ORIGINAL RESEARCH PAPER
Volume - 10 | Issue - 09 | September - 2021 | PRINT ISSN No. 2277 - 8179 | DOI : 10.36106/ijsr
INTERNATIONAL JOURNAL OF SCIENTIFIC RESEARCH

A COMPARATIVE ANALYTIC STUDY TO DISCERNMENT OF SARS-CoV-2 BY FOUR DIFFERENT MULTIPLE MOLECULAR In VITRO DIAGNOSTIC ASSAY.

Microbiology

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ABSTRACT

INTRODUCTION: In December 2019, a novel pneumonia related to the 2019 Coronavirus unexpectedly developed in Wuhan, Hubei provinces, China. The clinical presentation of Covid-19 is fairly nonspecific, symptoms overlap those of other seasonal respiratory infections, concurrently circulating in the population. Timely and accurate laboratory diagnosis of SARS-CoV-2 can significantly impact patient management, which is critically important to infection control measures aimed to curb the pandemic within communities & hospitals.

AIMS & OBJECTIVE: TO evaluate the clinical performance of different molecular assay for the detection of Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2).

Assessment of the analytical performance & comparison of different kits by using same extraction methodology and viral genomic material

METHODS: In the present study ,we evaluated the analytical sensitivity & clinical performance of the four SARS-CoV-2 molecular diagnostic assay in Nasopharyngeal Specimen under this study, to compare the validation as well as analytic performance of four different molecular assay : TruPCR® SARS-CoV-2 RTqpcr, COVIDSURE Multiplex Real time RT-PCR, VIRALDTECT-II Multiplex Real time PCR, COVISURE Genetix COVID19 Real time PCR .

RESULT & CONCLUSION: This study indicates a comparable analytical performance of the four different assay for the detection of SARS-CoV-2 through Real time RT-PCR. A sensitivity and specificity of 100% showed by VIRALDTECT-II panel while 96.29% sensitivity & 96.87% specificity was observed by Trupcr. 92.59 & 100% 0f sensitivity & specificity showed by Covisure, 96.29% of sensitivity & 96.96% specificity was showed by Covidsure molecular diagnostic assay.

KEYWORDS

Covid -19, SARS-CoV-2, Analytical performance, nasopharyngeal specimen.

INTRODUCTION:

An outbreak of viral pneumonia with severe acute respiratory syndrome corona virus-like (SARS-CoV-2) symptoms emerged in the Hubei province ,Wuhan (Huanan seafood market) across China in December 2019 & it's became the major public health threats globally [1,2] with the starting of January in the year of 2020 . "W.H.O stated that the epidemic of the Severe Acute Respiratory Syndrome Coronavirus2 (SARS-CoV-2) become as a public health emergency of International concern (PHEIC)" [3]. On 11 March 2020, the classification of the SARS-CoV-2 epidemic was escalated to the level of a global pandemic by the WHO [4].At the time of writing this according to the worldwide statistics the death rate was > 4.6%.

The clinical presentation of COVID-19 encompasses a broad spectrum of illness, ranging from the asymptomatic to those with mild to severe respiratory illness. Symptoms at the onset can include fever, cough, shortness of breath, chills & loss of taste / smell[5] ,other than SARS-CoV-2 ,their are SARS-CoV, Middle East respiratory syndrome-CoV (MERS-CoV) and SARS-CoV-2 seems to be more efficiently &rapidly transmitted b/w Humans, resulting in a massive global pandemics,[6–9] whereas seasonal corona virus HKU1, NL63, OC43,229E are associated with mild symptoms [10]. Corona virus can be transmitted through several different routes, including aerosol, & fecal to oral routes , with fomites often playing a key role in the infection cycles[11] the SARS-CoV-2 spike protein recognizes binds to Human cells receptor angiotensin- converting enzyme 2.(ACE2 receptor found on epithelial cell of lung & small intestine) & subsequently mediates fusion of the viral & host cell membranes, allowing the virus to gain entry [12] [13].

Timely and accurate laboratory diagnosis of SARS-CoV-2 can significantly impact patient management, which is critically important to infection control measures aimed to curb the pandemic within communities & hospitals. Many clinical laboraties have implemented multiple molecular testing of SARS-CoV-2. In present study , we evaluated the clinical performance of 4 SARS-CoV-2 assay, TruPCR SARS-CoV-2 RTqpcr, COVIDSURE Multiplex Real time RT-PCR, VIRALDTECT-II Multiplex Real time PCR, COVISURE Genetix COVID19 Real time PCR using nasopharyngeal swabs from symptomatic patients. The analytical sensitivity of the 04 assay was compared using the same quantified genomic materials , our study may help to health care providers as well as public health officials. The overall accuracy of these four assay was compared using patient clinical specimen and the reproducibility was studied using contrived specimens.

