Method Validation Study for SARS-CoV-2 Viral RNA Detection in Cervical, Rectal, Amniotic Fluid, Placental, Umbilical Cord Blood, and Breastmilk Specimens in a Cohort of Unvaccinated Women in Manila, Philippines

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ABSTRACT

Objectives. To validate a method in detecting SARS-CoV-2 via RT-qPCR in pregnant and non-pregnant samples other than nasopharyngeal swabs and/or oropharyngeal swabs such as cervical, rectal, amniotic fluid, placental, umbilical cord blood, and breastmilk.

Methods. We performed a validation experiment using MGI easy extraction kits and BGI PCR kits on non-conventional specimens, including cervical, rectal, amniotic fluid, placental, umbilical cord blood, and breastmilk to detect and confirm the presence of SARS-CoV-2. In addition, we tested the validated method on 572 purposively sampled field-collected non-conventional specimens from a cohort of 109 unvaccinated pregnant and 47 unvaccinated non-pregnant women to assess which candidate non-conventional maternal- and fetal-associated specimens may contribute to maternal-fetal viral vertical transmission.

Results. Positive detection of SARS-CoV-2 viral RNA in non-conventional specimens was demonstrated and verified. Of the 572 non-conventional samples tested, 1.8% (10/572) were positively validated by RT-qPCR for SARS-CoV-2 in the maternal-associated specimens particularly the rectal (5), placental (1), and cervical (4) swabs among six pregnant and four non-pregnant individuals. In contrast, no SARS-CoV-2 viral RNA was detected in fetal-associated specimens.

Conclusion. The results of the validation study may serve as an additional diagnostic screening layer to support maternal-child care. Furthermore, viral detection in these non-conventional maternal specimens may also be utilized to provide guidance in the clinical management of neonates, and pregnant women during delivery.

Keywords: Philippines, SARS-CoV-2, PCR, pregnant women, umbilical cord, placenta, amniotic fluid, breastmilk

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INTRODUCTION

The COVID-19 pandemic caused by SARS-CoV-2 accounts for approximately 638,175,811 confirmed cases and 6,612,970 deaths globally as of November 2022.[1] In the Philippines, 4,035,487 SARS-CoV-2 cases were confirmed, and 64,620 deaths were reported during the time of writing.[2] The National Capital Region (NCR) ranks first in confirmed COVID-19 cases (n=1,297,776).[2] COVID-19 infection threatens the general population and a higher severity risk has been observed in individuals with co-morbidities and other underlying health conditions. According to the American College of Obstetricians and Gynecologists (ACOG), "pregnant women may be at higher risk of severe illness, morbidity, or mortality compared with the general population".[3] Moreover, the possibility of SARS-CoV-2 transmission from an infected mother to the newborn upon delivery has also raised concerns among families and clinicians worldwide.

Viral diseases can result in intrauterine transmission when occurring during pregnancy.[4] Furthermore, the presence of the virus in human milk is also a possible route for vertical transmission from mother to child.[5] Considering the numerous studies conducted regarding the genomic and epidemiologic information of SARS-CoV-2, the possibility of the virus being transmitted vertically remains unanswered.

Currently, there are limited cases of reported maternal-fetal transmission of SARS-CoV-2 and little evidence supporting the vertical transmission of the virus. To date, cases of maternal-fetal transmission of SARS-CoV-2 were found to be rare among pregnant women that tested positive for the virus prior to delivery. It may be due to a few reported cases, low rates of SARS-CoV-2 detection in non-conventional maternal samples, and neonates born from COVID-positive mothers.

