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ORIGINAL ARTICLE

Comparative evaluation of cost effective extraction free molecular technique for detection of SARS-CoV-2 with reference to standard VTM based RT-qPCR method

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ABSTRACT

Background and Objectives: The entire globe is undergoing an unprecedented challenge of COVID-19. Considering the need of rapid and accurate diagnostic tests for SARS-CoV-2, this study was planned to evaluate the cost effective extraction free RT-PCR technique in comparison to the standard VTM based RT-qPCR method.

Materials and Methods: Paired swabs from nasopharynx and oropharynx were collected for SARS-CoV-2 testing, from 211 adult patients (≥18 years) in VTM and plain sterile tubes (dry swabs). These samples were processed and RT-qPCR was carried out as per standard protocols.

Results: 54.5% of the patients were females and 45.5% were males with sex ratio 1:1.19 (M: F). 38.86% were symptomatic, of which fever (86.59%), cough (79.23%) and breathlessness (46.34%) were the most common symptoms. The positivity by VTM based method and index method was 31.27% and 13.27% respectively. Of the 27 inconclusive results from index method, 37.04% were positive, 48.15% were negative by VTM based method. However, in 40 inconclusive results by VTM based method, 90% were negative and rest remained inconclusive by index method. The sensitivity and specificity of the index method were 39.39% and 85.71% respectively. The overall agreement between VTM based method and index method was 49.59% with estimated Kappa value of 0.19.

Conclusion: VTM based method showed higher sensitivity compared to the index method. The higher positivity by VTM based method, suggests that VTM based method could plausibly be a better detection method of SARS-CoV-2. Still, the index method might add value in a resource limited setups for detection of SARS-CoV-2.

Keywords: COVID-19; SARS-CoV-2; Coronavirus; Molecular diagnostic; Real time polymerase chain reaction

INTRODUCTION

Coronavirus disease (COVID-19), which originated in the Wuhan province of China, was declared as a global pandemic by the World Health Organization (W.H.O) (1), remains a significant problem involving health systems worldwide. COVID-19 is caused by severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2), which is a new member of the coronavirus family (2).

Till date nearly 19.7Cr confirmed cases and 42L deaths of COVID-19 have been reported worldwide. In India, till last week of July 2021, more than 3.16Cr cases and 4.23L deaths have been recorded due to SARS-CoV-2. Rapid transmissibility of SARS-CoV-2 reached globally and the disease necessitates prompt and precise laboratory diagnosis, so that appropriate containment measures as well as manage-

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ment practices could be ensured (3).

Public health experts have emphasized testing as many persons as possible, tracking infected people, and tracing their contacts as an efficient strategy to diminish the spread of the virus (4). Most of the governments across the globe are exercising this practice to variable extent using an array of testing methods (4). Accurate and early detection of SARS-CoV-2 infection is critical for minimizing spread and initiating treatment (5).

The molecular detection methods involve the laboratory analysis of nucleic acids present in the sample to identify the virus (4). Nucleic acid amplification based molecular tests are more specific and hence is preferred for diagnosis of SARS-CoV-2 (6). Currently, the most commonly used laboratory detection method for the clinical diagnosis of COVID-19 is RT-qPCR (4) as recommended by the World Health Organization (WHO) (7). Accurate diagnosis of SARS-CoV-2 depends on the stage of the disease, the quality of sampling, type of specimens collected and sample handling (collection, storage and transportation) (6).

The RT-qPCR method, presently followed in the on-going public health programs in India, involves RNA extraction. RNA extraction is a technologically demanding step, which requires expertise and is prone to cross contamination. RNA extraction procedure also demands time and resources. Different preliminary studies tested the performance of RT-qPCR approach eliminate the requirement for use of VTM at the sample collection site and RNA extraction step in the laboratory, showed comparable results with the reference method (8, 9).

Elimination of RNA extraction step in one-step RT-PCR method reduced the cost of test significantly, saved time and other resources and reduced the need for expertise pertaining to RNA extraction. The present study was designed to evaluate the performance of this newer approach, (henceforth termed index method) against the standard VTM based technique. The overall purpose was to critically examine the accuracy of the dry swab based RNA-extraction-free RT-qPCR technique. Therefore, the main objective of this study was to estimate the adequacy of RNA yield and to evaluate the feasibility/ease of use of Dry Swab based RT-qPCR (index method) compared with the standard viral transport medium (VTM) based RT-qPCR method (reference method).

