Differential gene expression analysis of common target genes for the detection of SARS-CoV-2 using real time-PCR

Reza Valadan[1,2], Soheila Golchin[3], Reza Alizadeh-Navaei[3], Mohammadreza Haghshenas[4], Mehryar Zargari[5], Tahoora Mousavi[1,6]* and Mohammad Ghamati[7]

Abstract

COVID-19 currently is the main cause of the severe acute respiratory disease and fatal outcomes in human beings worldwide. Several genes are used as targets for the detection of SARS-CoV-2, including the RDRP, N, and E genes. The present study aimed to determine the RDRP, N, and E genes expressions of SARS-CoV- 2 in clinical samples. For this purpose, 100 SARS-CoV-2 positive samples were collected from diagnostic laboratories of Mazandaran province, Iran. After RNA extraction, the real-time reverse transcription PCR (real-time RT-PCR) assay was performed for differential gene expressions' analysis of N, E, and RDRP. The threshold cycle (Ct) values for N, RDRP, and E targets of 100 clinical samples for identifying SARS-CoV-2 were then evaluated using quantitative real-time PCR (qRT-PCR). This result suggests N gene as a potential target for the detection of the SARS-CoV-2, since it was observed to be highly expressed in the nasopharyngeal or oropharynges of COVID-19 patients (P < 0.0001). Herein, we showed that SARS-CoV- 2 genes were differentially expressed in the host cells. Therefore, to reduce obtaining false negative results and to increase the sensitivity of the available diagnostic tests, the target genes should be carefully selected based on the most expressed genes in the cells.

Keywords: SARS-CoV-2, RT -PCR assay, RDRP, N, And E genes expressions

Introduction

COVID-19, which is currently known as the global pandemic of Coronavirus, is responsible for the severe acute respiratory disease and fatal outcomes in human beings worldwide (Korber et al. 2020). Coronaviruses as a group of enveloped viruses with positive-sense single-stranded RNA belong to the family Coronaviridae, which are able to spread between humans and animals (Holshue et al. 2020).

Unlike HCoV-229E, HCoV-OC43, HCoV-NL63, HCoV-HKU1 to cause moderate upper respiratory infection in human (Hu et al. 2014), three previous epidemics of

β-coronaviruses such as Middle East respiratory syndrome-related Coronavirus (MERS-CoV), severe acute respiratory syndrome Coronavirus (SARS-CoV), and severe acute respiratory syndrome Coronavirus 2 (SARS-CoV-2), have been potentially associated with acute respiratory distress syndrome (ARDS) with about 35.5%, 9.6%, and 6.76% mortality rates, respectively (Lu et al. 2020).

SARS-CoV-2 contains open reading frames (ORFs) that encode four structural proteins, including S-spike, M-membrane, E-envelope, and N-nucleocapsid. Of note, several genes are used as the targets gene for detection of SARS-CoV-2 such as the (RDRP and S), (N and S), and E genes. In this regard, studies have previously shown that N protein is produced in large quantities in infected cells, which is related to the processes of replication, translation, and transcription. Moreover, it causes

*Correspondence: stm.jmums@gmail.com

[6] Molecular and Cell Biology Research Center, Hemoglobinopathy Research Institute, Mazandaran University of Medical Sciences, Sari, Iran
Full list of author information is available at the end of the article

Valadan *et al. AMB Express*   (2022) 12:112

Page 2 of 7

cell cycle deregulation, consequently inhibiting interferon production and inducing apoptosis (Astuti 2020). The RNA-dependent RNA polymerase, called ORF1ab, is responsible for viral transcription and replication. Therefore, RT-PCR based 2 target genes (ORF1ab and N) are the crucial targets for SARS-CoV-2 detection (Shen et al. 2021).

In order to have the best RT-PCR performance, the components of these targets should be optimized (Tombuloglu et al. 2021). Accordingly, reverse transcription polymerase chain reaction (RT-PCR) using fluorescent dyes is considered as a gold standard method for detecting bacterial and viral nucleic acid (DNA / RNA). RT-q PCR can also be used as a rapid and accurate assay for screening SARS-CoV-2 in throat samples, nasopharyngeal swabs, and feces (Chaimayo et al. 2020). A cohort study has shown that RT-PCR with sensitivity and specificity values of 70% and 95% could detect viruses in patients, even in those showing no symptoms (Arevalo-Rodriguez et al. 2020; Rutuja Sunil and Vasudeo Pandharinath 2021). However, a successful detection of this virus depends on some factors such as test time, early or late detection time, viral load, and sample collection procedure (Vickers 2017).

