothers with positive SARS-CoV-2 NP results. In addition, a direct comparison study showed that NP specimens performed better than paired nasal specimens. In conclusion, by analyzing real-time RT-PCR test results for these non-traditional specimen types, two benefits were realized. Health care providers gained additional epidemiologic information (since information was not to be used for managing or treating patients), and the laboratory gathered important information about specimen types for which complete method validation studies could be pursued in the future.

Introduction

In the early stages of the coronavirus disease 2019 (COVID-19) pandemic, it was clear that the coronavirus could be spread by the respiratory route and cause respiratory infection, but questions remained about other routes of transmission and other infection sites. There were questions about lower respiratory specimens, stool, and breast milk and pregnancy-related specimen types, leading researchers to test a variety of specimen types to understand possible routes of transmission.[1] In addition, unusual presentations such as headaches/anosmia and testicular torsion for some patients hospitalized with suspected COVID-19 led to testing of non-traditional specimen types such as cerebrospinal fluid (CSF) and testicular tissue, respectively.[2, 3] Because the University of Louisville (UofL) Infectious Diseases Laboratory was performing real-time reverse transcription-polymerase chain reaction (RT-PCR) testing under U.S. Food & Drug Administration (FDA) Emergency Use Authorization (EUA) in March 2020, non-traditional specimen types were tested by special request as "research use only" and for epidemiologic purposes. Most of the specimens submitted for this study did not have paired, positive nasopharyngeal (NP) specimens.

The primary aim of this study was to determine whether SARS-CoV-2 could be detected in a variety of respiratory specimen types and other non-traditional specimen types. Additional goals were to publish the protocols used for pre-treatment of various specimen types, to determine whether the non-traditional specimen types were inherently inhibitory, and to compare the limits of detection (LOD) for nasopharyngeal (NP), breast milk, sputum, and other respiratory specimen types of interest.

Materials and Methods

Specimen collection and storage

All specimens submitted for research use only testing were collected as part of standard of care testing by the UofL Hospital under UofL Institutional Review Board (IRB) #18.0643 except as noted for breast milk specimens and nasal swab studies. No informed consent was required because these were considered waste specimens by the UofL IRB and thus not human subject research. Most of the specimens submitted did not have paired, positive NP specimens submitted for testing except for the breast milk specimens (consented and collected under UofL IRB #20.0257 and IRB #05.0556) and nasal swabs (consented and collected under UofL IRB #20.0786.). Specimens were stored in the refrigerator (4 °C) until testing was performed within 72 hours of collection.

Real-time RT-PCR assay for SARS-CoV-2

The UofL Infectious Diseases Laboratory validated a TaqMan real-time RT-PCR assay for SARS-CoV-2 with NP specimens in March 2020. The test was developed and its performance characteristics determined by the UofL Infectious Diseases Laboratory.[4] The assay was intended for use by Clinical Laboratory Improvement Amendments (CLIA)-certified, high-complexity laboratories with experience in developing molecular diagnostics and was only for use under FDA EUA. The reagents, instrument, and assay settings for different specimen types were the same as for NP specimens as previously described for the Luminex ARIES®. Briefly, a Luminex ARIES® TaqMan real-time RT-PCR was validated, targeting the N1 and N3 nucleocapsid genes, as well as the human RNase P gene, to create a multiplex molecular diagnostic assay. Results were for the qualitative detection of SARS-CoV-2 RNA in NP swab specimens from individuals suspected of COVID-19. For specimen types other than NP, the SARS-CoV-2 RNA assay was not cleared or approved by the FDA. Data shown in this study were intended to be used for research and epidemiological purposes, not for patient management.

Respiratory and non-traditional specimens tested by real-time RT-PCR SARS-CoV-2 assay

The UofL Infectious Diseases Laboratory received respiratory and non-traditional specimens during the early stages of the COVID-19 pandemic. These included upper respiratory specimens, such as nasal and oropharyngeal; lower respiratory specimens, such as sputum, bronchoalveolar lavage (BAL), and bronchial wash; stool; whole blood; cerebrospinal fluid (CSF); breast milk; amniotic fluid; and tissue specimens, such as placenta, umbilical cord, and testicle.