MATERIALS AND METHODS

Study design. This study was conducted at a tertiary care centre of New Delhi, India from 11th August to 7th September 2020, for the assessment of the index method compared to the reference standard method of sample followed by RT-qPCR reaction. The study participant was clinic attendees came for SARS-CoV-2 infection testing.

Ethic approval. Ethical clearance for this study was obtained from the Central Ethics committee for Human Research (CECHR) and Institutional Ethics Committee (IEC).

Sample size. Sample size was calculated by following exact binomial confidence limit method. The binary outcome (yes/no), in the form of presence or absence of SARS-CoV-2 infection generated by the index method was assessed. Assuming that the index method would have expected sensitivity of 0.95 and a lower 95% confidence limit would be allowed at 0.85 with 0.95 probability. Thus the total number of patients required was ~250.

Masking of samples. This study was blinded and no information made available to any of the testing laboratory technician, neither to any of the site investigators. Each patient was registered and a unique random code was generated by the link staff at the collection centre used for the dry tube. Only assigned link staffs were privy to such information that could unmask the blinding during final analysis.

Enrolment and sample collection. Each consenting clinic attendee was registered on ICMR portal and invited to participate in the investigation. Adult patients (≥18 years) came for SARS-CoV-2 infection testing was included and those, not willing to provide two additional nasopharyngeal/oropharyngeal samples were excluded from the study. Two nasopharyngeal swabs and two oropharyngeal swabs were collected from each enrolled patient. Autoclavable, unbreakable and leak proof 10 ml screw capped sample collection tubes/vials were used for dry swabs. The participant information sheet (PIS) was handed over and a written informed consent (CIF)was obtained. Demographic information along with brief clinical history was collected. “Specimen Collection, Packaging and Transport according to standard guidelines

for 2019 Novel Coronavirus (2019-nCoV)", developed by ICMR was followed.

Specimen processing. The labelled VTM tubes and dry tubes were processed on the same day as per study protocol. Fig. 1 schematically presents the steps followed in two methods that were compared in the present investigation.

Dry swab based RNA extraction free method (index method). The index test use samples collected as dry nasopharyngeal swabs transported without VTM. The swab was transferred to the testing laboratory for addition of 400µl of TE buffer and incubated for 30 minutes at room temperature. After 30 minutes, 50 µl of TE buffer was aliquoted from each sample tube into PCR plate/PCR tubes. PCR plates sealed and heated at 98°C for 6 minutes in the RT-PCR machine (CFX96, Biorad). This TE buffer extract from PCR plates/PCR tubes was directly used for RT-qPCR.

RNA extraction from VTM tube (reference method). RNA extraction from the VTM tube (contains Hanks Balanced Salt Solution (HBSS), heat-inactivated fetal bovine serum or serum protein components such as bovine albumin fraction and antibiotics) sample was carried out using "RNA extraction QIAmp viral RNA Mini Kit" (Qiagen India Pvt. Ltd.) as per manufacturer's protocol.

Real time–RT-qPCR. The RT-qPCR was carried out using the "ICMR NIV Multiplex Single Tube Real time –PCR-Version 3" kit as per the manufacturer's instructions. ICMR NIV Multiplex Single Tube Real time –PCR Kit-Version 3, contains a set of TaqManTM RT-PCR assays for the qualitative detection and characterization of SARS-CoV-2 RNA. The kit includes two targets of SARS-CoV-2 genes, and one house keeping gene B Actin gene. All the reactions were multiplexed and an amount of 7 µl of the template RNA was used per reaction. Discordant results between the VTM tube and dry tube samples,