The ORF1ab/RdRp, E, N, and S genes most commonly used targets for detection of SARS-CoV-2 so, there are some commercial RT-PCR kits for the diagnosis of COVID-19 such as Primer Design (England, RdRp), Seegene (Korea, RdRp, N, E), CerTest Biotec (Spain, ORF1ab, N), Altona Diagnostics (Germany, S, E), BGI (China, ORF1ab), KH Medical (Korea, RdRp, S), and R-Biopharm AG (Germany, E) with different qualities, which are available to be used for the diagnosis of SARS-CoV-2 (Puck et al. 2020). According to this point that diagnosis of SARS-CoV-2 infection with two or three targets lead to an increase in sensitivity and specificity and avoid a false negative result, so the present study attempted to analyze the RDRP, N, and E genes expressions of SARS-COV- 2 using qRT-PCR through specific primer pairs in the obtained clinical samples.

Materials and methods

Simplex primer and probe design

The specific qRT-PCR primers and probe for the diagnosis of the target regions of the SARS-CoV-2 were designed using the following programs: PrimerPooler, PrimerPlex, and Primer3 (Tombuloglu et al. 2021). Moreover, 5' Fluorescein amidites (FAM)-labeled probe was designed for the SARS-CoV-2 RdRp/ N/RP, as well as Hypoxanthine Phosphoribosyltransferase (HPRT) and Yakkima yellow-labeled probe for the viral E gene, which were then synthesized (Fig. 1). The sequence of each primer or probe is shown in Table 1.

RNA extraction from the clinical samples

The study was approved by the Mazandaran University of Medical Sciences, Iran, with the number IR.MAZUMS. REC.1399.8671. For the purpose of this study, Nasopharyngeal and oropharyngeal swabs were collected from symptomatic patients, immediately diluted with viral transfer medium (VTM), and finally transferred to the COVID-19 laboratory at Mazandaran University of Medical Sciences for the detection of SARS-CoV-2. RNA extraction was performed in 100 positive samples using the RNJia virus kit (Jivan, Iran) in terms of the
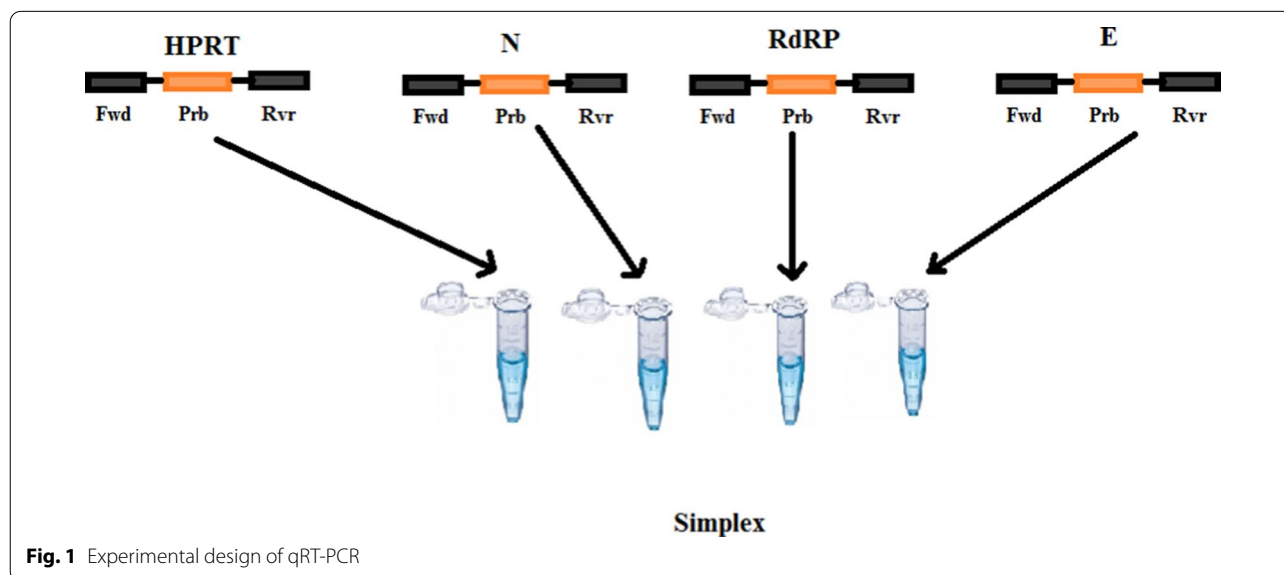
Fig. 1 Experimental design of qRT-PCR

Valadan *et al. AMB Express* (2022) 12:112

Page 3 of 7

Table 1 The sequences and concentrations of primer and probe sets used in the PCR reactions