Preparation of nasal swab and oropharyngeal swab specimens

Nasal and oropharyngeal specimens were processed in the same manner as an NP specimen. Briefly, after vortexing the specimen tube, 195 μL of the specimen was transferred to a microcentrifuge tube containing 5 μL of 1 μg/1 μL carrier RNA in AVE buffer (QIAGEN Catalog number 1026956, Germantown, MD). Next, this mixture was placed in an ARIES® cassette connected to Ready Mix® tubes containing 5 μL of primer/probe set for SARS-CoV-2 real-time RT-PCR testing.

Pre-treatment of sputum, BAL, and bronchial wash specimens

Bertin tubes (Precellys Lysis Kit, Reference number KT03961-1-006.2, Bertin Corp, Rockville, MD) were used for processing specimens that were considered viscous. Specimen processing for these specimen types was previously published for detection of *Pneumocystis jirovecii* in lower respiratory tract specimens.[5]

Briefly, 400 μL of sputum, BAL, or bronchial wash were added to the Bertin tube with 400 μL of AL buffer (QIAGEN Catalog number 19075, Germantown, MD). For thicker specimens, a wider mouth sterile transfer pipette was used. The Bertin tube was placed on a vortexer with an adaptor and shaken at 10,000 rpm for five minutes to liquify the sputum. After incubating at room temperature for ten minutes to reduce the foam, the tubes were centrifuged for two minutes at 14,000 rpm at room temperature. Finally, 200 μL of supernatant was added to tubes containing 5 μL of carrier RNA and 40 μL of 25 mg/mL Proteinase K (Sigma-Aldrich, Reference number P2308, St. Louis, MO), followed by vortexing; then, the entire volume of specimen with carrier RNA and Proteinase K mixture was used for the real-time RT-PCR assay.

Pre-treatment of stool specimens

The protocol for pretreatment of stool specimen was the same as for the Luminex xTAG® gastrointestinal pathogen panel (GPP).[6] For stool specimens that were more liquid (Bristol scores 6–7), 100 μL of stool was added to 1 mL of QIAGEN AL (lysis) buffer in a Bertin tube. For stool specimens that were more solid (Bristol scores 1–5), 100–150 mg of stool was added to 1 mL of AL buffer in a Bertin tube. The Bertin tubes were placed on a vortexer with an adaptor, then shaken at 10,000 rpm for five minutes to liquify the stool. After incubating at room temperature for ten minutes to reduce the foam, the tubes were centrifuged for two minutes at 14,000 rpm at room temperature. Finally, 200 μL of supernatant was added to tubes containing 5 μL of carrier RNA and 40 μL of 25 mg/mL Proteinase K, followed by vortexing. The entire volume of specimen with carrier RNA and Proteinase K was used for the real-time RT-PCR assay.

Preparation of CSF, amniotic fluid, and breast milk specimens

These specimen types were processed in the same manner as NP specimens. Initial testing compared cycle threshold (Ct) values between positive NP specimens and positive control material spiked into these specimen types. The Ct values were in the same range (data not shown).

Pre-treatment of tissue specimens (placenta, umbilical cord, and testicular tissue)

To optimize the tissue processing protocol for the real-time assay, Ct values were compared between positive NP specimens and positive control material spiked into tissue specimens. The protocol was adjusted until the Ct values were similar between the specimen types (data not shown). For all fresh tissue types, 40 μg was placed in a Biomasher II pestle/tube (Fisher Scientific catalog number 50-136-7690, Pittsburg, PA) with 80 μL ATL buffer (QIAGEN catalog number 19076, Germantown, MD) and pulsed 30 times using the Biomasher II pestle/pestle motor mixer (Fisher Scientific catalog number NC0493674, Pittsburg, PA). If tissue was received in fixative, such as formalin, then it was washed twice with 1 mL sterile 0.9% saline. The pestle was removed from pestle motor mixer and left in the tube. Pestles were then washed with 100 μL ATL buffer. Next, 40 μL of 25 mg/mL Proteinase K was added to the tube, which was pulse-vortexed for 5–10 seconds to thoroughly mix and briefly centrifuged to bring the contents to the bottom of the tube before incubating at 56 °C in a shaking bath or thermomixer (Eppendorf catalog number 5384000020, Enfield, CT) at 600 rpm for two hours to overnight. Cap locks were used over the top of the tubes to prevent them from opening during incubation. Tubes were pulse-vortexed for 5–10 seconds every 20 minutes for the first 1–2 hours of incubation time.