MATERIAL & METHODS:

1. STUDY Design :- A TOTAL OF 91 Nasopharyngeal specimen for routine COVID19 testing were selected for this study, out of 91 specimen analyzed -27 of the samples were positive & 64 were negative. The study population included patients of all ages & both genders, presenting with sign / symptoms of COVID19 infections. Specimen evaluation done by the manufacturer's specification.

2. SPECIMEN COLLECTION & STORAGE :- Sterile nylon , Dacron , rayon swabs with flexible plastic shafts were used to collect Nasopharyngeal specimen (NPS)/ Oropharyngeal specimen (OPS) from symptomatic patients , After collection swabs were placed into 3ml of sterile viral transport medium & tested as soon as possible after collection, the specimen can be stored at 4 degree upto 48 hrs. after collection if any delay is expected it's recommended to store the specimens at -20degree or lower. Before testing samples were subjected to vortex , mixing for 3 to 5 seconds and a calibrated pipette was used to transfer the specimen volume specified by each manufacturer's instruction for use, following routine testing samples were aliquoted and stored in a freezer at -80degree until comparator testing.

3. Viral RNA Extraction:- Genomic viral RNA, isolated from nasopharngeal/ oropharyngeal swabs by using HipurA Super II pre-filled plates for Insta NX Mag96 in just 11 minutes. The user can process 96 samples by using this

- HipurA Super II pre-filled plates for Insta NX Mag 96:- This kit carries out efficient extraction of viral RNA from wide range of

viral strains. Sample is first lysed under the highly denaturing conditions provided by lysis solution (c1) to inactivate RNase & to ensure isolation of intact viral RNA .All the specimen were processed in BSL-2 using BSL-2 biosafety measures and after the initial binding of RNA , impurities like proteins, polysaccharides , low molecular weight metabolites & salts are removed by short washing steps. High quality RNA is finally eluted in the elution solution provided with the kit.

- ELUTION:- The yield of RNA depends on the sample type & the number of cells in the sample. A single elution with 50µl of Elution solution will provide sufficient RNA to carryout multiple amplification reactions.
- Storage of the eluate with purified RNA:- The recommended storage temperature for the eluted RNA is -80©. Avoid repeated freezing & thawing of the sample , which may cause denaturing of the RNA.

4. AMPLIFICATION & DETECTION:-

S.N	Molecular assay	Sample type	Target gene	RNA extraction	Volume of RNA template/ Each PCR Reaction(µl)	Thermal cycling condition	Reaction time (min.)	Total cycles	Ct value
1.	VIRALDTECT-II Multiplex RTPCR	OS/ Sputum	E/RdRp/N gene	Automated	9µl/20µl	10min. at 55©,3min.at 95©and 15s at 95©,60s60©	92min.	40	37
2.	TRUPCR®SARS-CoV-2 RTqPCR	NS/OS,BAlN/ O aspirates	E,RdRP+N gene	Automated	10µl/25µl	15 min. at 50©,5min.at 95©,5s at 95©, 40s at 60©, 15s at 72©	89min.	38	35
3.	COVIDsure Multiplex Realtime RT-PCR	NS/OS, Oropharyngeal wash, sputum, E.Aspirates, BAL	E, Orf1ab	Automated	5µl/20µl	15min. at 46©,2min at 95©,10sat 95©,30s at 58©	81min.	40	36
4.	COVISure Genetix covid 19 Real Time PCR Kit	NS/OS, Sputum	E,RdRP	Automated	5µl/20µl	15 min at 50©,3min at 95©,10s at 95©,30s at 60©	89min.	45	36

Ct; cycle threshold, OS; Oropharyngeal swab, NS; Nasopharyngeal swab, BAI ; Broncho alveolar lavage, N/O; Nasopharyngeal/ Oropharyngeal.

