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Rapid and sensitive diagnostic procedure for multiple detection of pandemic *Coronaviridae* family members SARS-CoV-2, SARS-CoV, MERS-CoV and HCoV: a translational research and cooperation between the Phan Chau Trinh University in Vietnam and University of Bari "Aldo Moro" in Italy

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Abstract. – OBJECTIVE: A new pandemic coronavirus causing coronavirus disease-2019 (COVID-19), initially called 2019-nCoV and successively named Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2). The COVID-19 refers to the disease while the SARS-CoV-2 refers to the virus and is characterized by a rapid contagious capacity able to spread worldwide in a very short time. The rise in the number of infected patients and deaths is of great concern especially because symptoms are vague and similar to other forms of flu infection and corona syndrome infections characterized by fever, fatigue, dry cough, and dyspnea. According to the latest guidelines published by the World Health Organization (WHO), the diagnosis of COVID-19 must be confirmed by quantitative reverse transcription polymerase chain reaction (rRT-PCR) or gene sequencing of specimen obtained from throat, sputum and blood samples. However, the limitations due to logistics, as well as low sensitivity and specificity diagnostic tools currently available have been reported as the main cause of high incidence of either false-negative or positive results.

PATIENTS AND METHODS: The purpose of the present translational research protocol is to discuss and present the original findings from our research team on new diagnostic technique to detect four *Coronaviridae* family members (SARS-CoV-2, SARS-CoV, HCoV and MERS-CoV), highlighting the methodology, the procedure and the possible advantages.

Moreover, the authors review the current epidemiology, precautions and safety measures for health personnel to manage patients with known or suspected COVID-19 infection.

RESULTS: Implementation of an effective and rapid plan of diagnosing, screening and checking is a key factor to reduce and prevent further transmission. This procedure based on rRT-PCR could be of great help to decisively validate the results obtained from more conventional diagnostic procedures such as chest computed tomography (CT) imaging and chest ultrasound.

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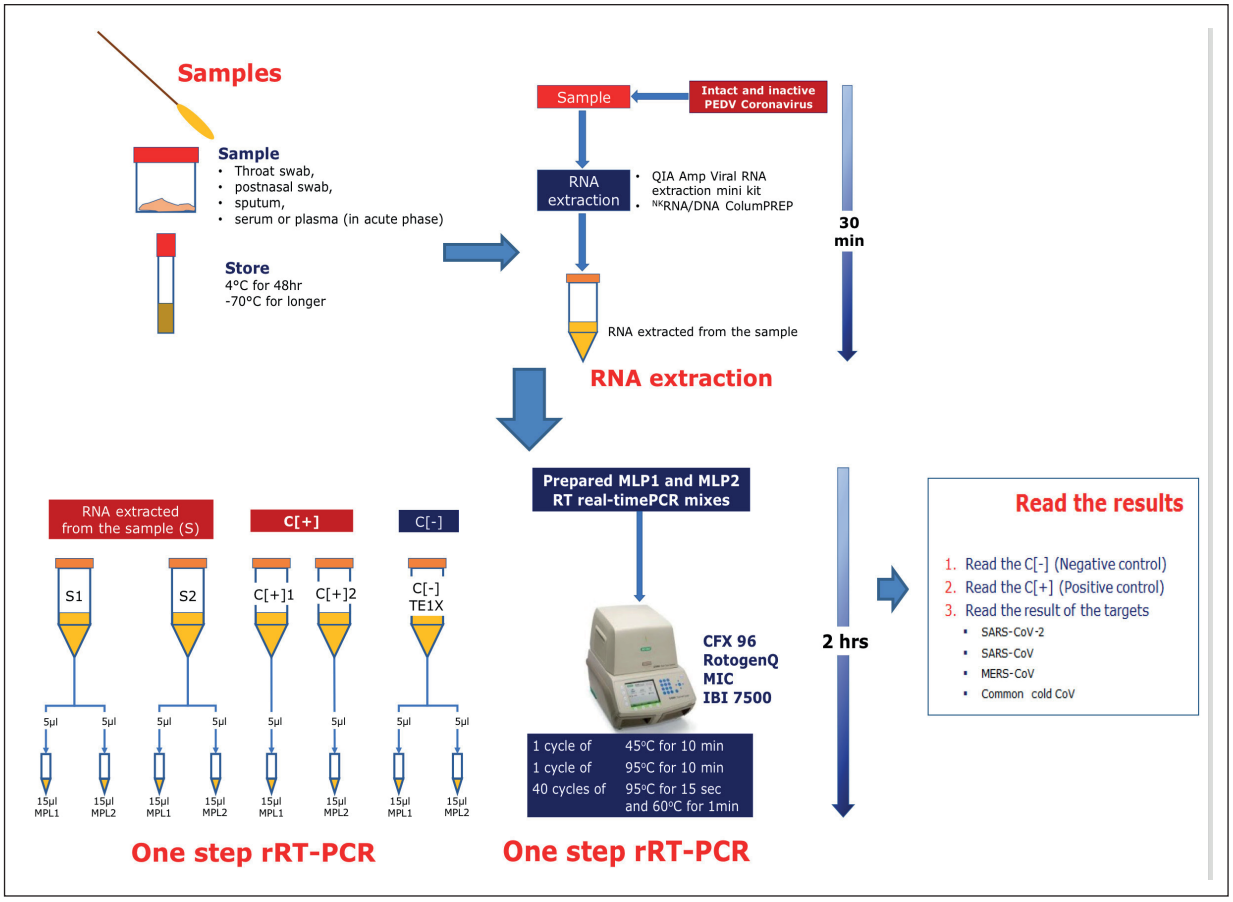
GRAPHICAL ABSTRACT: Diagnostic Kit composition and preparation of the kit before using.



CONCLUSIONS: This translational diagnostic tool will assist emergency and primary care clinicians, as well as out-of-hospital providers, in effectively managing people with suspected or confirmed SARS-CoV-2.

Key Words:
Coronavirus family, Coronavirus Disease 2019 (COVID-19), Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2), Coronavirus and severe acute respiratory syndrome (SARS-CoV), Middle East respiratory syndrome (MERS), Human Coronavirus (HCoV), Porcine epidemic diarrhea virus (PEDV), Translational research.