After two hours or overnight incubation, tubes were vortexed and then centrifuged quickly to collect any condensation. Two hundred (200) μL of AL buffer was added to the tubes, which were vortexed and centrifuged as before and incubated for ten minutes at 70 °C in a heat block. If any precipitate formed, the tube was incubated at 70 °C for one minute, then vortexed, and the specimen was placed back in the heat block at 70 °C for ten minutes. The entire volume was transferred to a QIAshredder column (QIAGEN catalog number 79656 Germantown, MD) and centrifuged two minutes at maximum speed (14,000 rpm). Eluate was transferred from the collection tube to a new 5.0 mL tube. Next, 500 μL QIAGEN AL buffer was applied to the column, which was centrifuged for two minutes at maximum speed (14,000 rpm); the resulting eluate was reapplied to the column, and the centrifuge step was repeated. The two eluates for each specimen were then pooled into the same 5.0 mL tube and pulse-vortexed. Finally, 195 μL of this lysate was added to new tubes containing 5 μL of carrier RNA and quickly vortexed. The resulting total volume was then used for the SARS-CoV-2 real-time RT-PCR assay.

LOD studies for sputum and breast milk

To determine the LOD for sputum and breast milk specimens, quantitated amounts of heat-inactivated virus were spiked into known negative sputum and breast milk specimens. Sputum and breast milk specimens were tested first by the Luminex ARIES® real-time RT-PCR assay and, if negative for SARS-CoV-2, were used for spiking experiments. Heat-inactivated viral culture fluid (Zeptometrix Catalog number 0810587 CFHI-0.5 mL, Buffalo, NY) at a concentration of 1.50×10^6 tissue culture infective dose-50% ($TCID_{50}$)/mL was tested using 10-fold serial dilutions in known negative pooled sputum or breast milk specimens (concentrations ranging from 1.50×10^4 to 1.50×10^{-1} $TCID_{50}$). Serial dilutions of the spiked specimen were performed using the negative sputum supernatants as diluent. These dilutions were tested in triplicate, and the lowest concentration that gave positive results 100% of the time was defined as the preliminary LOD. For sputum specimens, triplicates were all run on the same day, but three runs were divided between different instruments and different technologists. Breast milk specimens were tested in triplicate on two different days, using different instruments and different technologists.

Paired nasal and NP specimens for comparison study

Nasal specimens were self-collected by participants under clinical supervision, while NP specimens were collected by clinical staff at the UofL Division of Infectious Diseases Travel Clinic (UofL IRB 20.0786). Briefly, anterior nares nasal swab specimens were obtained first after explaining the study and providing details in a standard preamble to the participant. Consented participants were instructed to gently insert the flocked swab inside the nostril (anterior nares), firmly rotate the swab three times, and leave the swab in place for 10–15 seconds. This was repeated in the other nostril with the same swab. The NP swab specimens were obtained using the Centers for Disease Control and Prevention (CDC)–recommended collection procedure.[7] Specimens were tested using the Luminex ARIES® real-time RT-PCR assay for SARS-CoV-2 to determine the analytic sensitivity of the specimen type. One hundred and forty-one (141) paired nasal and NP swab specimens were collected until reaching 20 positive NP specimens. Corresponding nasal specimens were not tested if the NP specimen was negative. Positive paired nasal and NP specimens were tested on the same day.

Table 1. Variety of specimen types tested during the COVID-19 pandemic.

Specimen type	Number tested	Number positive (%)
Sputum	137	27 (19.7)
Bronchoalveolar lavage	5	1 (20.0)
Bronchial wash	2	0
Nasal swab	62	7 (11.3)
Stool	14	1 (7.14)
Cerebrospinal fluid	7	0
Breast milk*	16	0
Amniotic fluid	2	0
Placenta	2	0
Umbilical cord	1	0
Testicular	1	0
Total	249	36 (14.5)

* Only specimen type with paired nasopharyngeal specimen results for SARS-CoV-2.

Table 2. Limit of detection for sputum specimens using real-time RT-PCR SARS-CoV-2 assay.