Target	Sequence(5′-3′)	Label	Reference
2019-nCoV_N1-F	GAC CCC AAA ATC AGC GAA AT	None	(Jung et al. 2020)
2019-nCoV_N1-R	CT GGT TAC TGC CAG TTG AAT CTG	None	(Jung et al. 2020)
2019-nCoV_N1-P	FAM-ACC CCG CAT TAC GTT TGG TGG ACC-BHQ1	FAM, BHQ1	(Bruce et al. 2020)
E_Sarbeco_F1	ACAGGTACGTTAATAGCGT	None	(Corman et al. 2020a)
E_Sarbeco_R1	ATATTGCAGCAGTACGCACACA	None	(Corman et al. 2020a)
E_Sarbeco_P	Yakkima yellow-ACACTAGCCATCCTTACTGCGCTTCG-BHQ1	Yakkima yellow, BHQ1	(Corman et al. 2020a)
2019-nCoV_ RDRP-F1	GTGARATGGTCATGTGTGGCGG	None	(Corman et al. 2020a)
2019-nCoV_ RDRP-R	CARATGTTAAASACACTATTAGCATA	None	(Corman et al. 2020a)
2019-nCoV_ RDRP-P	FAM-CAGGTGGAACCTCATCAGGAGATGC-BHQ1	FAM, BHQ1	(Corman et al. 2020a)
HPRT-F	GGACTAATTATGGACAGGACTG	None	(Valadan et al. 2015a)
HPRT-R	GCTCTTCAGTCTGATAAAATCTAC	None	(Valadan et al.2015a)
HPRT-P	FAM-CCTCCCATCTCCTTCATCACATCTC–BHQ1	FAM, BHQ1	(Valadan et al. 2015a)
RP-F	AGA TTT GGA CCT GCG AGC G	None	(Gregianini et al. 2019)
RP-R	GAG CGG CTG TCT CCA CAA GT	None	(Gregianini et al. 2019)
RP-P	FAM – TTC TGA CCT GAA GGC TCT GCG CG – BHQ-1	FAM, BHQ1	(Gregianini et al. 2019)

manufacturer's instructions. Subsequently, the differential gene expressions of N, E, and RdRp were performed using qRT-PCR.

Real-time RT-PCR assay

In this study, 20-μL reaction containing 4 μL of RNA, 10 μL of one step RT-PCR kit(add bio, korea), 2 μL of enzyme mixture, 0.5 μL of forward and reverse primers, 0.5 μL of each probe, and RNase/DNase-free ddH2O up to 20 μL, was setup. Final primers and probes concentrations in the reaction were adjusted using the following steps:

1. 1.0.25 pM for RdRP-F, and 0.25 pM for RdRP-R.
2. 2. 0.25 pM for E-F, and 0.25 pM for E-R.
3. 3. 0.25 pM for N-F, and 0.25 pM for N-R.
4. 4. 0.25 pM for HPRT-F, and 0.25 pM for HPRT-R.

The reaction was dispensed in 96-well microplates (MicroAmp™ Fast Optical 96-well reaction Plate 0.1 mL, Applied Biosystems) and then sealed with optical film (MicroAmp™ Optical Adhesive Film, Applied Biosystems). Of note, a negative control reaction (RNase/DNasefree ddH2O) was used to check the presence of any contamination. In addition, HPRT and RP genes were used as internal controls (Valadan et al. 2015b).

Thereafter, Quantitation experiments were conducted using RT-PCR instrument (StepOne™ Real-Time PCR System).

As well, qPCR was performed as follows:

1) Reverse transcription was performed for 20 min at 50 °C,
2) Inactivation of the reverse transcriptase was done for 10 min at 95 °C.
3) PCR amplification was performed with 40 cycles for 15 s at 95 °C and for 30 s at 58 °C using StepOne™ Real-Time PCR.

Statistical analysis

The obtained results were examined by determining the amplification curve of the target gene and the housekeeping gene. Continuous variables are indicated as means (standard deviation, SD). All the statistical analyses were performed using GraphPad Prism 8 software and p-values less than 0.001 were considered as statistically significant.

Results

In the present study, 100 respiratory samples were collected from nasopharyngeal (NP) and throat swabs in health-care centers of Mazandarn, Iran, from December 2020 to September 2021. Thereafter, Real-time RT-PCR, using E, RDRP, and N targets, was performed for genome detection of SARS-CoV-2. Firstly, all the primers and probes were analyzed by simplex qRT-PCR. Prior to preparing the reactions, the qRT-PCR instrument was properly calibrated in order to achieve the best fluorescent signal. The simplex reactions were then performed in triplicate for three viral E, N, and RDRP genes as well as internal control genes (HPRT and RP). The criteria for the diagnosis of positive, negative, and suspicious

Valadan *et al. AMB Express*   (2022) 12:112

Page 4 of 7

Table 2 The Ct value emerged in RT-PCR assay for the SARS-COV-2

Characteristics	Result
Ct-value of E Mean ± SD (min, max)	26 ± 6.53 (14.24, 39.24)
Ct-value of N Mean ± SD (min, max)	20.19 ± 5.93 (9.72, 33.82)
Ct-value of RDRP Mean ± SD (min, max)	26.92 ± 7.04 (13.85, 40)
Ct-value of HPRT Mean ± SD (min, max)	31.34 ± 2.75 (26.94, 40)
Ct-value of RP Mean ± SD (min, max)	24.43 ± 1.63 (20.56, 27.94)

Table 3 The ΔCt value emerged in RT-PCR assay for the SARS-COV-2.