- **VIRAL DTECT-II MULTIPLEX Real Time PCR Kit for CoVID-19**

This product is designed as a multiplex real time reverse –transcription PCR system, containing specific primers & fluorescent probes targeting Rdrp, E, N gene of SARS-CoV-2 . For each specimen , a single multiplex set that included E gene, Rdrp gene, N gene & RNase P were prepared and 11µl of each master mix was dispensed into appropriate wells followed by 9µl of extracted sample RNA, Each run also included a no- template control (NTC) & a SARS-CoV-2 Positive control. The detectors used are FAM(E), Texas Red (Rdrp),Cy5(N), HEX(RNaseP) Amplification was performed on Biorad CFX-96,at the following cycling conditions : 1 cycle at 55© for 10 minutes,1 cycle at 95© for 03 minutes and 40 cycles at 95.0©for 15s,then 60© for 60 s. The result interpretation algorithm for reporting a positive specimen requires E gene , Rdrp gene, N gene targets to be detected.

- **COVISure Genetix Covid 19 Real Time PCR Kit:-**

Covisure is a real time PCR based diagnostic kit for the qualitative detection of novel Corona virus SARSCoV-2 RNA from nasopharyngeal specimen/ oropharyngeal specimen , sputum samples from patients. It's recommended to use a standard viral RNA extraction kit (Magnetic bead or spin colmun based) for viral RNA isolation, testing was perfomed according to the manufactrer's instruction for use. A 6µl Covisure RT master mix was added followed by PPMix A(3µL), PPMix B (3µl) and PPmix C (3µl), so the final volume of master mix (per reactions) was 15µl Dispense 15µl from above master mix in separate wells and add 5µl of isolated RNA from patient's sample separately to each tube , The detectors used FAM (E), Quasar670(Rdrp), HEX (Internal control) .Amplification was performed on Biorad CFX96 , reverse transcription activity at 50© for 15 min., initial denaturation at 95© for 3 min, denaturation at 95© for 10 s, annealing at 60© for 30s all over 45 cycles. Each run also included a no- template control (NTC) & a SARS-CoV-2 Positive control the result interpretation algorithm for reporting appositive specimen requires both E & Rdrp or Rdrp to be detected.

- **TRUPCR®SARS-CoV-2 RTqpcr kit:**

It's an invitro nucleic acid amplification test for the qualitative detection of severe acute respiratory syndrome Corona virus 2 specific RNA from respiratory specimen. NS/OS , bronchoalveolar lavage, tracheal aspirates, N/O aspirates & sputum.For 15µl reactions mix preparation add 10µl of master mix followed by 0.35µl add enzyme mix & 4.65 µl primer probe mix. Transfer this 15µl of prepared reaction mix in 0.2ml pcr tubes and close them , add 10µl of RNA samples /pc/nc to make up the final volume 25µl. Detectors used are Texas Red/ROX/Orange (E gene), FAM/Green (Rdrp+N gene),HEX(RNaseP). Biorad CFX 96 Real time RTPCR system was used at the following conditions: 1 cycles at 50© for 15 min., 1 cycles at 95© for 05 min., and 38 cycles at 95© for 05s, 60© for 40s, then 72© for 15s. The result interpretation algorithm for reporting a positive specimen requires both target E & Rdrp or only Rdrp should be detected, under threshold cutoff cycle ct≤35.

- **COVIDSURE MULTIPLEX Real time RT-PCR KIT BY TRIVITRON LABSYSTEMS DIAGNOSTICS.:-**

This kit uses real time PCR technology based upon synthetic DNA primers & fluorescence labeled hydrolysis probes for 15 µl reaction mix preparation add 10µl 2Xrt-pcr Mix, followed by 2µl of primer-probe mix (Orf1ab-Fam, E-Hex, RPP30-Rox) and 3µl of RNase free water. Transfer 15µl of master mix in each well of 96 well plate or PCR tubes , avoid formation of bubbles while dispensing the master mix , add 5µl of sample RNA in each well. The detectors used in Biorad CFX96 was FAM (Orf1ab), ROX (RPP30 as house keeping gene / I.C.), HEX(E) and the cycling conditions as follow: Reverse Transcription at 46© for 15 min. Initial activation at 95© for 02 min. Annealing and signal acquisition at 58© for 30s in 40 cycles.