Sample ID	SARS-CoV-2 heat-inactivated $TCID_{50}$/mL	N1 Ct value*	N3 Ct value*	RNaseP Ct value*
2	1.50×10^{4} (15,000)	18.27±0.40	18.20±0.46	18.53±0.32
3	1.50×10^{3} (1,500)	21.10±0.62	21.00±0.35	20.53±0.35
4	1.50×10^{2} (150)	24.98±1.16	23.87±0.91	21.95±0.82
5	1.50×10^{1} (15)	31.70±0.82	30.47±0.64	23.10±1.01
6	**1.50×10^{0} (1.5)**	**34.20±2.36**	**33.97±2.54**	**22.87±0.67**
7	1.50×10^{-1} (0.15)	34.67±2.67	32.65±1.48†	23.90±1.61

Abbreviations: Ct, cycle threshold; $TCID_{50}$, tissue culture infective dose-50%.
* Mean±standard deviation.
† Only detected 2 out of 3 times.

Data analysis

All data were entered into a secure Excel (Microsoft, Redmond, WA) spreadsheet, and basic means and standard deviations were calculated for replicate data points.

Results

The UofL Infectious Diseases Laboratory received a variety of specimen types and determined the number of specimens that were positive by real-time RT-PCR SARS-CoV-2 assay (**Table 1**). Based on these data, respiratory specimens were the best specimen types for detecting SARS-CoV-2. Notably, the UofL ID Laboratory received many sputum specimens to test for the presence of SARS-CoV-2 RNA early in the pandemic, although most did not have paired, positive NP specimens submitted. A total of 137 sputum specimens were tested, and 27 specimens were positive by SARS-CoV-2 assay (**Table 1**). As a result, an LOD study was performed to establish that sputum was not inherently inhibitory (**Table 2**). Results confirmed that sputum was not inhibitory, and the LOD was similar to the LOD for NP specimens established previously.[4]

The UofL ID Laboratory received breast milk specimens from nursing mothers who were positive for SARS-CoV-2 based on their NP specimens (UofL IRB #20.0257 and IRB #05.0556). A total of 15 breast milk specimens were tested, and all were negative by SARS-CoV-2 assay (**Table 1**). Another LOD study was performed to establish that breast milk was not inherently inhibitory (**Table 3**). Results confirmed that breast milk was not inhibitory, and the LOD was similar to the LOD established previously for NP specimens.[4]

Because NP swab collection is invasive, uncomfortable, and requires extra personal protective equipment, a comparison study with nasal swab specimen collection was performed. Nasal swab collection is associated with less patient discomfort and less need for personal protective equipment

Table 3. Limit of detection for breast milk specimens using real-time RT-PCR SARS-CoV-2 assay.

Sample ID	SARS-CoV-2 heat inactivated $TCID_{50}$/mL	N1 Ct value*	N3 Ct value*	RNase P Ct value*
3	1.50×10^{3} (1,500)	20.88 (0.95)	20.38 (0.96)	21.20 (0.85)
4	1.50×10^{2} (150)	24.93 (2.68)	24.28 (2.64)	24.60 (2.67)
5	1.50×10^{1} (15)	28.60 (2.49)	27.85 (2.34)	27.00 (1.59)
6	**1.50×10^{0} (1.5)**	**32.70 (2.49)**	**31.83 (1.69)**	**29.10 (1.13)**
7	1.50×10^{-1} (0.15)	35.00†	34.7†	30.18 (2.63)
8	1.50×10^{-2} (0.015)	ND	ND	31.48 (5.17)

Note: All patients had positive nasopharyngeal results.
Abbreviations: Ct, cycle threshold; ND, not detected; $TCID_{50}$, tissue culture infective dose-50%.
* Mean±standard deviation.
† Detected 1 out of 4 times.

Table 4. Comparison of paired nasopharyngeal and nasal swab specimens for gene targets N1 and N3.