In China, 38 pregnant women with COVID-19 and their newborns were examined and there was no evidence of SARS-CoV-2 intrauterine or transplacental transmission from a positive mother to their fetus.[4] A study in the UK among pregnant women, demonstrated only 3% possibility of vertically acquired infection among newborns tested 12h after birth.[6] An analysis conducted by Gajbhiye et al. suggested an 8% possibility of intrauterine maternal-fetal transmission among cases of neonates diagnosed with COVID-19 by RT-PCR or exhibited IgM antibodies against SARS-CoV-2 in the first 48h of life.[7] In addition, a prospective study by Maeda et al. established that vertical transmission is possible because maternal compartments can be a source of exposure for the neonate.[8] A case report of Kulkarni strongly suggests vertical transmission. A neonate tested positive for SARS-CoV-2 with the presentation of symptoms whose mother, though SARS-CoV-2 negative before delivery, is antibody positive and her placenta and cord stump is positive at birth.[9] In the US, a case report confirmed the detection of SARS-CoV-2 in the placental samples collected from mothers positive for the virus upon delivery; however, none of the infants tested positive for SARS-CoV-2.[10] Yu et al., detected IgG in one breastmilk sample, thus indicating a protective effect from a COVID-19-infected mother.[11] Moreover, Wu et al., detected the presence of SARS-CoV-2 in the milk sample collected one day postpartum from a COVID-19-positive mother suggesting that breastfeeding can also be a possible route for vertical transmission.[12]

In the Philippines, although there have been cases of infants that tested positive for SARS-CoV-2 right after birth, vertical transmission has not been definitively ruled out owing to the inadequacy of evidence. Furthermore, in a study conducted at the Philippine General Hospital, SARS-CoV-2 viral RNA was not detected in the amniotic fluid, placental tissue, umbilical cord, breast milk, and anal swab samples collected after birth of the 14 COVID-19-positive patients.[13]

Detection of SARS-CoV-2 virus in maternal samples other than breastmilk and placenta must be further investigated to determine other potential sources of vertical transmission from mother to child during delivery. This study aimed to validate a method for SARS-CoV-2 detection among swabs collected from the cervix, rectum, amniotic fluid, placenta, umbilical cord blood, and breast milk using a validated PCR method for respiratory specimens as part of the longitudinal study on risk factors, clinical characteristics, and transmission of COVID-19 in both non-pregnant and pregnant women in the Philippines.[14]

METHODS

Preparation of Contrived SARS-CoV-2 Positive Samples

To perform the spike-recovery method, collected samples from SARS-CoV-2 negative patients were pooled according to sample type to meet the required specimen volume for dilution and spiked with SARS-CoV-2 positive analyte. A SARS-CoV-2 transcript was prepared for spiking the pooled specimens. Six aliquots of the pooled negative specimens were prepared. The first aliquot contained 200 µL of the negative pooled specimens and was used as the true negative sample. The five remaining aliquots were used for the serial dilution to prepare the contrived SARS-CoV-2 positive samples. Five contrivance levels were prepared (1:10 to 1:100,000) to perform the serial dilution wherein 50 µL of the SARS-CoV-2 transcript was added to 450 µL of negative pooled specimens. The SARS-CoV-2 transcript was further diluted into 10-fold using nuclease-free distilled water to reference cycle threshold (CT) values. SANLI Universal Transport Media (UTM) (Catalog No. SL901B Liuyang SANLI Medical Technology Development Co., Ltd.) was also spiked with the SARS-CoV-2 transcript to determine the stability of the SARS-CoV-2 viral transcript in the universal transport media used in the study. All contrived samples were tested in triplicates except for the 1:10,000 and 1:100,000 dilutions.