Introduction

The emerging of recent or old mutated harmful pathogens always causes a global threat[1,2]. The last two decades have seen the menace of particularly dangerous mutated flu-virus strains, 6 were identified, four of them are known to cause mild respiratory symptoms in immune-competent individuals, Human Coronavirus (HCoV) HCoV-229E, HCoV-NL63, HCoV-OC43 and HCoV-HKU1 whereas the other two, Middle East respiratory syndrome (MERS-CoV) coronavirus and severe acute respiratory syndrome (SARS) coronavirus (SARS-CoV), have caused epidemics with high mortality rate[3-5]. The whole members of Coronavirus family are characterized by a unique biochemical structure composed of non-segmented, Porcine Epidemic Diarrhea Virus (PEDV), positive-sense, single-strand ribonucleic acid viruses (RNA)[6]. Novel mutated strains represent a severe risk due to the wide distribution of coronavirus wide genetic diversity with frequent genomic recombination. In addition, their wide distribution together with increased human-animal interactions are the cause of newly frequent cross-species emerging casualties[7-9].

Between November and December 2019, a new type of coronavirus named SARS-CoV-2, the causative agent of Coronavirus Disease 2019 otherwise known as COVID-19, rapidly has be-

gun spreading uncontrollably in Wuhan province in China mainland. The virus extracted from the lower respiratory tract samples of several infected patients confirmed the unavoidable coronavirus family genetic marks. The patients presented with typical symptoms of severe pneumonia that included fever, physical weakness and fatigue, dry cough, and respiratory distress.

SARS-CoV-2 aggressive nature is particularly seen in individuals with preexistent comorbidities. Evidence has shown that SARS-CoV-2 uses angiotensin converting enzyme 2 (ACE2) as a preferred receptor to get in and start infecting, and the cellular protease type II transmembrane serine proteases (TMPRSS2) for entry into target cells (Figure 1). The ACE2 messenger RNA is highly expressed and stabilized by neutral amino acid transporter (B0AT1) in gastrointestinal system providing a prerequisite for SARS-CoV-2 infection.

The viral load related to the number of viral particles actively present in an organism is linked to a high unfavorable prognosis in elderly, allergic and immune compromised patients. The early phases of the infection resulted often asymptomatic and, the early clinical symptoms might be easily confused with common cold disease symptoms. The evidence showed the ability of the virus to contaminate cells and tissues elsewhere in the body such as the guts, liver, stomach and kidneys. Recently, Rothe et al[10] report showed SARS-CoV-2 RNA in a stool specimen, alerting on new possible viral spreading route via fecal-oral-gastrointestinal tract. In addition, the incubation period may take up to several days before the disease is fully manifested. It is not rare to encounter people becoming entirely symptomatic between 10 to 15 days after the first exposure.

The virus quickly has spread worldwide from the East to West as of this writing, with fast human-to-human diffusion and nowadays it represents a potential pandemic affliction[11,12]. The incubation period is still a matter of debate because it ranges from 2 to 14 days (mean 5.2 days) after exposure. For this reason, travel of asymptomatic persons is a potent force in spreading the disease globally. Virus can move from an affected though asymptomatic individual during the incubation period. Moreover, high sputum viral loads

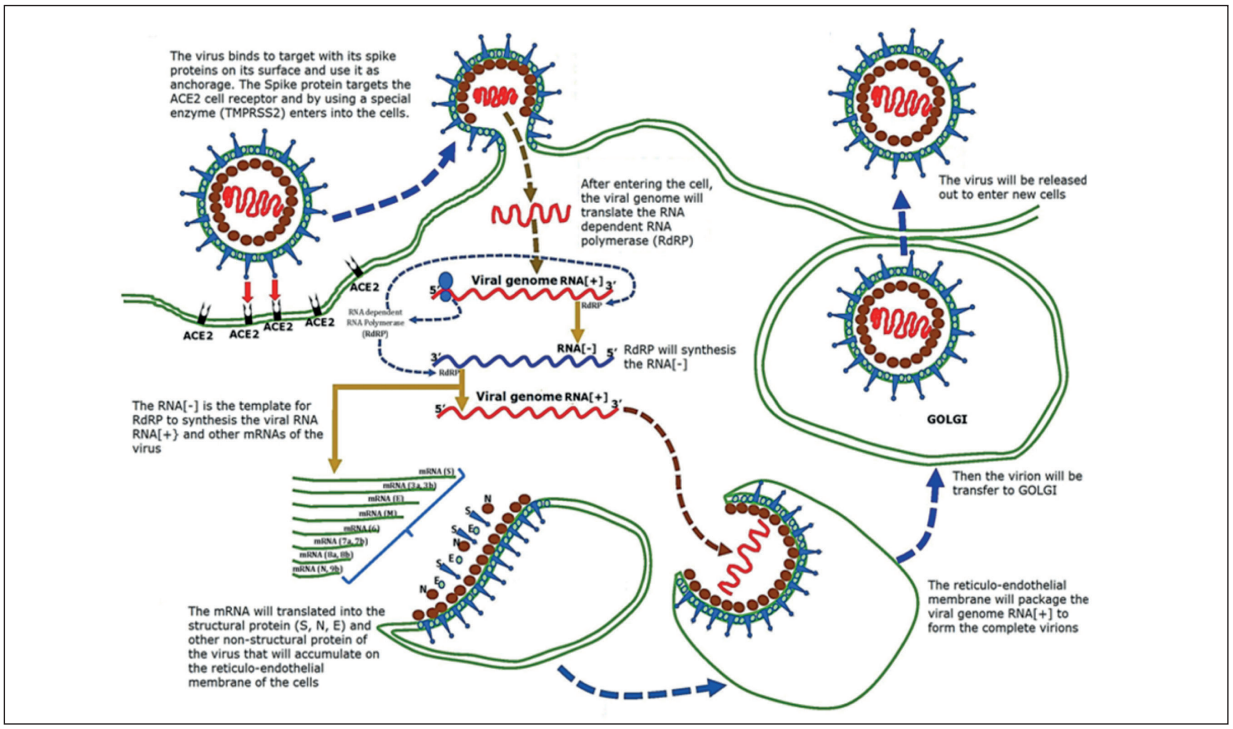


Figure 1. Replication of SARS-CoV-2. The SARS-CoV-2 infectious pathway depends upon the virus RNA. The virus binds to target with its spike proteins on its surface and use it as anchorage. The Spike protein targets the ACE2 cell receptor and enters into the cells by using a special enzyme (TMPRSS2). Once the virion is quietly accommodated, it releases its RNA. From host cell DNA machinery, it is then used to produce virion proteins that are used to replicate more infectious RNA. Proteins and RNA are used in the Golgi apparatus to produce more virus and to be released out.

were found in a patient with COVID-19 infected pneumonia (NCIP) during the recovery phase[13]. The World Health Organization (WHO), on 11th March 2020, declared COVID-19 as pandemic and single Governments had announced a World health state of emergency.