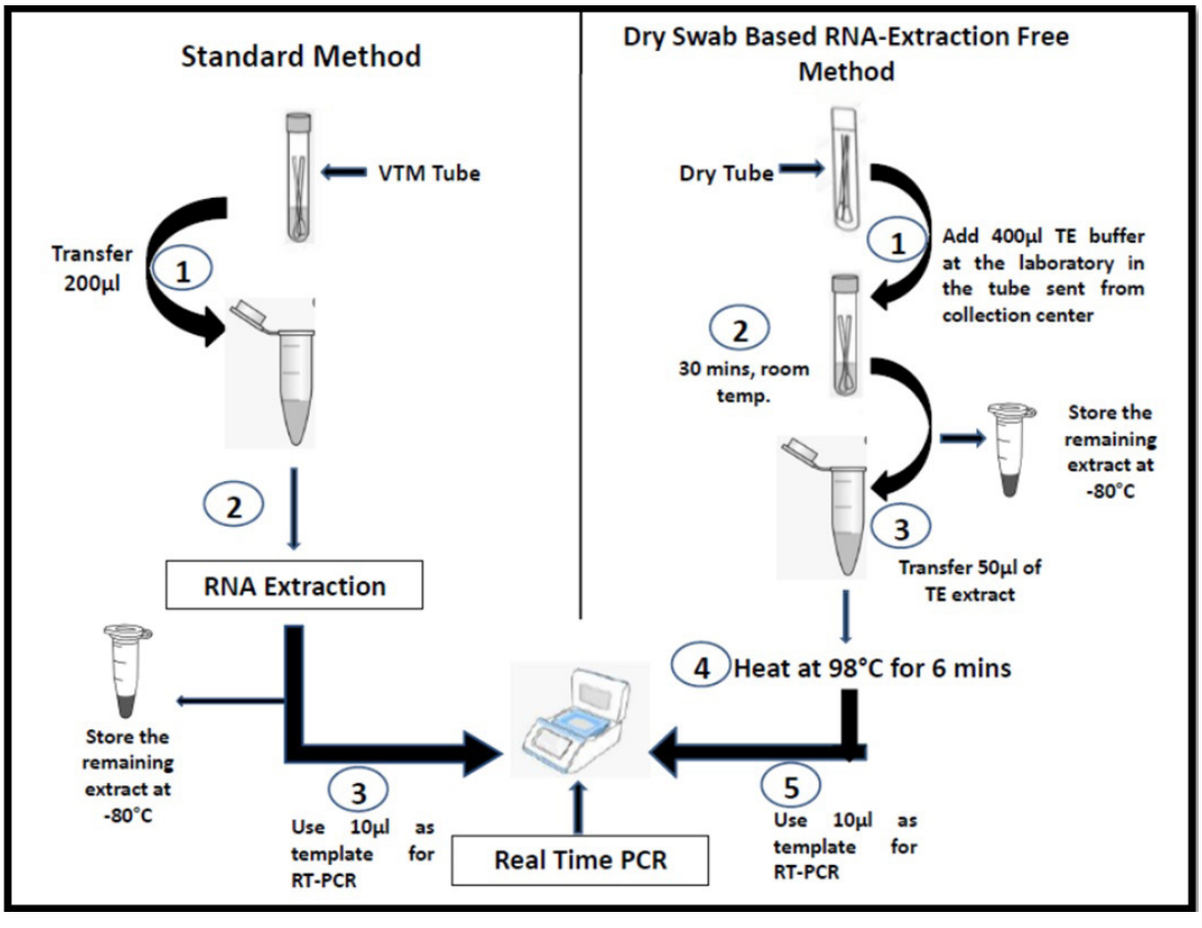

Fig. 1. Comparison of VTM tube and dry tube based techniques

RT-qPCR was repeated for negative samples using the ICMR-NIV kit by "Single tube Uniplex assay" using E gene and ORF gene primers in separate PCR tubes (Fig. 2).

Interpretation of RT-qPCR results. The RT-qPCR data generated as cycle threshold (Ct) values, were interpreted as per the ICMR SOP "Detection of 2019 novel coronavirus (2019-nCoV) in suspected human cases by Multiplex Single Tube Real Time – PCR: Version 3", as positive, negative, inconclusive or invalid (Table 1). The results were recorded in a blinded manner. The following algorithm was used for interpretation of outcome.