Study Population

All women whether non-pregnant or pregnant who attended consultations from November 2020 to April 2021 at the five study sites in Metro Manila, Philippines namely, Dr. Jose Fabella Memorial Hospital (FMMC), Jose R. Reyes Memorial Medical Center (JRRMMC), Ospital ng Maynila Medical Center (OMMC), Sta Ana Hospital (SAH), and Justice Abad Santos General Hospital (JASGH) were recruited for the study. Consultations for any disease except, malignant lesions and congenital anomalies of the reproductive tract, and tested for SARS-CoV-2 were prospective participants. Non-pregnant and pregnant women were screened and recruited to be participants. For the non-pregnant women, all were eligible participants apart from those women with genital tract malignancy considerations, less than 18 years of age, and who were unable to consent. For pregnant women, any age of gestation was included in recruitment except for those women <18 years old, with genital tract malignancy considerations, and unable to provide consent. During the recruitment of participants, hospitals were mainly getting participants in their third trimester or about to deliver as pregnant women do not even go out for prenatal care due to the pandemic. In addition, all participants were unvaccinated for COVID-19 at the time of recruitment. Their pregnancy, obstetric, and neonatal outcomes were collected postpartum before discharge. The final eligible participants included 109 unvaccinated pregnant and 47 unvaccinated non-pregnant women; the sample size was based on the study protocol published elsewhere.

Specimen Collection

Five hundred seventy-two purposively sampled site-collected non-conventional clinical specimens were analysed. Clinical specimens such as rectal (RS) and cervical (CS) swabs were collected from non-pregnant and pregnant individuals. In addition, umbilical cord blood (CB), amniotic fluid (AF), placenta (PL), and breast milk (BM) swabs were collected from pregnant individuals who were enrolled in the study. The swabs were then stored in SANLI UTM for further analysis.

SARS-CoV-2 Detection by RT-qPCR

RNA extraction from the contrived and collected samples was done using the MGI Easy extraction kit (Cat No. 1000021043, Wuhan MGI Tech Co., Ltd.) with the Kingfisher Flex Purification System (Thermo Scientific) automated extraction machine. Extraction reagents were prepared following the manufacturer's instructions. Before the actual sample run, a trial run was performed using the installed program for MGI extraction for process familiarity of the user, and to check for possible errors and issues.

Moreover, RT-qPCR detection was carried out using BGI Real-Time PCR fluorescent RT-PCR Kit for detecting 2019-nCoV (BGI. Cat. No. MFG030010. Europe A/S) following the manufacturer's instructions.[15] The SARS-CoV-2 PCR kit used in this study detects ORF1ab gene of the target pathogen. To evaluate the validity of extraction, an endogenous internal control (IC) which detects the human Beta-actin gene is also included as gene target in the PCR kit. Analysis was performed using ABI 7500 Fast machine and software wherein the ORF1ab and IC were detected using the FAM and VIC/HEX channels, respectively.

Clinical samples were tested after the spike and recovery method using the contrived SARS-CoV-2 samples. Since this is a method validation study, a revised result interpretation was used wherein samples with CT ≤37 in the SARS-CoV-2 gene target were considered as positive and a CT ≤35 in the internal control (IC) target is considered valid. Furthermore, samples that showed indeterminate results in the initial testing, such as those that demonstrated a sigmoidal curve (S-shape curve) but the Ct >37 in the SARS-CoV-2 gene target were retested in duplicates to increase confidence in the results. The consistent S-shape curve with CT >37 in the sample was considered positive.[15,16]

In addition, 572 non-conventional clinical specimens from pregnant and non-pregnant participants were analyzed for SARS-CoV-2 detection, including amniotic fluid swabs, breastmilk swabs, cord blood swabs, placental swabs, cervical swabs, and rectal swabs.

Data Analysis

Descriptive statistics were calculated and tabulated in means and standard deviations (SD) for the continuous variable cycle threshold (CT) value, and frequencies and percentages for the categorical variable confirmed SARS-CoV-2 positive. All analyses were done in Microsoft Excel software.