Currently, in mid-April 2020, the worldwide contaminated cases raised to (about) 1,500,000 with 350,00 recovered and 90,000 deaths, in Italy the number is 140,000 infected cases, 27,000 recovered and 18,000 deaths.

As for SARS-CoV the examination procedure of SARS-CoV-2 also needed the collection of specimens preferably from respiratory system as the nasopharyngeal aspirates, plasma or serum to be analyzed[13]. At the present time, the most precise and reliable diagnosis procedure of COVID-19 still relies on quantitative reverse transcription polymerase chain reaction (rRT-PCR) that can confirm the presence of viral RNA in clinical samples assessing either activity of the virus or the disease progression[13].

Therefore, aware of the unpredictable manifestations of the novel infection the main target of this research was to propose a new analytical methodology for the rapid and certain diagnosis of the COVID-19. In this translational protocol/technical note, we discussed the main traits of this new diagnostic tool with a focus on procedures and possible advantages.

The intended use of this protocol was based on rRT-PCR test, for the quantitative detection of nucleic acid from SARS-CoV-2, SARS-CoV, MERS-CoV and HCoV in upper and lower respiratory specimens (such as nasopharyngeal or oropharyngeal swabs, sputum, lower respiratory tract aspirates, bronchoalveolar lavage, and nasopharyngeal wash/aspirate or nasal aspirate) collected from individuals suspected of COVID-19 by their healthcare provider.

The SARS-CoV-2 RNA is generally detectable in respiratory specimens during the acute phase of infection. In this protocol either positive or negative results are suggestive of SARS-CoV-2 status infection; the clinical correlation with symptoms and clinical information are necessary to confirm patients' infection condition. In case of positive results, the procedure does not rule out bacterial infection, however it can be assessed the co-infection with other viruses belonging to Coronavirus family and the pathogen detected may be defined as the cause of the disease.

The present procedure through multiplex (MPL) rRT-PCR required a negative (no template) control in order to eliminate the possibility of sample contamination when the assay run and it should be used every time on each test sample. This control should not be amplified as it is nuclease-free water. In the MPL1, all target genes N1, N2, N3 of the SARS-CoV-2 must be amplified with the amplification signal in the channel FAM (N1), TexasRED (N2), and HEX (N3). The amplification of Ribonuclease P (RNase P-RP) (CY5) that belongs to host epithelial cells may occur depending on the amount of the SARS-CoV-2 revealed in the tested sample. In the MPL2, the E gene (FAM) of SARS-CoV and SARS-CoV-2, the N gene (HEX) of PEDV must be amplified. Thus, positive templates are needed (COVID-19 N 1-2-3) as control verifies that the assay run is performing as intended and it is used on every assay plate starting from master mix addition at a concentration of 10 copies/uL.

The DNA positive controls were made from synthesized fragments of targeted DNA specifically designed for the real-time PCR. The main intent of using positive controls N1-C[+], N2-C[+], N3-C[+], E-C[+], upE-C[+], HCoV-C[+] and PEDV-C[+], was to evaluate exactly the grade of sensitivity related to the amplification of targeted DNA. The sequences of these C[+] were synthesized by Integrated DNA Technologies (IDT) in a scale of 100 nM. An internal control targeting Ribonuclease P (RNase P-RP) is needed to verify that nucleic acid is present in every sample and is used for every sample processed. This also serves as the extraction control to ensure that samples resulting as negative contain nucleic acid for testing. The RNA extraction ^{NK}DNA-RNA-prep-COLUMN for 50 preparations (Nam Khoa Biotek. Co., LTD, Hồ Chí Minh, Vietnam) is a kit that includes the spin column, a ready to use proteinase K, a binding buffer solution, a washing buffer 1st solution, a washing buffer 2nd solution, a washing buffer 3rd solution, and the elution buffer.

Materials and Methods

The Multiplex rRT-PCR Master Mix

The synthetic DNA fragments of the targeted viruses and the RNA extraction of the sputum were used in this study to control and evaluate the sensitivity and specificity of the designed kit in the testing.

Two multiplex (MPL) rRT-PCR master mix, MLP1 and MLP2 were prepared with the reagents and formula listed in Table I (Applied Biosystems, Thermo Fisher, Hillsboro, OR, USA).

The test used three primers and probes sets to detect 3 regions in the SARS-CoV-2 nucleocapsid

Table I. The formula to prepare the multiplex rRT-PCR master mix.

MPL1 rRT-PCR master mix	MPL2 rRT-PCR master mix	Amount (pm) per 1 reaction	Stock (pm/µl)	Volume (µl) per 100 reactions
2019-nCoV_N1-F	E_Sarbeco_F1	10	100	10
2019-nCoV_N1-R	E_Sarbeco_R2	10	100	10
2019-nCoV_N1-P (FAM/BHQ1)	E_Sarbeco_P1 (FAM/BHQ1)	5	100	5
2019-nCoV_N2-F	upE_TqF	10	100	10
2019-nCoV_N2-R	upE_tqR	10	100	10
2019-nCoV_N2-P (TexasRED/BHQ2) *	upE_TqPR (TexasRED/BHQ2)	5	100	5
2019-nCoV_N3-F	PEDV-NF	10	100	10
2019-nCoV_N3-R	PEDV-NR	10	100	10
2019-nCoV_N3-P (HEX/BHQ1)	PEDV-PR (HEX/BHQ1)	5	100	5
RP-F	HCoV-HKU-1-F	2	100	2
RP-R	HCoV-HKU-1-Redit	2	100	2
RP-P (CY5/BHQ3)	HCoV-HKU-1-Pr (CY5/BHQ3)	5	100	5
Apath-ID RT-PCR buffer*		10 µl		1000
Apath-ID RT-PCR enzyme*		0.8 µl		80
Enzyme stabilizer*		1 µl		100
DNAse/RNAse free DW		to 15 µl		236
Total		15 µl		1500

*The "path-ID RT-PCR buffer 2X", the "Apath-ID RT-PCR enzyme 25X" were from the "AgPath-ID™ One-Step RT-PCR" (Applied Biosystems-Thermo Fisher, Hillsboro-Oregon, USA), Primers and Probes (IDT, Coralville, IA, USA). The "Enzyme stabilizer" was supplied by Nam Khoa Biotek. Co., LTD. (Ho Chi Minh City-Vietnam), this is to stabilize the enzyme in the prepared the rRT-PCR mix.