S. No.	SARS-CoV-2 RT Qpcr kit	Catalogue No.	Reaction Volume required	Limit Of Detection (LOD)
1.	VIRALDTECT-II Multiplex Real time PCR(Gene2me)	G2M020220	20µI	10 Copies/µI
2.	TRUPCR®SARS CoV-2 RTqPCR (V-3.2)	3B306	25µI	≤5 Copies/µI
3.	COVISURE (Genetix)RTPCR	COVIZONE-100	20µI	1X10³Copies/ µI
4.	COVIDSURE Multiplex RTPCR (Trivitron)	500-30201284	20µI	10 Copies/µI

RESULT: A total 91 samples were included in the study . In the following table was showing number of male & female , out of 91 samples by the four different kits.

- **Demographic analysis of total 91 samples included in the study:**

N=91	VD-2		Trupcr		COVISURE		Covidsure	
	POS.	NEG	POS.	NEG.	POS.	NEG.	POS.	NEG
MALE	14	43	15	42	13	44	14	43
FEMALE	13	19	14	20	12	22	13	21
TOTAL	27	62	29	62	25	66	27	64
N=	91		91		91		91	

- **Comparison of clinical performance to all four invitro diagnostic molecular assay for Covid-19 detection:-**

A sensitivity and specificity of 100% showed by VIRALDTECT-II panel while 96.29% sensitivity & 96.87% specificity was observed by Trupcr. 92.59 & 100% 0f sensitivity & specificity showed by Covisure, 96.29% of sensitivity & 96.96% specificity was showed by Covidsure molecular diagnostic assay.

S.No.	SARS CoV-2 Molecular assay	SENS. SP.	Percentage of false negative	Percentage of false positive
1.	VIRALDTECT-II Multiplex Real time PCR(Gene2me)	100% 100%	0	0
2.	TRUPCR®SARS CoV-2 RT qPCR (V-3.2)	96.29% 96.87%	3.7%	3.1%
3.	COVISURE (Genetix)RTPCR	92.59% 100%	7.4%	0
4.	COVIDSURE Multiplex RTPCR (Trivitron)	96.29% 96.96%	3.7%	3%

SENS., sensitivity; SP., specificity

Percentage of false negative & positive was found zero for the VD-2 , 3% of false positiveness were observed by TRUPCR & COVIDSURE assay respectively. While as maximum % of false negative were observed by COVISURE 7.4 after that 3.7% by TRUPCR and COVIDSURE assay respectively.

- Value obtained by at least 3 of the 4 molecular assays (reference standard value)

α These two samples had Ct (cycle threshold) values of 33 & 33.7 by the TRUpcr assay.

S.No.	Molecular assay and result	No. of results by reference standard Positive	Negative	PPA	NPA
1.	VIRALDTECT-II Multiplex Real time PCR(Gene2me) Positive Negative	27 0	0 64	100	100
2.	TRUPCR®SARS CoV-2 RT qPCR (V-3.2) Positive Negative	26 1β	2α 62	92.85%	98.41%
3.	COVISURE (Genetix)RTPCR Positive Negative	25 2γ	0 64	100%	96.96 %
4.	COVIDSURE Multiplex RTPCR (Trivitron) Positive Negative	26 1ε	2δ 62	92.85%	98.46 %

β Cycle threshold (ct) value undetermined.

γ These 2 samples had not detected RdRp through Covisure assay.

δ These 2 samples had ct value for Orf1ab 31.25 & 33.69 by the Covisure assay.

ε These sample had ct value for Orf1ab , E 36.74 &31.26 respectively.

PPA :positive predictive analysis
NPA:negative predictive analysis

- Following testing of 91 clinical specimens, the VIRALDTECT-II and Covisure assay demonstrated a PPA of 100% (27/27) & (25/25) respectively, while Trupcr & Covidsure both assay were showed 92.85 (26/28). A NPA of 100% observed by VIRALDTECT-II, 98.41% (62/63), 96.96%(64/66) and 98.46%(64/65) were observed for TRUPCR , Covisure , Covidsure assays respectively

- Details of discordant sample analysis results are shown in the following Table. A total five discordant samples were found among two or three of the four platforms.