Characteristics	ΔCt value (HPRT control)	ΔCt value (RP control)
E gene Mean ± SD (min, max)	−5.34 ± 6.76 (−15.15, 0)	1.57 ± 6.74 (−8.71, 19.07)
N gene Mean ± SD (min, max)	−11.15 ± 6.28 (−22.45, 0.43)	−4.23 ± 6.13 (−13.23, 13.65)
RDRP gene Mean ± SD (min, max)	−4.42 ± 7.27 (−16.91, 11.42)	2.49 ± 7.32 (−6.84, 17.59)

COVID-19 samples were as follows: $(0 < Ct < 37.00)$, (NO Ct or $Ct \geq 40.00$), and $(37.00 \leq Ct < 40.00)$, respectively.

The average cycle threshold (Ct) and ΔCt value with standard deviations (SD) are shown in Tables 2 and 3, and the comparative Ct performances of each assay are shown in Figs. 2 and 3. In this research, HPRT and RP genes were used as internal controls. Indeed HPRT and RP had significantly increased expression level compared to other targets (including N, E, and RDRP) $(P < 0.0001)$. Our findings showed that no detectable difference exists between HPRT and RP internal controls. According to the comparison of ΔCt values among N, E, and RDRP targets, the N gene expression level was found to be higher than that of E and RDRP genes. $(P < 0.0001)$. As shown in Fig. 4, there is no significant difference between E and N targets (0.611). The result of our study suggest N gene as the most sensitive target compared to E and RDRP for SARS-CoV-2 detection using RT-PCR.
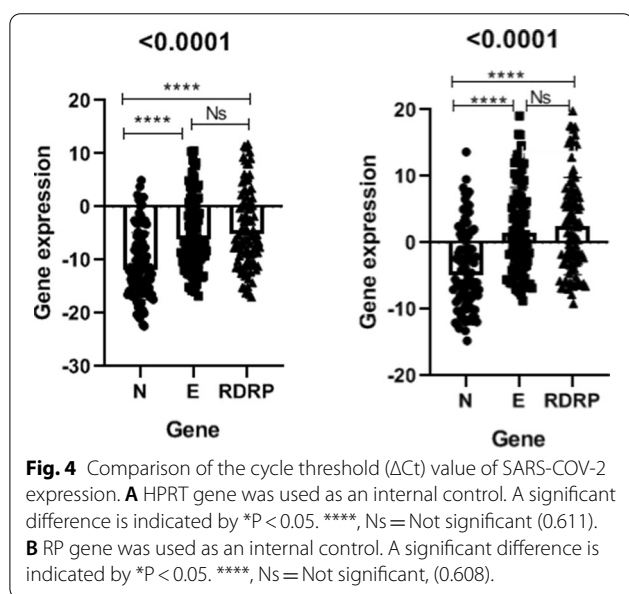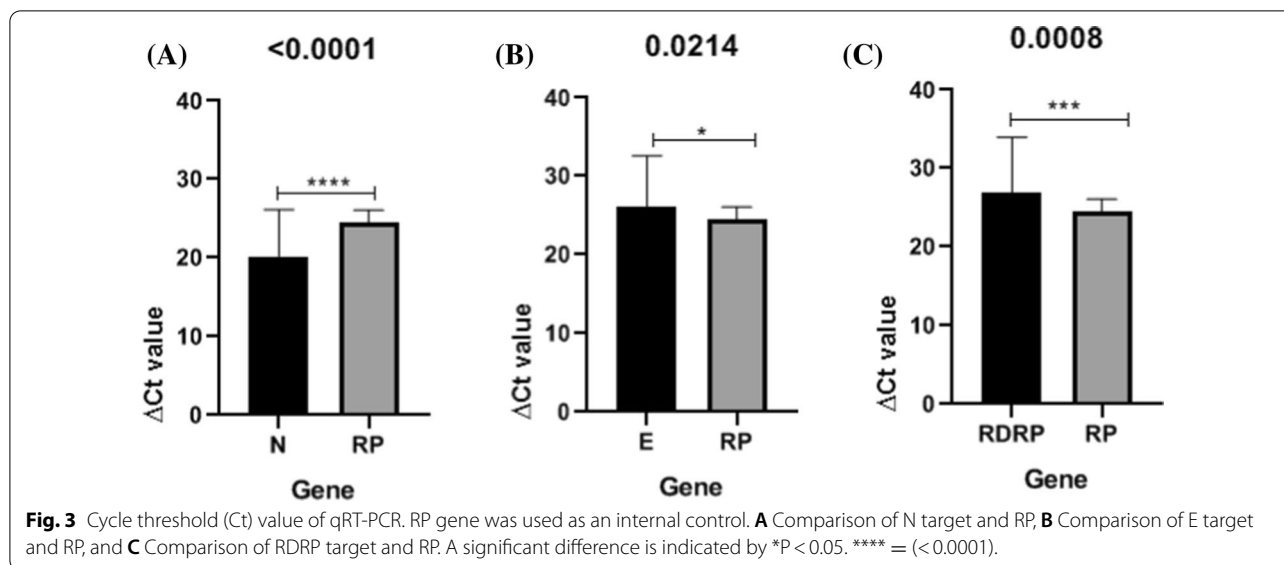
Discussion