(N1-F,R,P; N2-F,R,P; N3-F,R,P) gene, 1 region in SARS-CoV upE (E-F1,R2,P1) gene, 1 region in MERS-CoV HKU (HKURP-1F, RP-1R, RP-1Pr) gene, 1 region in PEDV-Virus N (NF, NR, PR) gene; the test used primer and probe set to detect human RNase P (RP) in a clinical sample. RNA isolated from respiratory specimens was reverse transcribed to cDNA and subsequently amplified using AgPath-ID™ One-Step RT PCR (Applied Biosystems, Thermo Fisher, Hillsboro, OR, USA). During the amplification process, the probes annealed to the specific target sequence located between the forward and reverse primers. During the extension phase of the PCR cycle, the 5' nuclease activity of Taq polymerase (Applied Biosystems, Thermo Fisher, Hillsboro, OR, USA) degrades the bound probe, causing the reporter dye (FAM/HEX/TexasRED/CY5) to separate from the quencher dye (BHQ1/BHQ2/BHQ3), generating a fluorescent signal. Fluorescence intensity is monitored at each rRT-PCR by CFX-96 Touch Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA).

The MPL1 was implemented to detect targets SARS-CoV 2 and control for the presence of the host epithelial cells in the samples; the MPL2 was used to detect the SARS-CoV, SARS-CoV-2, HCoV, MERS-CoV, and also as control for the recognition of integral coronavirus (PEDV).

The prepared MPL1 and MPL2 were shared in dark low-binding screw caps, each received 120 µl for 8 PCR mix (15 µl/mix) and stored at -20°C until use.

The primers and probes listed in Tables II-III-IV were used to prepare the MPL1-2 master mix for rRT-PCR. These primers and probes were synthesized by IDT (Singapore and Coralville, IA, USA) in a scale of 100 nM.

Positive Controls

The DNA positive controls were obtained from synthesized fragments of the targeted DNA specifically for the rRT-PCR analysis. The positive controls, N1-C[+], N2-C[+], N3-C[+], E-C[+], upE-C[+], HCoV-C[+] and PEDV-C[+] were used to check the sensitivity of the amplification of the target DNA. The sequences of these C[+] were synthesized by IDT at the scale of 100 nM. All of the DNA positive control was prepared into the stock solution of 100 pm/µl and then dilute to working solution of 10

Table II. Primers and probes used to prepare the rRT-PCR master mix.

Name	Target gene	Reference
2019-nCoV_N1-F 2019-nCoV_N1-R 2019-nCoV_N1-P (FAM/BHQ1)	N1 (SARS-CoV-2)	From: 2019-Novel Coronavirus (2019-nCoV) RT-PCR Panel Primers and Probes. Center for Diseases and Control (CDC) USA[14]
2019-nCoV_N2-F 2019-nCoV_N2-R 2019-nCoV_N2-P (TexasRED/BHQ2)	N2 (SARS-CoV-2)	
2019-nCoV_N3-F 2019-nCoV_N3-R 2019-nCoV_N3-P (HEX/BHQ1)	N3 (SARS-CoV-2)	
E_Sarbeco_F1 E_Sarbeco_R2 E_Sarbeco_P1 (FAM/BHQ1)	E (SARS-CoV & SARS-CoV-2)	Diagnostic detection of 2019-nCoV by RT-PCR Corman et al[15]
upE_TqF upE_tqR upE_TqPR (TexasRED/BHQ2)	upE (MERS-CoV)	Diagnostic detection of 2019-nCoV by RT-PCR Corman et al[15]
HCoV-HKU-1-F HCoV-HKU-1-Redit HCoV-HKU-1-Pr (CY5/BHQ3)	Replicase (HCoV)	From: R. Rockett[16]
RP-F RP-R RP-P (CY5/BHQ3)	RNAseP (human)	From: 2019-Novel Coronavirus (2019-nCoV) RT-PCR Panel Primers and Probes. Center for Diseases and Control (CDC) USA[14]
PEDV-NF PEDV-NR PEDV-PR (HEX/BHQ1)	N (PEDV*)	From: Yu et al[17]

*PEDV Porcine Epidemic Diarrhea virus.

Table III. MPL1 primers and probes sequences used to prepare the rRT-PCR master mix.

N1-F2019-nCoV	GACCCCAAAATCAGCGAAAT
N1-R2019-nCoV	TCTGGTTACTGCCAGTTGAATCTG
N1Probe	FAM-ACCCCGCATTACGTTTGGTGGACC-BHQ1
N2-F2019-nCoV	TTACAAACATTGGCCGCAAA
N2-R2019-nCoV	GCGCGACATTCCGAAGAA
N2Probe	TexasRED-ACAATTTGCCCCCAGCGCTTCAG-BHQ2
N3-F2019-nCoV	GGGAGCCTTGAATACACCAAAA
N3-R2019-nCoV	TGTAGCACGATTGCAGCATTG
N3Probe	HEX-AYCACATTGGCACCCGCAATCCTG-BHQ1
RP-F	AGATTTGGACCTGCGAGCG
RP-R	GAGCGGCTGTCTCCACAAGT
RP-P	CY5–TTCTGACCTGAAGGCTCTGCGCG–BHQ3

Table IV. MPL2 primers and probes sequences used to prepare the rRT-PCR master mix.

E_Sarbeco_F1	ACAGGTACGTTAATAGTTAATAGCGT
E_Sarbeco_R2	ATATTGCAGCAGTACGCACACA
E_Sarbeco_P1	FAM-ACACTAGCCATCCTTACTGCGCTTCG-BHQ1
PEDVNFedit3	GCGCAAAGACTGAACCCACTA
PEDVNR	TTGCCTCTGTTGTTACTTGGAGAT
PEDV-HEX(RV)	HEX-TGTTGCCATTGCCACGACTCCTGC-BHQ1
upE_TqF	GCAACGCGCGATTCAGTT
upE_tqR	GCCTCTACACGGGACCCATA
upE_TqPR (TexasRED)	TexasRED-CTCTTCACATAATCGCCCCGAGCTCG-BHQ2
HCoV-HKU-1-F	CCTTGCGAATGAATGTGCT
HCoV-HKU-1-R	TTGCATCACCACTGCTAGTACCAC
HCoV-HKU-1-PR	CY5–TGTGTGGCGGTTGCTATTATGTTAAGCCTG-BHQ3

mM Tris: 0.1 mM EDTA; pH 8.0 and obtain a final solution of TE 1x (10 fm/µl). All DNA positive control was aliquot in low-binding Eppendorf tube (EP; Eppendorf, Hamburg, Germany), 200 µl/each, and kept at -20°C until used.