- **Details of discordant sample analysis**

SARS –CoV-2 molecular assayresult (ct)						
Sample ID	Reference standard result	Covisure	Covidsure	Trupcr	Viraldtect /gene2me	Comment
A	Pos.	Neg.	Neg.	Pos. (33.57/29 .74)	Pos.(35.72 /33.52)	Sample assay was repeated(Covisure & Covidsure) & determined pos.
B	Pos.	Neg.	Pos.(3 3.59/3 1.54)	Pos.(32.9 7/29.63	Pos.(35.13 /33.40)	Sample assay was reprocessed by Covisure & determined pos.
C	Neg.	Neg.	Pos.(3 1.25/2 9.82)	Pos.(33/2 8.52)	Neg.	After re-extract & process sample was determined as Neg. by Covidsure & Trupcr.
D	Neg.	Neg.	Pos.(3 3.69/2 9.92)	Pos.(33.7 2/29.76)	Neg.	Sample was reprocessed & found Negative.
E	Pos.	Pos.(2 8.44/2 4.99)	Pos.(2 4.11/2 3.76)	Undeter mined	Pos.(25.72 /26.86)	Sample assay re-extracted & repeated determined pos. with Trupcr.

CT : Cycle threshold; ID, identifier; Neg., Negative; Pos, positive Sample A had a higher ct value of36.74 for Orf1ab genes & found negative for Rdrp genes on initial testing by Covidsure & Covisure assay respectively, After repeating extraction & retesting the sample was determined to be positive.

Sample B was considered false negative by Covisure , after retesting Covisure assay was able to detect as positive.

Two samples (Sample ID C& Sample ID D) were considered false positive by Trupcr & Covidsure after reprocessing the assay was able to detect both samples as Negative.

Sample E have found undetermined through Trupcr , although ct value was 5.86 forE & 27.94 Rdrp but Relative fluorescence unit (RFU) found not properly & the samples has found more chances to come towards Negative (Internal control was proper so run is valid).

DISCUSSION:-

We compared four different molecular assay for SARS-COV-2 detection in this study, patient specimen collected during April & May 2021. We were able to make several observation including workflow comparison , this study were useful for hospitalized patients, where result are very crucial as well as critical for treatment , clinical management, infection control, cross infection, & cohorting for bed management . False negative result serve as potential thread because they inevitably lead to more exposures, particularly in outpatient setting as the basis for social distancing measure to slow the rate spread of infections[14] [15] Also very essential for health professional need to rapid results to ensure that they are not exposing the patients , whom they are treating the levels of personal protective equipment & health care facility required by health care professional, depends on greater extent whether a patient is positive for SARS-CoV-2 or not. A false negative results serve as potential thread because they inevitably lead to more exposures particularly in outpatient setting as the basis of social distancing measure to slow the rate spread of infections, also very essential for health professional need to rapid result to ensure that they are not exposing the patients, whom they are treating, the levels of personal protective equipment & health care facility required by health care workers, depends on greater extent whether a patient is positive for SARS-CoV-2 or not . A false negative result can be devasting especially in case of immune competent individual , people required long term care facility. Both VIRALDTECT & COVIDSURE assay had 100% PPA & detect all specimen deemed positive by the consensus standard (interpretation of three of four evaluated assays as the gold standard), COVISURE missed 2 positive specimen(which were subsequently detected by COVISURE after repeat testings). NPA 100% for VIRALDTECT while TRUPCR & COVISURE showed 2 & 1 discordant results respectively. After retesting the 3 samples showed that for TRUPCR , sample C & D as negative and sample E was positive upon repeat

testing this could have previously been a false negative as well. This study had several limitations that should be mentioned, firstly this was a single centre study & the majority of the specimens were frozen after initial testing on VIRALDTECT secondarily the number of specimen included were only 91 , the patient samples spanned the entire range of clinical positives (including inclusion of specimens with low viral loads) and reflected our overall true positivity rate which was b/w 30% to 40% during that time of covid pandaemic, considering the design of all four invitro diagnostic assay differences that could affect assay performance, could include characteristic such as input volume of initial specimen , RNA purification , elution, volume difference overall difference in gene targets. All these parameters along with patient care needs ,may assist clinical laboraties to identify & choose the correct testing platform that best fits the needs for the diagnosis of patient infected with this SARS-CoV-2 virus. Many questions remains to be answered about the clinical sensitivity of PCR assays for the diagnosis of COVID-19, the minium acceptable analytical sensitivity, different studies have shown different detection pattern of the viral RNA based on the specimen type & the specimen collection time in relation to the onset of symptoms. A different group consistently showed that the sputum , BAL specimens showed the highest positivity rate (15). Additional studies from other groups showed lower sensitivity of PCR for early detection in highly suspected patients in comparison to CT scan(16) or serology(17). Our study show that the four Invitro diagnostic SARS-CoV-2 assay are comparable, although the clinical sensitivity of PCR in Covid -19 diagnosis is still an area of the more investigation.

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