In this study, Ct values for the N, RDRP, and E targets were evaluated using qRT-PCR in order to detect SARS-CoV-2 in 100 clinical samples. It was observed that N gene has less Ct values (23.73 ± 6.99) than those of E and RDRP. Moreover, our results show a significant difference among the E, N, and RDRP groups.

The diagnosis of SARS-CoV-2 using molecular tests is known as the gold standard method for the diagnosis of COVID-19 infection. Of note, the RT-PCR is a sensitive assay for the detection of SARS-CoV-2 RNA in clinical specimens (Chaimayo et al. 2020). The study showed that after the onset of the disease's symptoms, the SARS-CoV-2 viral load can be immediately observed in the upper respiratory tract and the antigen can also be detected in the first phase. However, some factors such as clinical manifestations, duration of disease to laboratory test, type of clinical sample, and sample collection procedure (technique process) can be effective on interpreting the results (Zou et al. 2020).
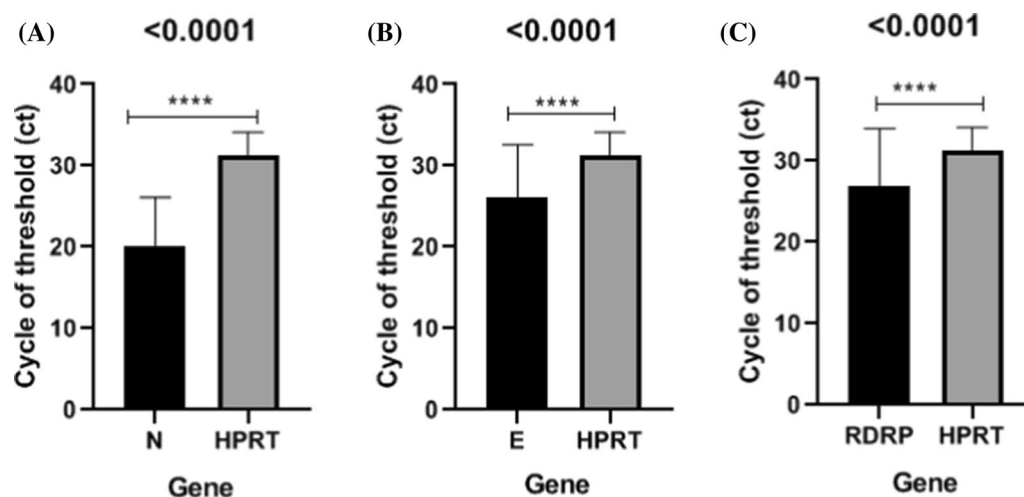
Fig. 2 Cycle threshold (Ct) value of qRT-PCR. HPRT gene was used as an internal control. **A** Comparison of N target and HPRT, **B** Comparison of E target and HPRT, and **C** Comparison of RDRP target and HPRT. A significant difference is indicated by *P < 0.05. **** = (< 0.0001).

Valadan *et al. AMB Express*   (2022) 12:112

Page 5 of 7

Fig. 3 Cycle threshold (Ct) value of qRT-PCR. RP gene was used as an internal control. **A** Comparison of N target and RP, **B** Comparison of E target and RP, and **C** Comparison of RDRP target and RP. A significant difference is indicated by *P < 0.05. **** = (< 0.0001).

Fig. 4 Comparison of the cycle threshold (ΔCt) value of SARS-COV-2 expression. **A** HPRT gene was used as an internal control. A significant difference is indicated by *P < 0.05. ****, Ns = Not significant (0.611). **B** RP gene was used as an internal control. A significant difference is indicated by *P < 0.05. ****, Ns = Not significant, (0.608).