The PEDV C[+] is the intact and inactive of the Porcine epidemic diarrhea virus in the sample. This positive control plays the role of internal control for capacity of the detection of the real coronavirus in the samples. It has been used a lyophilized product, stored at -20°C. It was diluted in 200 µl of TE 1X, then aliquot into 20 µl and keep at -20°C.

The RNA extraction kit included the spin column, a ready to use proteinase K, the binding buffer, the washing buffer 1st, the washing buffer 2nd, the washing buffer 3rd, and the elution buffer. This kit supplied was NKDNARNAprep-COLUMN for 50 preparations (Nam Khoa Biotek. Co., LTD, Hồ Chí Minh, Vietnam), kept at room temperature.

The thermal cycle was set-up in the machine as following: 1 cycle at 45°C for 10 minutes; 1 cycle at 95°C for 10 minutes; and 40 cycles at 95°C for 15 seconds and at 60°C for 1 minute. Four dye channels were also selected: FAM, HEX, TexasRED and CY5.

Methods and Results

The In-Vitro Sensitivity of the MPL rRT-PCR Mix

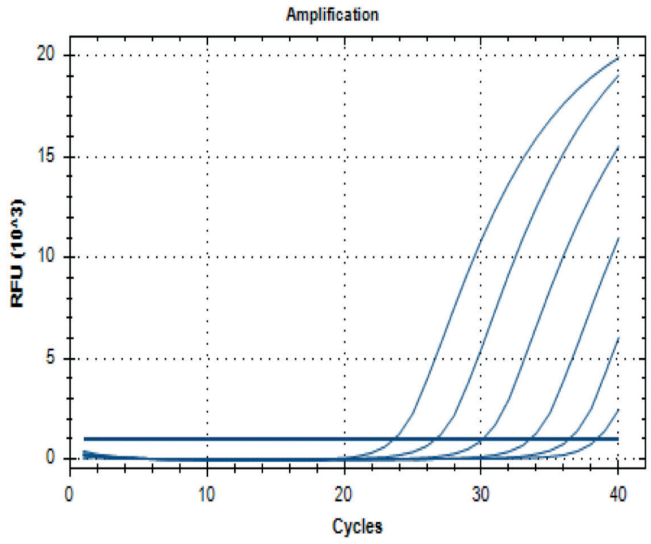
The procedure based on the use of 3 tubes of MPL1 and 3 tube (0.1 ml) of MPL2 master mix to be inserted into rRT-PCR and then thawed at room temperature. The tubes were decisively shacked up and down for 8-10 times, once finished each MLP real-time PCR master mix was shared into 24 PCR tube 0.1 low profile white (Eppendorf, Hamburg, Germany) and stored in cold environment.

It was prepared 10 X dilutions gradient of the DNA positive controls, then it was added 5 µl of each dilution between 10^{-7} to 10^{-14} to the MPL real-time PCR mix. The MPL1 received the N1-C[+], N2-C[+], N3-C[+]. The MPL2 received the E-C[+], upE-C[+], HCoV-C[+]. The PCR tubes were closed and run, once finished the all PCR tubes were moved into CFX-96 thermal-cycler and proceeded. The results are shown in Figures 2 to 7.

The sensitivity of the rRT-PCR step for the detection of all target genes of the coronavirus was summarized in Table V.

The In-Vitro Sensitivity of the MPL in rRT-PCR for the Detection of the Intact Coronavirus in the Sputum Sample

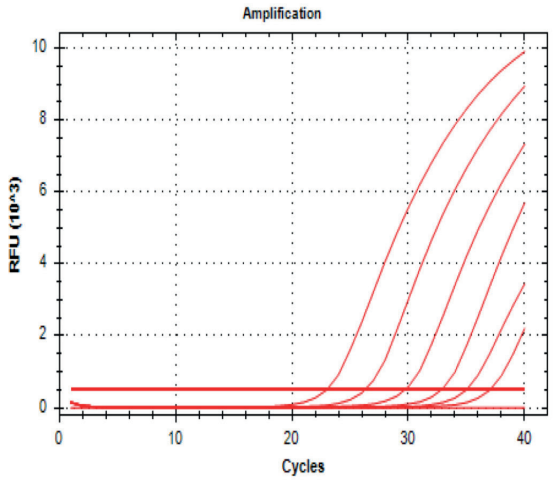
Two tubes, 1 of MPL1 and 1 tube of MPL2 real-time PCR master mix were thawed at room temperature. The samples were strongly shacked for about 8 to 10 times and let lay for few moments and shared into 8 PCR tube 0.1 (low profile white). The MPL real-time PCR mix was kept in cold environment. The lyophilized PEDV was rehydrated with 200 µl of TE 1X and was mixed well and diluted (10 folds dilution) in TE 1X from 10^{-6} to 10^{-13}. Each 20 µl dilution was added into 200 µl of spu-



N1 in MPL1 (FAM)

DILUTION	Sq	CT
N1 10^{-7}	85,849	23.62
N1 10^{-8}	10,425	26.66
N1 10^{-9}	946	30.12
N1 10^{-10}	88	33.54
N1 10^{-11}	12	36.38
N1 10^{-12}	3	38.39
N1 10^{-13}	N/A	N/A
N1 10^{-14}	N/A	N/A

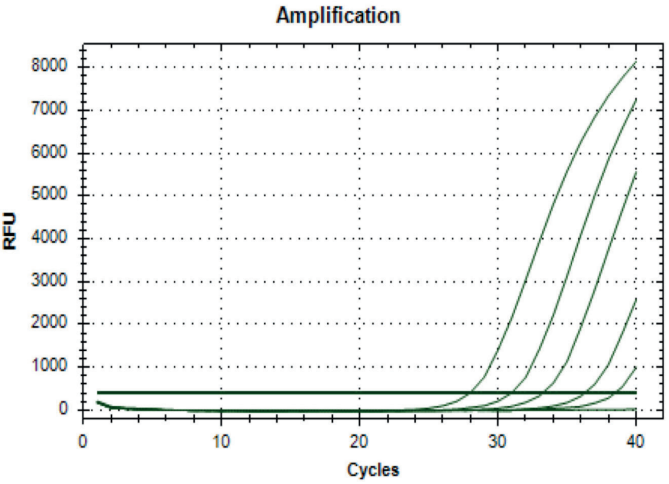
Figure 2. The sensivity of rRT-PCR step for the detection of N1 gene of SARS-CoV-2.