Statistical analysis. Sensitivity, specificity, concordance, discordance, positive predictive value and negative predictive values were estimated. Both

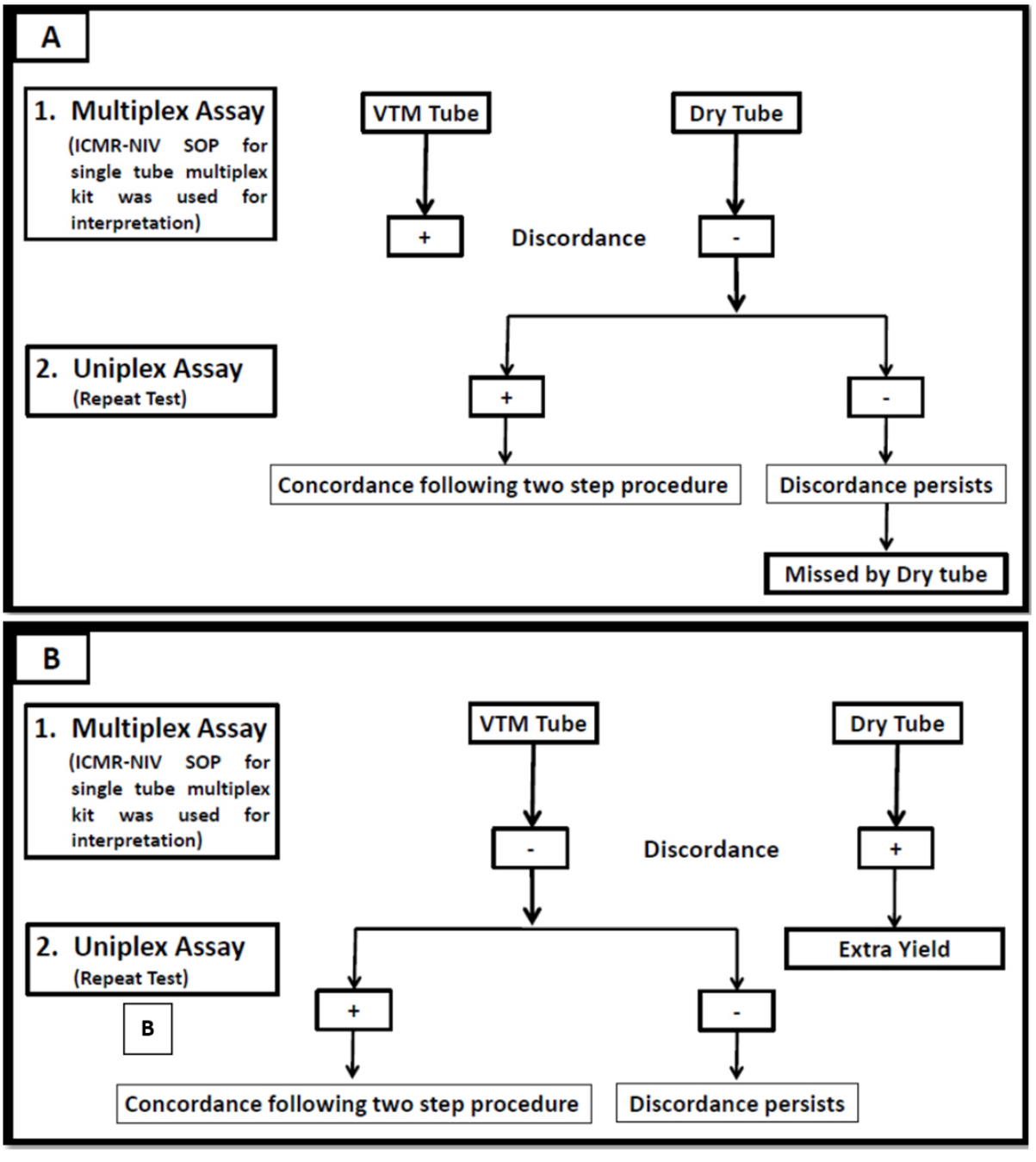

Fig. 2. Algorithms used for resolving; (A) discrepant condition 1, (B) discrepant condition 2.

Table 1. Interpretation of RT-PCR results

Beta Actin	E gene	ORF gene	Result Interpretation
+	+	+	Positive Positive
-	+	+	Inconclusive
+	+	-	Inconclusive
+	-	+	Negative
+	-	-	Invalid
-	+	-	Invalid
-	-	+	Invalid
-	-	-	

parametric and non-parametric tests were used an appropriate using STATA (version 13.1). Standards for Reporting of Diagnostic Accuracy Studies (STARD) guidelines were followed to present the study findings.

RESULTS

Two hundred and fifty (250) participants were enrolled in the study. Out of 250 samples, 244 were analysed, 33 were invalid by Dry Swab based RT-qPCR and 3 were invalid by VTM based RT-qPCR. One sample was invalid by both methods. Hence, results from 211 samples were included for descriptive analysis. Fig. 3 presents a schematic diagram of summary sample flow chart.