RESULTS

Evaluation of PCR Detection Protocol in Testing Non-conventional Samples

The detection of SARS-CoV-2 in the contrived non-conventional specimens serially diluted ten-fold is summarized in Table 1. The SARS-CoV-2 RNA transcript was detected in all replicates (3/3) in the contrived non-conventional specimens at 1:10 dilution. At the hundred-fold dilution (1:100), the amniotic swab only generated 67% (2/3) replicate detection while the rest of the contrived specimens still demonstrated 100% (3/3) replicate detection. In the thousand-fold dilution (1:1000), 100% (3/3) detection in all replicates was only exhibited by the cord-blood and rectal swabs, however; the mean CT value for the contrived rectal swab (mean CT = 38.09) replicate testing was already beyond the accepted CT value cut-off (Ct ≤37) of the PCR assay. Based on this validation study using diluted SARS-CoV-2 RNA transcript, low viral load or high Ct values in both SARS-CoV-2 and endogenous internal control targets were observed from non-conventional samples. Therefore, those

exhibiting CT values beyond the established cut-off values of the study for positivity were retested to increase confidence in the results, especially for testing actual samples. Based on the product IFU (BGI-STP-WHO-01-14v.6)[15], samples yielding CT values beyond the cut-off for positivity but with a sigmoidal-shaped amplification curve upon repeat-testing were considered positive for SARS-CoV-2 viral RNA but with a note that these possibly contain the low viral load. They can be reported as positive if the repeat result has a CT value greater than 38 and with S-shape curve. The consistent occurrence of amplifications in the duplicate testing indicates that the sample contains a low viral load concentration beyond the detection limit. Moreover, the endogenous internal control target's cut-off values were adjusted from CT ≤32 to CT ≤35[16] due to the non-conventional specimens' observed low viral RNA yield.

During the specimen sampling optimization stage, direct body fluid samples of the amniotic fluid and breast milk were also tested. However, variable results were obtained (data not shown); thus, the decision to adopt swabs across the different specimens.

The endogenous internal control (IC) of the BGI PCR kit uses the human housekeeping gene β-actin to monitor sample quality and extraction procedure. The MGI Easy Extraction kit used in this experiment which utilizes magnetic beads to extract RNA material from respiratory samples demonstrated that it can also be used to extract nucleic acids from non-conventional swab samples used in this study. All the non-conventional samples showed valid IC with CT values less than the cut-off (CT ≤35).

SARS-CoV-2 PCR detection in Cervical (CS), Rectal (RS), Amniotic Fluid (AF), Placental (PL), Umbilical Cord Blood (CB), and Breastmilk (BM) Swabs

A total of 572 non-conventional samples collected from 109 pregnant and 47 non-pregnant participants were analyzed. Of these 156 participants, only ten pregnant (6.4%) and 26 non-pregnant (16.7%) were confirmed PCR-positives in their NPS/OPS samples. Table 2 shows the distribution of non-conventional samples collected from the five study sites. The highest number of non-conventional samples collected were rectal swabs followed by cervical swabs. In contrast, breastmilk swabs were the lowest number of non-conventional specimens collected (Table 2). Of the 572 non-conventional samples collected, ten (1.8%) tested positive for SARS-CoV-2, including five (0.9%) rectal swabs, one (0.2%) placental swab, and four (0.7%) cervical swabs.

Table 3 shows the summary profile of the seven patients that showed positive results in this RT-qPCR method and their disease status. Among the seven patients, four were pregnant and three were non-pregnant. Notably, one patient who was NPS/OPS COVID-19 negative had a positive result for a rectal swab. In addition, positive non-conventional samples generated high CT values in most rectal and cervical swabs indirectly indicating that SARS-CoV-2 viral RNA is present in low concentrations (weak positives). However, two samples from patient 4 showed a high viral concentration in the cervical and placental swabs that tested positive for SARS-CoV-2.

Table 1. SARS-CoV-2 Detection in Contrived Non-conventional Specimen Results of the Validation Study

Specimen Swab	1:10			1:100			1:1000		
	Mean CT	SD	Replicate Detection	Mean CT	SD	Replicate Detection	Mean CT	SD	Replicate Detection
Cord Blood	32.83	0.90	3/3	35.93	1.22	3/3	36.63	0.60	3/3
Amniotic Fluid	33.37	0.40	3/3	37.53*	0.56	2/3	38.33*	0.62	2/3
Placenta	32.68	0.49	3/3	34.84	0.52	3/3	N/A	N/A	0/3
Breast milk	32.04	0.37	3/3	36.31	0.13	3/3	37.93*	N/A	2/3
Rectal	31.75	0.72	3/3	35.68	0.19	3/3	38.09*	0.22	3/3
Cervical	33.68	0.40	3/3	36.90	0.74	3/3	N/A	N/A	0/3