N2 in MPL1 (TexasRED)

DILUTION	Sq	CT
N2 10^{-7}	132,891	22.99
N2 10^{-8}	13,854	26.25
N2 10^{-9}	1,275	29.69
N2 10^{-10}	145	32.82
N2 10^{-11}	32	35
N2 10^{-12}	8	37.02
N2 10^{-13}	N/A	N/A
N2 10^{-14}	N/A	N/A

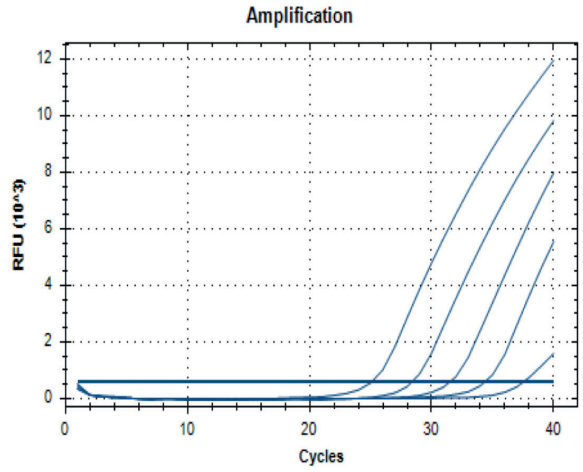
Figure 3. The sensitivity of the rRT-PCR step for the detection of N2 gene of SARS-CoV-2.



N3 in MPL1 (HEX)

DILUTION	Sq	CT
N3 10^{-7}	4,202	27.97
N3 10^{-8}	525	30.97
N3 10^{-9}	110	33.22
N3 10^{-10}	13	36.26
N3 10^{-11}	3	38.44
N3 10^{-12}	N/A	N/A
N3 10^{-13}	N/A	N/A
N3 10^{-14}	N/A	N/A

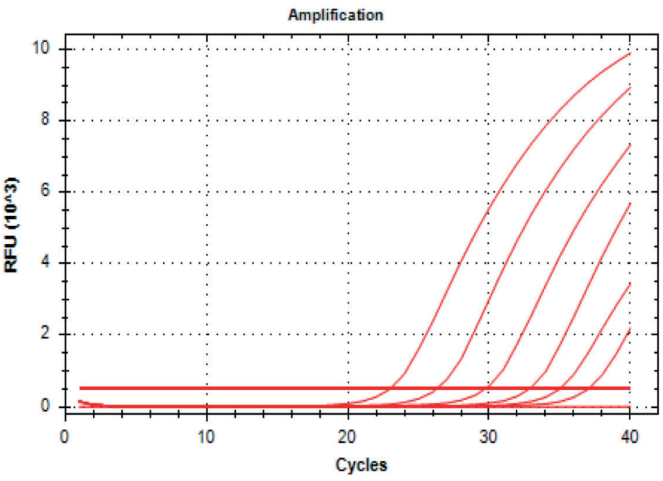
Figure 4. The sensitivity of the rRT-PCR step for the detection of N3 gene of SARS-CoV-2.



E in MPL2 (FAM)

DILUTION	Sq	CT
E 10^{-7}	30,758	25.1
E 10^{-8}	3,140	28.39
E 10^{-9}	361	31.51
E 10^{-10}	50	34.37
E 10^{-11}	6	37.54
E 10^{-12}	N/A	N/A
E 10^{-13}	N/A	N/A
E 10^{-14}	N/A	N/A

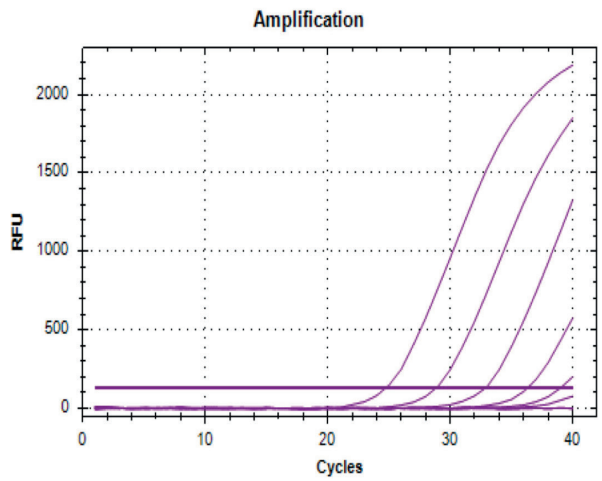
Figure 5. The sensitivity of the rRT-PCR step for the detection of E gene of SARS-CoV and SAR-CoV-2.



upE in MPL2 (TexasRED)

DILUTION	Sq	CT
upE 10^{-7}	322,876	21.71
upE 10^{-8}	30,334	25.12
upE 10^{-9}	2,071	28.99
upE 10^{-10}	185	32.47
upE 10^{-11}	14	36.19
upE 10^{-12}	N/A	N/A
upE 10^{-13}	N/A	N/A
upE 10^{-14}	N/A	N/A

Figure 6. The sensitivity of the rRT-PCR step for the detection of upE gene of MERS-CoV.



HCoV in MPL2 (CY5)