About 54.5% of the study participants were females (115/211) and 45.5% were males (96/211). The median age was 35 years for males (mean=38, SD=13.6) and 30 years for females (mean=35, SD=13.2). While the minimum age was 18 for males and 19 for females, the maximum age was 68 for males and 85 for females. Twenty-fifth percentile age was 26 years for males and 30 years for females and the 75th percentile was 51 years for males and 43 years for females. Table 2 presents frequency distribution of study participants across various age class intervals (Table 2). Symptomatic participants represented 38.86%, of which fever (86.59%), cough (79.23%) and breathlessness (46.34%) were the most commonly presenting symptoms.

About 31.27% (66/211) of the participants tested positive by VTM based RT-qPCR method, whereas about 13.27 % (28/211) were detected to be positive by the index method. Fig. 4 presents a comparative bar chart of outputs obtained through both the methods.

Of the 27 samples showing inconclusive results from dry swab, 37.04% were detected as positive (10/27), 48.15% were negative (13/27) and 14.81%

Table 2. Age wise distribution of study participants (n=211)

Age class interval (year)	Frequency (%)
18-30	94 (45)
31- 40	45 (21)
41-50	33 (16)
50-60	28 (13)
>60	11 (5)
Total	211

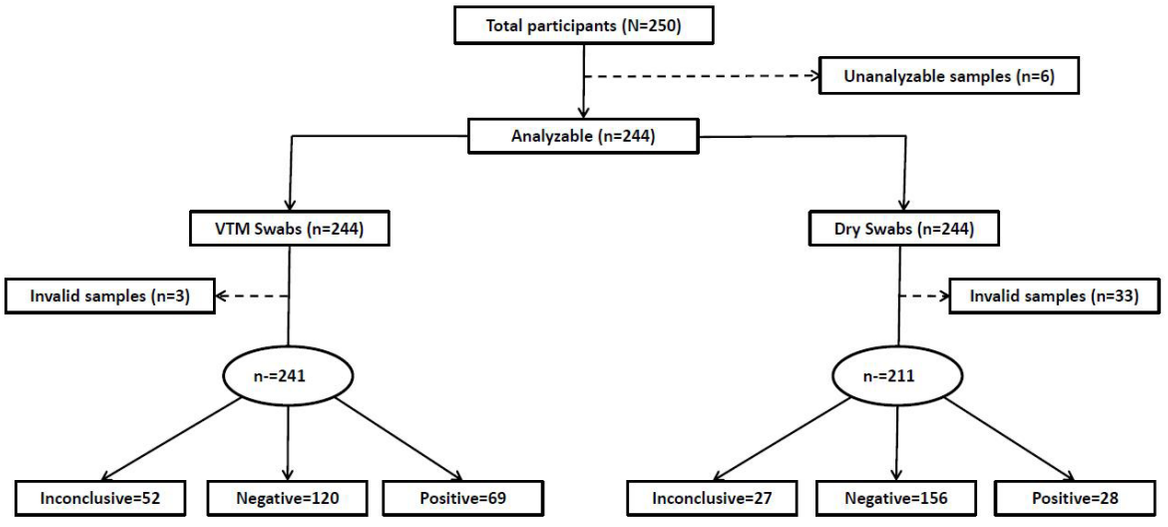

Fig. 3. Schematic diagram of summary sample flow chart

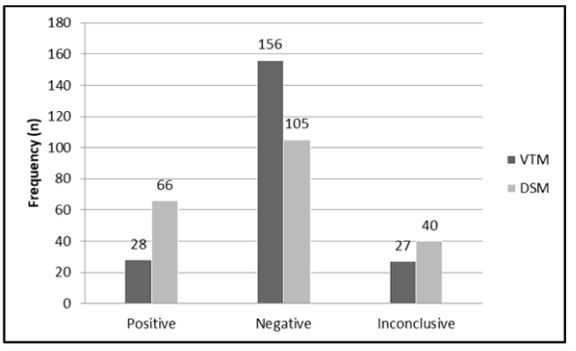

Fig. 4. Comparison of VTM and dry swab method

(4/27) were inconclusive by VTM based RT-qPCR method. However, out of the 40 inconclusive results by VTM based RT-qPCR method, 90% were negative (36/40) and 10% (4/40) were inconclusive by dry Swab based RT-qPCR method (Table 3).