**Samples were re-tested to confirm positive results*

Table 2. Distribution of Non-conventional Samples Collected from the Five Study Sites (N=572)

Non-conventional Samples	Hospital					Total
	FMMC	JASGH	JRMMC	OMMC	SAH	
Amniotic Fluid Swab (AF)	27	13	22	13	19	94 (16.4%)
Breastmilk Swab (BM)	19	6	15	7	17	64 (11.2%)
Cord Blood Swab (CB)	27	12	22	13	18	92 (16.1%)
Cervical Swab (CS)	33	11	24	9	23	100 (17.5%)
Placental Swab (PL)	24	13	20	9	19	85 (14.9%)
Rectal Swab (RS)	46	21	32	13	25	137 (23.9%)
Total	**176**	**76**	**135**	**64**	**121**	**572**

Table 3. Summary Profile of the Seven Patients that Showed Positive Results in RT-qPCR Testing of Non-conventional Samples, and their Disease Status

Sample No.	Pregnant Status	NPS/OPS COVID-19 Status	Positive Non-conventional Sample	Mean CT values (SARS-CoV-2) Orf1ab gene
Patient 1	Pregnant	Positive	Rectal swab	36.21
Patient 2	Non-pregnant	Positive	Rectal swab	37.94*
Patient 3	Pregnant	Negative	Rectal swab	32.08
Patient 4	Pregnant	Positive	Rectal swab	33.76
			Cervical Swab	27.03
			Placental Swab	25.93
Patient 5	Non-pregnant	Positive	Rectal Swab	38.24*
			Cervical swab	33.12
Patient 6	Non-pregnant	Positive	Cervical swab	35.74
Patient 7	Pregnant	Positive	Cervical swab	33.77

**Samples were re-tested to confirm positive results*

DISCUSSION

This study conducted a method validation wherein the Real-time RT-PCR protocol for detection of SARS-CoV-2 in NPS/OPS was verified if it is also applicable for detection of the virus in non-conventional samples such as cervical, rectal, amniotic fluid, placental, umbilical cord blood, and breastmilk swabs. In molecular diagnostics, assay validation or verification is required before a detection method is being used for routine testing. Concordance testing, determining the assay's analytical and diagnostic sensitivity and specificity, or testing several samples can be performed to validate the applicability of an assay.[17-20] An initial method validation study was conducted which includes testing of contrived non-conventional samples in various relative viral concentrations to estimate the assay's analytical sensitivity and testing a certain number of non-conventional samples to verify the assay's applicability.

Our study provided that SARS-CoV-2 transcript spiked in non-conventional samples such as rectal, cervical, cord blood, amniotic fluid, placental, and breast milk swabs in SANLI UTM could be detectable in the Real-time reverse transcriptase polymerase chain reaction testing. The SARS-CoV-2 transcript was detectable at 1:100 dilution in all the non-conventional samples. The non-conventional samples exhibited higher CT values than to the dilutions in nuclease-free water, possibly indicating that PCR inhibitors may be present in non-conventional samples which may contribute to the decrease in viral load or RNA degradation. Nevertheless, our method validation study results suggest that SARS-CoV-2 viral RNA can be detected in non-conventional specimens, and more studies are warranted to validate our findings.

In addition, patient samples also showed that SARS-CoV-2 viral RNA could be detected in non-conventional specimens such as rectal, placental, and cervical swabs. Our finding of SARS-CoV-2 positive results in the rectal swabs was in agreement with a study that detected the virus 27% (9/34) of the rectal swabs collected from pregnant women in the third trimester.[21] Furthermore, SARS-CoV-2 detection in stool specimens has already been confirmed by numerous studies that investigated other routes of viral transmission.[4,22-24] These findings suggest a higher likelihood of SARS-CoV-2 detection in rectal swabs, and anorectum can be a potential route for mother-to-child transmission during normal delivery.