In general, many developed laboratory methods use various tools, reagents, and targets in order to identify SRRS-COV-2 (LeBlanc et al. 2020). RDRP, E, and N are three targets proposed by WHO for the SARS-COV-2 identification (Corman et al. 2020a). As well, the E gene is the first line screening, the RDRP gene is used as confirmatory test, and the N gene is used for a confirmatory testing, all of which are used in identifying the coronavirus. A previous study has shown that the RdRP_SARSr-P2 target could be specific for the coronavirus, and other probes are suitable for the detection of other types of coronavirus, and if false positive results are obtained

regarding the diagnosis of Covid-19, it may possibly indicate that patients with mild symptoms are infected with other types of corona virus (Kakhki et al. 2020b). Besides, evidence suggests that other targets such as ORF8 and specific primers / probes, may act as additional confirmatory tests in the diagnosis of SARS-COV-2 (kamali Kakhki et al. 2020a).

Houda et al. in their study have evaluated three genes of RDRP, N, and E in 187 COVID-19 samples and found gene expression as 22% and 40% in N and N, E genes, respectively. They have also shown that 6% of patients with both E and N genes and 14% of those with N gene still remained positive after a 12-day treatment period (Benrahma et al. 2020). In addition, a study of 114 respiratory specimens has revealed that the N Ct value was more specific for laboratory diagnosis of SARS-CoV-2 (Abbasi et al. 2022).

However RT-qPCR has a high levels of specificity and sensitivity, but sensitivity of COVID-19 RT-PCR diagnostic kits could be associated to the specimen conditions such as transportation or storage, sample preservation times, and the quality of the kits (Bezier et al. 2020). COVID-19 RT-PCR diagnostic kits with high analytical specificity and sensitivity could help reduce the impact of false-negative results and significantly improve the identification of COVID-19 patients (Shen et al. 2021).

Another study has shown that the one-step real-time RT-PCR can detect SARS-CoV-2 RNA in clinical specimens with a low detection sensitivity (Michel et al. 2021). Since January 2020, protocols, tests, and reagents have been developed and introduced for the detection of SARS-COV-2. These laboratory tests that use SARS-CoV-2 RNA for the detection of COVID-19, were

Valadan *et al. AMB Express* (2022) 12:112

Page 6 of 7

compared with commercial kits. A previous study using RT-PCR and two primers (N1 and N2) for SARS-COV-2 identification (Shirato et al. 2020) has shown that N2 primer has high specificity and sensitivity in this regard. These primers were also assessed using the following commercial kits: LN S & W-E, LN S & W-N, and LMW & RDRP (Hoehl et al. 2020). The results showed that the commercial LN S & W-N kit containing N primer was able to detect the virus better than the LN S & W-E (25 copies detected) and LMW & RDRP kits. It was observed that the LN S & WE targets are strongly conserved in the E gene region on SARS-COV and SARS-COV-2, while the N2 targets are a single region of N gene on SARS-COV-2 virus, so N2 is highly sensitive and specific for the detection of SARS-CoV-2 (Corman et al. 2020b).

This study showed that selection of different targets with high expression lead to increased sensitivity of diagnostic kits, therefore, to reduce false negative results and to increase the sensitivity, diagnostic tests should be designed based on the targets that have the most differential expression. Correspondingly, RT-PCR method using of N, E, and RDRP targets is known as a reliable and accurate method for SARS-CoV-2 identification that can be used in infection's prevention and control, and in diagnostic laboratories and medical centers.

Author contributions
RV participated in experimental design, SG carried out real time PCR, R A-N performed the statistical analysis, MH participated in data collection, MZ and MGH participated in manuscript preparation, TM contributed to experimental design and manuscript revision. All authors read and approved the final manuscript.

Data Availability
The data and materials are mentioned in the manuscript.

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
This research has been approved with the number of (Grant No. IR.MAZUMS. REC.1399.8671) in the ethics committee of Mazandaran University of Medical Sciences, Sari, Iran.

Competing interests
Author declares no conflict of interest.

Author details
[1]Molecular and Cell Biology Research Center, Faculty of Medicine, Mazandaran University of Medical Sciences, Sari, Iran. [2]Department of Immunology, Faculty of Medicine, Mazandaran University of Medical Sciences, Sari, Iran. [3]Gastro-intestinal Cancer Research Center, Non-communicable Diseases Institute, Mazandaran University of Medical Sciences, Sari, Iran. [4]Department of Microbiology, Molecular and Cell-Biology Research Center, Faculty of Medicine, Mazandaran University of Medical Sciences, Sari, Iran. [5]Department of Clinical Biochemistry and Genetics, Molecular and cell biology research center, Faculty of Medicine, Mazandaran University of medical sciences, Sari, Iran. [6]Molecular and Cell Biology Research Center, Hemoglobinopathy Research Institute, Mazandaran University of Medical Sciences, Sari, Iran. [7]Medical student, Mazandaran University of Medical Sciences, Sari, Iran.