The sensitivity and specificity of the index method (Dry Swab based RT-qPCR method) were found to be 39.39% (26/66) and 85.71% (90/105) respectively (Table 3). The positive predictive value of Dry Swab based RT-qPCR method was 92.86% (26/28) and negative predictive value was 57.69% (90/156).

The performance of the index method (Dry Swab based RT-qPCR method) was assessed separately in a subgroup of 82 symptomatic participants. The sensitivity and specificity of the index method (Table 4) were found to be 51.43% (18/35) and 81.08% (30/37) respectively. The positive predictive value was 90% (18/20) and negative predictive value was 62.5% (30/48).

The overall agreement between VTM based RT-qPCR method and Dry Swab based RT-qPCR method was estimated using the weighted table (Table 5). The observed agreement was 49.59% (121/244) and the estimated Kappa value was 0.19 (95% CI 0.11 to 0.28, SE=0.042).

DISCUSSION

The ongoing COVID-19 pandemic has put exceptional strain on public health, hospital and commercial laboratories as they attempt to keep up with demands for SARS-CoV-2 testing (8). To address this issue, different preliminary studies tested the performance of a RT-qPCR approach eliminate the requirement for use of VTM at sample collection site and RNA extraction step in the laboratory (8, 9). Therefore, this study was designed to estimate the adequacy of RNA yields and to evaluate the feasibility/ease of use of Dry Swab based RT-qPCR (index method) compared with the standard VTM based RT-qPCR method (reference method).

To best of our knowledge there are paucity of such studies, however we tried our best to discuss this study with available few studies. According to avail-

Table 3. Performance of the index method in all participants

		Reference method (VTM)*			Total
		Positive	Negative	Inconclusive	
Index method	Positive	26	2	-	28
(Dry swab)	Negative	30	90	36	156
	Inconclusive	10	13	4	27
Total		66	105	40	211

* For calculation of sensitivity and specificity, the positive and negative test results obtained from VTM based detection method were considered as denominators respectively.

Table 4. Performance of index method in symptomatic clinic attendees

		Reference method (VTM)*			Total
		Positive	Negative	Inconclusive	
Index method	Positive	18	02	-	20
(Dry swab)	Negative	11	30	7	48
	Inconclusive	06	05	3	14
Total		35	37	10	82

Table 5. Agreement statistics for both diagnostic methods (N=244)

		Reference method (VTM)				Total
		Positive	Negative	Inconclusive	Invalid	
Index method	Positive	26	-	2	-	28
(Dry swab)	Negative	10	4	13	1	28
	Inconclusive	30	36	90	1	157
	Invalid	3	12	15	1	31244
Total		69	52	120	3	

able literature, in most of the studies SARS-CoV-2 found more in males than females (10-12). However, in our study females (54.5%) were more with sex ratio 1:1.19 (M: F). As most of the studies were male dominant indicated that the infection in females were lower than males ranging from 32.3% to 49.3% (12-16) whereas, our study suggesting slight female preponderance.

SARS-CoV-2 infects people of all ages. However, there are two main groups at a higher risk of developing severe disease: older people and people with underlying co-morbidities such as diabetes mellitus, hypertension, cardio-respiratory disorders, chronic liver diseases and renal failure (17, 18). The mean age of the patients in this study was 36.5 years (SD=13.4 years), ranging from 18 years – 85 years. In a study, evaluating data from 1,099 patients with confirmed COVID-19, observed that the mean age of the patients was 47 years (13). Another study by Chen et al., (2020) (12) observed that the average age of the patients was 55·5 years (SD=13·1).

COVID-19 presents varied clinical features, ranging from asymptomatic to ARDS. The most common symptoms at onset of COVID-19 include fever, cough, and shortness of breath (19). In this study majority of patients (61.14%) are asymptomatic and often presented without fever and many had normal X-ray findings. Symptomatic participants in our study represented 38.86%, in which fever (86.59%), cough (79.23%) and breathlessness (46.34%) were the most common presenting symptoms concordant with a study in which the main symptoms were fever (83%), cough (82%) and breathlessness (31%) (20). Another study by Jin et al, 2020 (16) also reported, fever (95.3%) and cough (65.1%) to be the most common symptoms.