ID	Nasopharyngeal Swab Results				Nasal Swab Results				Agreement
	N1 Ct	N3 Ct	RNaseP Ct	Result	N1 Ct	N3 Ct	RNaseP Ct	Result	
1	25.8	24.7	25.3	DETECTED	28.2	27.3	ND	DETECTED	Yes
2	23.9	22.8	ND	DETECTED	29.9	29	ND	DETECTED	Yes
3	23.2	22.1	22.6	DETECTED	35	35.2	27.8	DETECTED	Yes
4	35.9	34.9	30.2	DETECTED	ND	ND	34.2	NOT DETECTED	No
5	31.1	30.1	28.3	DETECTED	ND	ND	33.9	NOT DETECTED	No
6	31.1	30.1	28.9	DETECTED	25.5	24.1	25.2	DETECTED	Yes
7	31.4	30.2	29.1	DETECTED	ND	ND	31.6	NOT DETECTED	No
8	20.4	19.4	ND	DETECTED	26.4	25.9	26.3	DETECTED	Yes
9	26.8	25.7	25.9	DETECTED	32.9	32.8	32.9	DETECTED	Yes
10	22.5	21.4	ND	DETECTED	30.3	29.8	29.4	DETECTED	Yes
11	23.4	22.1	ND	DETECTED	23	22	ND	DETECTED	Yes
12	36.3	36.3	26.7	DETECTED	ND	ND	35.6	NOT DETECTED	No
13	18.6	17.1	ND	DETECTED	22.7	21.2	ND	DETECTED	Yes
14	31.7	30.6	28.1	DETECTED	ND	ND	36	NOT DETECTED	No
15	20.3	19.4	ND	DETECTED	24.7	23.4	24.8	DETECTED	Yes
16	25.7	23.8	24.7	DETECTED	24.8	23.2	ND	DETECTED	Yes
17	25.7	24.7	25.3	DETECTED	32.2	31.3	31.5	DETECTED	Yes
18	24.8	24.4	25	DETECTED	30.3	29.8	28.2	DETECTED	Yes
19	32.4	31.6	30.8	DETECTED	ND	ND	31.4	NOT DETECTED	No
20	27.2	26.1	25.8	DETECTED	27.6	26.3	26.3	DETECTED	Yes

Abbreviations: Ct, cycle threshold; ND, not detected.

by health care workers, but analytic sensitivity may be reduced. Thus, paired NP and nasal swab specimens from 20 SARS-CoV-2-positive participants were tested on the Luminex ARIES® real-time RT-PCR assay for SARS-CoV-2. We observed that 14 specimen results were positive for both specimen types (**Table 4**). Ten of the NP specimens out of 14 had lower cycle threshold (Ct) values than the nasal swabs, suggesting a higher viral RNA concentration. Only one nasal swab specimen had a lower Ct value than the corresponding NP swab specimen, while three patients had the same Ct values for both specimen types. The remaining six patients had discrepancies in the results. The NP specimens were all positive for these patients, while the nasal specimens were negative for SARS-CoV-2. These NP specimens had higher Ct values, close to the established LOD, suggestive of lower viral RNA concentrations. The corresponding nasal specimens either did not have enough viral RNA at the time of specimen collection, or the amount of viral RNA was below the detection range in the real-time RT-PCR assay.

Discussion

The primary aim of this study was to determine whether SARS-CoV-2 RNA could be detected in a variety of respiratory specimen types and other non-traditional specimen types. Of 249 non-NP clinical specimens, we detected SARS-CoV-2 RNA in 36 (14.5%), with the highest percentage of detection in sputum and BAL specimens, consistent with other studies.[1, 8, 9] In addition, since the real-time RT-PCR assay used in this study included an internal control, we demonstrated that the various specimen types tested in this study were not inherently inhibitory because human RNase P was detected for each test specimen with a negative SARS-CoV-2 result.

The UofL Infectious Diseases Laboratory originally validated a real-time RT-PCR assay for SARS-CoV-2 with NP specimens in March 2020.[10] Additional LOD studies were performed for sputum and breast milk in this study using the same real-time RT-PCR assay as well as the same heat-inactivated control material on the Luminex ARIES® platform, identically to NP swabs. The LOD was 1.5 $TCID_{50}$/mL for NP swabs, sputum, and breast milk specimens. Regarding the 15 breast milk specimens tested using this SARS-CoV-2 real-time RT-PCR assay, there was no evidence of viral RNA present, suggesting that the risk of transmission by breastfeeding is minimal. This finding is supported by other published studies [11–13], yet some studies found SARS-CoV-2 in breast milk.[14, 15] Comparison of paired nasal and NP specimens confirmed that NP was a better specimen type for detecting SARS-CoV-2, also consistent with other studies [16]; however, during times when there is a limited supply of personal protective equipment for specimen collectors, nasal swabs may be acceptable substitutes.[16]

Strengths of this study include the use of a sensitive and specific real-time RT-PCR assay (approved as a diagnostic test under FDA EUA) to test NP specimen types in a sample-to-answer, closed system for research purposes. Comparison of real-time PCR tests can be challenging due to different reporting units, but these results demonstrated similar analytical sensitivity among various specimen types using the real-time RT-PCR assay described here. Because no FDA-authorized tests existed for non-traditional specimen types, these test results increased knowledge and provided physicians and hospital epidemiologists with additional information regarding unusual presentations of COVID-19.