To our knowledge, there are still no reported cases of SARS-CoV-2 detection in cervical swabs. Although SARS-CoV-2 detection in vaginal swabs and vaginal mucosa samples was reported, this detection suggests the likely persistence of the virus in the female reproductive tract.[25,26] The detection of SARS-CoV-2 in cervical swabs collected in our study provided additional evidence of the presence of the virus in the birthing pathway. Moreover, the ACE-2 receptor mode for host cell viral entry and replication is also found in the cervix in low concentrations.[27]

Our study detected SARS-CoV-2 in placenta (1.2%; 1/85), which was also previously reported by a study that found 3 out of 11 placental swabs tested positive for the pathogen collected from severe to critically ill mothers at the time of delivery.[10] Multiple studies confirmed the detection of SARS-CoV-2 in samples other than NPS/OPS. However, the positivity rates were low.[4,25-27] Interestingly, no fetal-associated specimens (i.e., amniotic fluid or cord blood swabs) turned out positive, given the sample size analyzed in our study. The ten samples that turned out positive in our study were all associated with the maternal specimens, including the rectal, placental, and cervical swabs. Furthermore, the ability of this RT-qPCR method to indirectly detect low concentration templates is comparable across the different sample types (See Table 1). Nonetheless, our finding of negative SARS-CoV-2 in fetal-associated specimens should be validated in future studies.

Our study is the first reported evidence in the Philippines wherein SARS-CoV-2 viral RNA was detected in the cervical, placental, and rectal swabs of COVID-19 patients.

Additionally, SARS-CoV-2 viral RNA may persist even in low concentrations in non-conventional samples such as rectal, placental, and cervical swabs, as indirectly evidenced by the results generated in our validation study. Whether the virus detected in the rectal, placental, and cervical swabs can be transmittable to the fetus during or post-delivery needs further validation. Although reports of fetuses being SARS-CoV-2 positive have been made[28], this study could not detect such, given our limited sample size. The case likely depends on several factors such as the event's rarity, timing, and course of infection during sample collection, its dependence on the viral load or the emergence of a more infectious variant.

CONCLUSION

The RT-qPCR method detected SARS-CoV-2 transcript spiked in all non-conventional samples, including the rectal, cervical, cord blood, amniotic fluid, placental, and breast milk swabs in SANLI UTM. SARS-CoV-2 transcript may be detectable at 1:100 dilution in all the non-conventional samples. The ability to indirectly detect low-concentration templates was comparable across the different sample types. Applying the RT-qPCR test to 572 patient samples suggest that SARS-CoV-2 viral RNA may be detected in non-conventional specimens particularly the rectal, placental, and cervical swabs. In addition, positive samples may be likely associated with the maternal specimens and not with fetal-derived specimens. Moreover, SARS-CoV-2 viral RNA may persist even in low concentrations in non-conventional samples such as rectal and cervical swabs.

The validation approach can be applied to similar RT-qPCR diagnostics utilizing non-conventional samples from maternal and fetal tissues/body fluids. Furthermore, the validated method can serve as an additional diagnostic layer to support maternal-child care during pandemic scenario.

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Institutional Review Board Statement

The study was conducted in accordance with the International Ethical Guidelines for Health-related Research Involving Humans, and approved by the UP-Manila Research Ethics Board and the Single Joint Research Ethics Board (SJREB 2020-30).

Informed Consent Statement

Informed consent was obtained from all subjects involved in the study.

Data Availability Statement

Data is available on request due to privacy restrictions. The data presented in this study are not available because they are in the process of analysis for results publication.

Statement of Authorship

All authors certified fulfillment of ICMJE authorship criteria.

Author Disclosure

All authors declared no conflicts of interest.

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