In this study, about 31.27% (66/211) of the participants tested positive by VTM based RT-qPCR method, whereas about 13.27 % (28/211) were detected to be positive by the Dry Swab based RT-qPCR method, which was too low in comparison to VTM based RT-qPCR method. One of the reasons of lower positivity in our study may be that the other studies were done on positive cases. Other reasons for lower positivity by Dry Swab based RT-qPCR method may be due to the viral RNA present in the more dilute swab sample can be concentrated in VTM based RT-qPCR method (21); Direct heating of samples at 95°C for 10 minutes may delayed the detection of viral RNA; Direct addition of unprocessed swab samples decreases the test sensitivity (21).

In this study, of the 27 samples showing inconclusive results from dry swab, 37.04% were detected as positive, 48.15% were negative and 14.81% were inconclusive by VTM based RT-qPCR method. However, out of the 40 inconclusive results by VTM based RT-qPCR method, 90% were negative and 10% were inconclusive by Dry Swab based RT-qPCR method. In Kiran et al. (2020) (9) study, both the methods showed consistent results for 33 out of 40 samples (19 positives and 14 negatives) and differed for 7 samples. Bruce et al. (2020) (8) reported that the direct RT-qPCR (nasopharyngeal swab VTM preheated for 5 minutes at 70°C prior to RT-qPCR) correctly identified 92% of samples (n = 155) as positive for SARS-CoV-2 RNA by RT-qPCR.

In this study, overall sensitivity and specificity of the Dry Swab based RT-qPCR method were found to be 39.39% and 85.71% respectively, whereas in symptomatic patients were found to be 51.43% and 81.08% respectively. However, in a study with direct RT-qPCR (nasopharyngeal swab VTM preheated for 5 min. at 70°C prior to RT-qPCR) approach has a sensitivity of 95% on samples (8). Another study with the Direct method (nasopharyngeal swab VTM without additives were subject to a direct 70°C incubation for 10 min.) yielded a sensitivity and specificity of 87.8% and 100% respectively (22).

In this study, the positive predictive value of Dry Swab based RT-qPCR method was 92.86% and neg-

ative predictive value was 57.69%. However, in a study by Bruce et al. (8) the positive predictive value was 100%, given that no false positives were observed and the negative predictive value was ranging from 97.4% to 99.8%.

The overall agreement (49.59%) between VTM based RT-qPCR method & Dry Swab based RT-qPCR method and the estimated Kappa value (0.19) of this study indicated a poor concordance. Although the Dry Swab based RT-qPCR method described here would not have sufficient overall agreement, kappa value and sensitivity to detect individuals most likely to be infectious, could also be easily adopted in more resource limited settings, together with large portions of the developing world that at present entirely lack access to RNA extraction. A testing method that uses patient samples directly without RNA extraction would open up possibly the only viable avenue for widespread testing in these regions (8).

We suggest that the Dry Swab based RT-qPCR method approach could be useful in regions of the world that have some degree of access to RNA extraction kits to perform the recommended CDC or WHO clinical RT-qPCR test, this approach could be used as a screening strategy to supplement testing capacity to those who are not currently receiving tests and use it as a means to implement additional testing capacity (8). Further studies required to ascertain optimal swab sample lysis, heating and storage conditions, as well as whether it could be employed in tests other than RT-qPCR (21).

Considering the present situation of SARS-CoV-2 pandemic, we need to have more robust diagnostic modalities with a low turnaround time. Molecular diagnostic methods demand high quality assurance with multiple steps in costly equipment & consumables during a sample processing. Using a molecular technique which is less time consuming such as the "dry swab method" in the present study might add value to the diagnosis of SARS- CoV-2 in terms of turnaround time and cost.

CONCLUSION

Our results showed that the VTM based RT-qPCR method had higher sensitivity compared to the Dry Swab based RT-qPCR method. The higher detection of infection by the VTM based RT-qPCR method, compared to the Dry Swab based RT-qPCR method, suggests that VTM based RT-qPCR method could plausibly be a better detection method at this point of time. Still, we see a possibility of using this Dry Swab based RT-qPCR method as an alternative in more resource limited settings, together with large portions of the developing world that at present entirely lack access to RNA extraction kits and machines. More studies are needed with large cohort to come to a conclusion for this promising dry swab based extraction method for SARS-CoV-2 detection.

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