The primary limitation of the study was that not all specimens submitted for research testing had a positive, paired NP specimen. In addition, small sample numbers for many of the non-traditional specimens, especially those related to pregnancy, such as breast milk, placenta, and amniotic fluid, were limiting factors. Due to the small sample numbers, no conclusions could be drawn about the likelihood of finding SARS-CoV-2 RNA in specimen types other than NP or sputum specimens. The timing of collection was beyond the control of the laboratory, so delays in collection after symptom onset may have affected the ability of the real-time RT-PCR assay to detect SARS-CoV-2 RNA in various specimen types. Finally, detection of SARS-CoV-2 RNA does not indicate the infectivity or viability of the virus.

Based on the data presented here, the laboratory will pursue complete validation studies on non-traditional specimen types, such as sputum and perhaps BAL specimens, to meet CLIA requirements for a diagnostic molecular test for SARS-CoV-2 pneumonia.

Received: September 22, 2022

Accepted: February 10, 2023

Published: February 15, 2023

Funding Source: The author(s) received no specific funding for this work.

Conflict of Interest: All authors declared no conflict of interest in relation to the main objective of this work.

References

1. Wang W, Xu Y, Gao R, Lu R, Han K, Wu G, Tan W. Detection of SARS-CoV-2 in Different Types of Clinical Specimens. JAMA. 2020;323(18):1843-4. doi: 10.1001/jama.2020.3786. PubMed PMID: 32159775; PubMed Central PMCID: PMC7066521.

2. Rogers JP, Watson CJ, Badenoch J, Cross B, Butler M, Song J, Hafeez D, Morrin H, Rengasamy ER, Thomas L, Ralovska S, Smakowski A, Sundaram RD, Hunt CK, Lim MF, Aniwattanapong D, Singh V, Hussain Z, Chakraborty S, Burchill E, Jansen K, Holling H, Walton D, Pollak TA, Ellul M, Koychev I, Solomon T, Michael BD, Nicholson TR, Rooney AG. Neurology and neuropsychiatry of COVID-19: a systematic review and meta-analysis of the early literature reveals frequent CNS manifestations and key emerging narratives. J Neurol Neurosurg Psychiatry. 2021;92(9):932-41. Epub 20210603. doi: 10.1136/jnnp-2021-326405. PubMed PMID: 34083395.

3. Shields LBE, Daniels MW, Peppas DS, White JT, Mohamed AZ, Canalichio K, Rosenberg S, Rosenberg E. Surge in testicular torsion in pediatric patients during the COVID-19 pandemic. J Pediatr Surg. 2022;57(8):1660-3. Epub 20210716. doi: 10.1016/j.jpedsurg.2021.07.008. PubMed PMID: 34392971; PubMed Central PMCID: PMC9282895.

4. Marimuthu S, Aliesky H, Connelly B, Malik D, Wolf LA. Development of a real-time Reverse-Transcription PCR for SARS CoV-2 on the Luminex ARIES® Platform. Univ Louisville J Respir Infect. 2021;5(1):a10. doi: 10.18297/jri/vol5/iss1/10.

5. Marimuthu S, Ghosh K, Wolf LA. Development of a Real-time PCR assay for *Pneumocystis jirovecii* on the Luminex ARIES® Platform. Univ Louisville J Respir Infect. 2019;3(1):a5. doi: 10.18297/jri/vol3/iss1/5.

6. Le M. Pre-treatment is Key in GPP [Internet]: Luminex Corporation; 2013 Feb 19 [cited 2023 Jan 24]. [about 3 screens]. Available from: https://www.luminexcorp.com/blog/pre-treatment-is-key-in-gpp/.

7. Centers for Disease Control and Prevention [Internet]. Atlanta: The Centers. Interim Guidelines for Collecting and Handling of Clinical Specimens for COVID-19 Testing; 2020 Feb 11 [updated 2020 July 8; cited 2020 Sep 4]; [about 10 screens]. Available from: https://www.cdc.gov/coronavirus/2019-nCoV/lab/guidelines-clinical-specimens.html.