References
Abbasi H, Tabaraei A, Hosseini SM, Khosravi A, Razavi Nikoo H (2022) Real-time PCR Ct value in SARS-CoV-2 detection: RdRp or N gene? Infection 50(2):537–540

Arevalo-Rodriguez I, Buitrago-Garcia D, Simancas-Racines D, Zambrano-Achig P, Del Campo R, Ciapponi A, Sued O, Martinez-García L, Rutjes W, Low A, Bossuyt NM, Perez-Molina PA, Zamora J J (2020) False-negative results of initial RT-PCR assays for COVID-19: a systematic review. PLoS ONE 15(12):e0242958

Astuti I, srafil Y (2020) Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2): An overview of viral structure and host response. Diabetes Metab Syndr 14(4):407–412

Benrhama H, Diawara I, Smyej I, Rahoui J, Meskaouni N, Benmessaoud R, Arouro K, Jaras Kh, Moujid FZ, Adam Z, Nahir S, Aouzal Z, Elguezzar H, Jeddane L, Ousti F, EL Bakkouri J, Nejjari Ch (2020) Epidemiological description and analysis of RdRp, E and N genes dynamic by RT-PCR of SARS-CoV-2 in Moroccan population: Experience of the National Reference Laboratory (LNR)-UM6SS. *medRxiv*

Bezier C, Anthoine G, Charki A (2020) Reliability of real-time RT-PCR tests to detect SARS-Cov-2: A literature review. Int J Metrol Qual Eng 11:13

Bruce EA, Tighe S, Hoffman J, Laaguiby Ph J, Gerrard L, Diehl DA, Leonard SGB, Huston Ch DD, Kirkpatrick D, Crothers BW, Dragon J, Botten J (2020) J RT-qPCR detection of SARS-CoV-2 RNA from patient nasopharyngeal swab using Qiagen RNeasy kits or directly via omission of an RNA extraction step. *BioRxiv*

Chaimayo Ch, Kaewnaphan B, Tanlieng N, Athipanyasilp N, Sirijatuphat R, Chayakulkeeree M, Angkasekwinai N, Sutthent R, Puangpunngam N, Tharmviboonsri T, Pongraweewan O, Chuthapisith S, Sirivatanauksorn Y, Kantakamalakul W, Horthongkham N (2020a) Rapid SARS-CoV-2 antigen detection assay in comparison with real-time RT-PCR assay for laboratory diagnosis of COVID-19 in Thailand. Virol J 17(1):177

Corman VM, Landt O, Kaiser M, Molenkamp R, Meijer A, Chu DKW, Bleicker T, Brünink S, Schneider J, Schmidt ML, Mulders DGJC, Haagmans BL, van der Veer B, van den Brink Sh, Wijsman L, Goderski G, Romette JL, Ellis J, Zambon M, Peiris M, Goossens H, Reusken Ch, Koopmans MPG, Drosten Ch (2020b) Detection of 2019 novel coronavirus (2019-nCoV) by real-time RT-PCR. Eurosurveillance 25(3):23

Corman V, Bleicker T, Brünink S, Drosten Ch (2020a) Diagnostic detection of 2019-nCoV by real-time RT-PCR. World Health Organization 17:1–13

Gregianini TS, Santos Varella IR, Fisch P, Garay Martins L, Veiga ABG (2019) Dual and triple infections with influenza A and B viruses: a case-control study in Southern Brazil. J Infect Dis 220(6):961–968

Hoehl S, Rabenau H, Berger A, Kortenbusch M, Cinatl J, Bojkova D, Behrens P, Böddinghaus B, Götsch U, Naujoks F, Neumann P, Schork J, Tiarks-Jungk P, Walczok A, Eickmann M, Vehreschild MJG, Kann T, Wolf G, Gottschalk T, Ciesek R S (2020) Evidence of SARS-CoV-2 infection in returning travelers from Wuhan, China. N Eng J Med 382(13):1278–1280

Holshue ML, Lofy KH, Spitters Ch, Tural A, Patel A, Tong S, Pallansch M-A, Biggs H-M (2020) First case of 2019 novel coronavirus in the United States. N Eng J Med 382:929–936

Jung YJ, Park GS, Moon JH, Ku K, Beak SH, Kim S, Changkyun Park E, Park D, Lee JH, Ch WB, Lee JJ, Maeng JS, Kim SJ, Il Kim S, Kim BT, Lee MJ, Kim HG (2020) Comparative analysis of primer–probe sets for RT-qPCR of COVID-19 causative virus (SARS-CoV-2). ACS Infect Dis 6:2513–2523

kamali Kakhki R, Aryan E, Meshkat Z, Sankian M (2020a) Development of a cost-effective line probe assay for rapid detection and differentiation of mycobacterium species: a pilot study. Rep Biochem Mol Biol 8(4):383–393

Valadan *et al. AMB Express*   (2022) 12:112

Page 7 of 7

Kamali Kakhki R, Kamali Kakhki M, Neshani A (2020b) COVID-19 target: A specific target for novel coronavirus detection. Gene Rep 20:100740