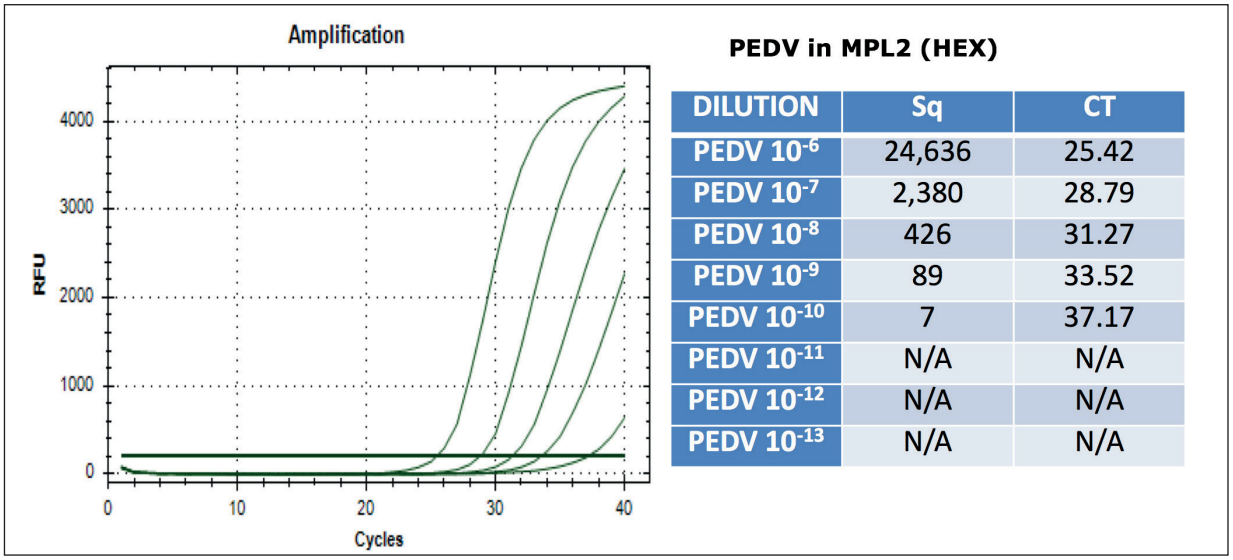
DILUTION	Sq	CT
HcoV 10^{-7}	41,733	24.66
HcoV 10^{-8}	2,396	28.78
HcoV 10^{-9}	146	32.81
HcoV 10^{-10}	14	36.24
HcoV 10^{-11}	2	38.93
HcoV 10^{-12}	N/A	N/A
HcoV 10^{-13}	N/A	N/A
HcoV 10^{-14}	N/A	N/A

Figure 7. The sensitivity of the rRT-PCR step for the detection of gene of HCoV (CY5).

tum. The sputum was stored in the laboratory after routinely bacterial examination. The RNA extraction of the sputum was carried out using the ^{NK}DNARNAprep-COLUMN kit (Nam Khoa Biotek. Co., LTD, Hồ Chí Minh, Vietnam) following the instruction of the kit. It was added 5 µl of each RNA extracted into 1 MPL1 real-time PCR mix and 1 MPL2 real-time PCR mix. Once PCR run was over, all tubes were put into CFX-96 thermal-cycler, then run (Figures 8 and 9).

Table V. The limit of detection (LOD) of the DNA positive controls to control the sensitivity of the RT-PCR step of the test.

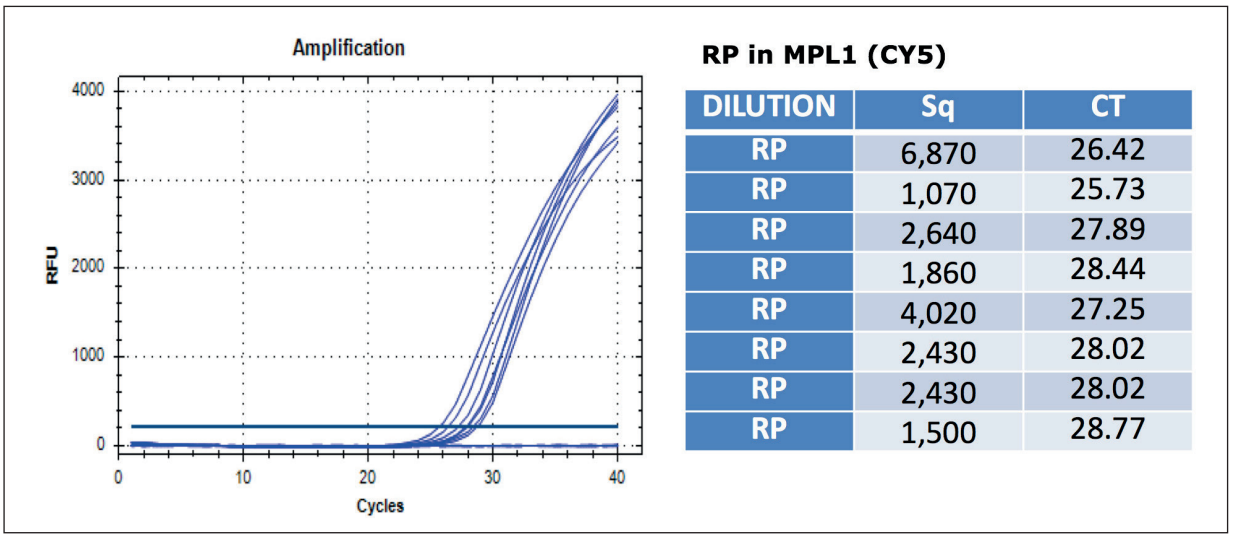
	N1 SARS-CoV-2	N2 SARS-CoV-2	N3 SARS-CoV-2	E gene SARS-CoV	upE gene MERS-CoV	Replcase HCoV
LOD in reaction volume (Copies number)	3	8	3	6	14	2



PEDV in MPL2 (HEX)

DILUTION	Sq	CT
PEDV 10^{-6}	24,636	25.42
PEDV 10^{-7}	2,380	28.79
PEDV 10^{-8}	426	31.27
PEDV 10^{-9}	89	33.52
PEDV 10^{-10}	7	37.17
PEDV 10^{-11}	N/A	N/A
PEDV 10^{-12}	N/A	N/A
PEDV 10^{-13}	N/A	N/A

Figure 8. The sensitivity of the rRT-PCR for the detection of the intact coronavirus from the sputum sample.



RP in MPL1 (CY5)

DILUTION	Sq	CT
RP	6,870	26.42
RP	1,070	25.73
RP	2,640	27.89
RP	1,860	28.44
RP	4,020	27.25
RP	2,430	28.02
RP	2,430	28.02
RP	1,500	28.77

Figure 9. All the RNAseP Gene were detected in MPL1.

The Detection Protocol for SARS-CoV-2, SARS-CoV, MERS-CoV and HCoV From the Sample

The analyzed specimens were collected from sputum, throat swab and post-nasal swab. However, in acute phase, blood could be also collected, in this case in ethylenediaminetetraacetic acid (EDTA) containers, used to isolate plasma. The samples were kept either at 4°C for up to 48 hours, or at -70°C for longer periods of storage.