8. Yu F, Yan L, Wang N, Yang S, Wang L, Tang Y, Gao G, Wang S, Ma C, Xie R, Wang F, Tan C, Zhu L, Guo Y, Zhang F. Quantitative Detection and Viral Load Analysis of SARS-CoV-2 in Infected Patients. Clin Infect Dis. 2020;71(15):793-8. doi: 10.1093/cid/ciaa345. PubMed PMID: 32221523; PubMed Central PMCID: PMC7184442.

9. Mohammadi A, Esmaeilzadeh E, Li Y, Bosch RJ, Li JZ. SARS-CoV-2 detection in different respiratory sites: A systematic review and meta-analysis. EBioMedicine. 2020;59:102903. Epub 20200724. doi: 10.1016/j.ebiom.2020.102903. PubMed PMID: 32718896; PubMed Central PMCID: PMC7380223.

10. Rao A, Wolk DM, Goldstein DY, Wolf LA. Development and Evaluation of Two SARS-CoV-2 RT-PCR Laboratory Developed Tests on the ARIES® Automated, Sample-to-Answer, Real-Time PCR System. White Paper. [Temple, TX]: [publisher unknown], 2020 March. 9 p. Report No.: WP242351.

11. Bäuerl C, Randazzo W, Sánchez G, Selma-Royo M, García Verdevio E, Martínez L, Parra-Llorca A, Lerin C, Fumadó V, Crovetto F, Crispi F, Pérez-Cano FJ, Rodríguez G, Ruiz-Redondo G, Campoy C, Martínez-Costa C, Collado MC. SARS-CoV-2 RNA and antibody detection in breast milk from a prospective multicentre study in Spain. Arch Dis Child Fetal Neonatal Ed. 2022;107(2):216-21. Epub 20210820. doi: 10.1136/archdischild-2021-322463. PubMed PMID: 34417223; PubMed Central PMCID: PMC8384494.

12. Bhatt H. Should COVID-19 Mother Breastfeed her Newborn Child? A Literature Review on the Safety of Breastfeeding for Pregnant Women with COVID-19. Curr Nutr Rep. 2021;10(1):71-5. Epub 20210104. doi: 10.1007/s13668-020-00343-z. PubMed PMID: 33394459; PubMed Central PMCID: PMC7780073.

13. Rodrigues C, Baía I, Domingues R, Barros H. Pregnancy and Breastfeeding During COVID-19 Pandemic: A Systematic Review of Published Pregnancy Cases. Front Public Health. 2020;8:558144. Epub 20201123. doi: 10.3389/fpubh.2020.558144. PubMed PMID: 33330308; PubMed Central PMCID: PMC7719788.

14. Groß R, Conzelmann C, Müller JA, Stenger S, Steinhart K, Kirchhoff F, Münch J. Detection of SARS-CoV-2 in human breastmilk. Lancet. 2020;395(10239):1757-8. Epub 20200521. doi: 10.1016/s0140-6736(20)31181-8. PubMed PMID: 32446324; PubMed Central PMCID: PMC7241971.

15. Pace RM, Williams JE, Järvinen KM, Belfort MB, Pace CDW, Lackey KA, Gogel AC, Nguyen-Contant P, Kanagaiah P, Fitzgerald T, Ferri R, Young B, Rosen-Carole C, Diaz N, Meehan CL, Caffé B, Sangster MY, Topham D, McGuire MA, Seppo A, McGuire MK. Characterization of SARS-CoV-2 RNA, Antibodies, and Neutralizing Capacity in Milk Produced by Women with COVID-19. mBio. 2021;12(1).

Epub 20210209. doi: 10.1128/mBio.03192-20. PubMed PMID: 33563823; PubMed Central PMCID: PMC7885115.

16. Péré H, Podglajen I, Wack M, Flamarion E, Mirault T, Goudot G, Hauw-Berlemont C, Le L, Caudron E, Carrabin S, Rodary J, Ribeyre T, Bélec L, Veyer D. Nasal Swab Sampling for SARS-CoV-2: a Convenient Alternative in Times of Nasopharyngeal Swab Shortage. J Clin Microbiol. 2020;58(6). Epub 20200526. doi: 10.1128/jcm.00721-20. PubMed PMID: 32295896; PubMed Central PMCID: PMC7269411.