Korber B, Fischer WM, Gnanakaran S, YoonH, Theiler J, Abfalterer W, Hengartner N, Giorgi EE, Bhattacharya T, Foley B, Hastie KM, Parker MD, Partridge DG, Evans CM, Freeman TM, I de Silva T, McDanal Ch, Perez LG, Tang H, Moon-WalkerA, Whelan SP, LaBranche CC, Saphire EO, Montefiori D-C (2020) Tracking changes in SARS-CoV-2 Spike: evidence that D614G increases infectivity of the COVID-19 virus. Cell 182(4):812–827

LeBlanc JJ, Gubbayc J-B, Lif Y, Needleg R, Radons Arnesonf S, Marcinof D, Charesth H, Desnoyersi G, Dustj K, Fattouhk R, Garceaui R, Germanl G, Hatchette TF, Kozak RA, Krajdenn M, Kuschakf T, Lango ALS, Levettn P, Mazzulli T, McDonaldo R, Mubareka S, Prystajeckyn N, Rutherfordq C, Smiejaq M, Yug Y, Zahariadisg G, Zelyas N, Bastienf N (2020) Real-time PCR-based SARS-CoV-2 detection in Canadian laboratories. J Clin Virol 128:104433

Lu L, Zhong W, Bian Z, Li Z, Zhang K, Liang B, Zhong Y, Hu M, Lin L, Liu J, Lin X, Huang Y, Jiang J, Yang X, Zhang X, Huang Z (2020) A comparison of mortality-related risk factors of COVID-19, SARS, and MERS: A systematic review and meta-analysis. J Infect 81(4):e18–e25

Michel J, Neumann M, Krause E, Rinner T, Muzeniek T, Grossegesse M, Hille G, Schwarz F, Puyskens A, Förster S, Biere B, Bourquain D, Domingo C, Brinkmann A, Schaade L, Schrick L, Nitsche A (2021) Resource-efficient internally controlled in-house real-time PCR detection of SARS-CoV-2. Virol J 18(1):110

Puck BVK, van der Veer B, van den Brink Sh, Wijsman L, de Jonge J, van den Brandt A, Molenkamp R, Reusken CBEM, Meijer A (2020) Comparison of seven commercial RT-PCR diagnostic kits for COVID-19. J Clin Virol 128:104412

Qin Hu, Lu R, Peng K, Duan X, Wang Y, Zhao Y, Wang W, Lou Y, Tan W (2014) Prevalence and genetic diversity analysis of human coronavirus OC43 among adult patients with acute respiratory infections in Beijing, 2012. PLoS ONE 9(7):e100781

Rutuja Sunil P, Vasudeo Pandharinath Z (2021) Development of RT-PCR Based Diagnosis of SARS-CoV-2. Biotechnology to Combat COVID-*19* (IntechOpen.96823)

Shen L, Cui Sh, Zhang D, Lin Ch, Chen L, Wang Q (2021) Comparison of four commercial RT-PCR diagnostic kits for COVID-19 in China. J Clin Lab Anal 35(1):e23605

Shirato K, Nao N, Katano H, Takayama I, Saito Sh, Kato F, Katoh H, Sakata M, Nakatsu Y, Mori Y, Kageyama T, Matsuyama Sh, Takeda M (2020) Development of genetic diagnostic methods for novel coronavirus 2019 (nCoV-2019) in Japan. Jap J of Infect Dis 73(4):304–307

Tombuloglu H, Sabit H, Al-Suhaimi E, Al Jindan R, Alkharsah R, Kh (2021) Development of multiplex real-time RT-PCR assay for the detection of SARS-CoV-2. PLoS ONE 16(4):e0250942

ValadanR, Amjadi O, Tehrani M, Rafiei A, Hedayatizadeh-Omran A, Alizadeh-Navaei R (2015a) 'Pseudogene-free amplification of HPRT1 in quantitative reverse transcriptase polymerase chain reaction. Anal biochem 485:46–48

ValadanR Hedayatizadeh-Omran A, Naghavi Alhosseini-Abyazani M, Amjadi O, Rafiei A, Tehrani M, Alizadeh-Navaei R (2015b) Data supporting the design and evaluation of a universal primer pair for pseudogene-free amplification of HPRT1 in real-time PCR. Data brief. 4:384–89

Vickers NJ (2017) Animal communication: when i'm calling you, will you answer too? Curr bio 27(14):R713–R15

Zou L, Ruan F, Huang M, Liang L, Huang H, Hong Zh, Yu J, Kang M, Song Y, Xia J, Guo Q, Song T, He J, YenH-L, Peiris M, Wu J (2020) SARS-CoV-2 viral load in upper respiratory specimens of infected patients. N Eng J Med 382(12):1177–1179

Publisher's Note