For the throat and post-nasal collected specimen, the swab was eluted in TE 1X in order to obtain 200 µl. The same procedure was performed with plasma. Each plasma sample was thus eluted in TE 1X in order to obtain 200 µl. Sputum specimen should be homogenized in ratio of 1:1, 1 volume of sputum to 1 volume of TE 1X and centrifuged for 15 seconds and collected. Then, 20 µl of the PEDV C [+] was added into 200 µl of prepared sample with an extra 20 µl proteinase K at 56°C for 15 minutes. At the end of 15 minutes the sample was ready for the RNA extraction using the ^{NK}DNA RNAprep-COLUMN kit, following the instruction of the kit.

Table VI. The input of RNA extracted samples (2 samples), the DNA C [+], the negative control into the MPL rRT-PCR mix.

PCR tube	1	2	3	4	5	6	7	8
MPL1 (µl)	15		15		15		15	
MPL2 (µl)		15		15		15		15
S1 RNA extracted (µl)	5		5					
S2 RNA extracted (µl)		5		5				
DNA-C[+]1* (µl)					5			
DNA-C[+]2** (µl)						5		
TE 1X (µl)							5	5
Total volume	20	20	20	20	20	20	20	20

*DNA-C[+] 1 is the mix of N1-C[+], N2-C[+], N3-C[+]; **DNA-C[+] 2 is the mix of E-C[+], PEDV-C[+], upE-C[+], HCoV-C[+].

The MPL real-time PCR master mix was melted at room temperature and mixed by shaking up and down several times for 15 seconds. The master mix was then poured into PCR tube (15 µl/mix) and kept in cold place. The RNA was added to the extracted samples together with the DNA C[+] the TE 1X as negative controls and inserted into real-time PCR mix (5 µl for each real-time PCR mix as indicated in Table VI). The PCR tubes were inserted into CFX-96 thermal-cycler.

Reading and Analysis of the Results

The negative control

The negative controls were not amplified (Figure 10). These results indicated that samples were not contaminated.

The positive control

In MPL1, the C[+]1 had the amplification in channel FAM (N1), TexasRED (N2) and HEX (N3). In MPL2, the C[+]2 had the amplification in channel FAM (E), TexasRED (upE), HEX (PEDV) and CY5 (HCoV). This result indicated that the amplification step is sensitive in the detection of all target DNA (Figure 11).

The negative results of SARS-CoV-2, SARS-CoV, MERS-CoV and HCoV

The target DNA pathogens were not amplified. However, in MPL1 the RP (CY5) was amplified to detect the presence of epithelial cells from the patient. In MPL2 the PEDV (HEX) was amplified to confirm that the kit could extract RNA of the intact coronavirus with the amplification procedure of rRT-PCR. This procedure of MLP1 and MLP2 confirmed the ability to exclude false negatives as indicated in Figure 12.

The samples tested positive to SARS-CoV-2

In the MPL1, all targeted genes N1, N2, N3 of the SARS-CoV-2 were amplified respectively in the channels FAM (N1), TexasRED (N2), and HEX (N3). The amplification of RP (CY5) that is related to the amount of the specimen collected detects, also in this case, the presence of epithelial cells from the patient. In the MPL2, the E gene (FAM) of SARS-CoV and SARS-CoV-2 and N gene (HEX) of PEDV were also amplified. The results are showed in Figure 13.

The samples tested positive to SARS-CoV

In the MPL1, the RP gene of epithelial cells was amplified. In the MPL2, the E gene (FAM) of SARS-CoV and SAR-CoV-2 was amplified and the N gene (HEX) of the PEDV was also amplified as showed in Figure 14.

The samples tested positive to MERS-CoV

In the MPL1 the RP gene to detect the host epithelial cells was amplified in the MPL2; the upE gene (TexasRED) of MERS-CoV and the N gene (HEX) of the PEDV were amplified as indicated in Figure 15.

The samples tested positive to HCoV

In the MPL1 only the RP gene of the host epithelial cells was amplified. In the MPL2 the replicase gene (CY5) of the HCoV and the N gene (HEX) of the PEDV was amplified. As the results are showed in Figure 16.

The current diagnostic test positive results were indicative of the presence of SARS-CoV-2 RNA, this procedure showed enough sensitivity and specificity to be potentially independent from clinical patient history and other diagnostic information to determine the patients' infection status. Positive results ruled out bacterial infection or

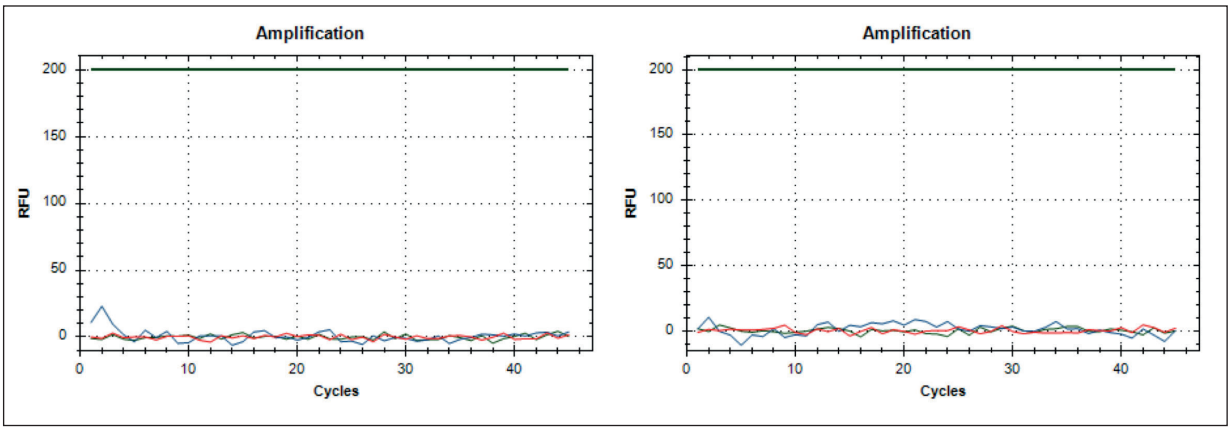


Figure 10. The negative control did not show any amplification signals in MPL1 and MPL2.

co-infection with other coronaviruses. In addition, negative results do unequivocally preclude the SARS-CoV-2 infection. However, results must be always combined with current clinical observations, patients' secondary co-morbidities, and epidemiological information. In Figures 17 and 18 we reported four *in-vivo* cases respectively positive and negative for SARS-CoV-2. The patients were diagnosed at one authorized laboratory (Vietnam) and the extracted RNA were re-checked by the test kit for *in-vivo* quality control.

Discussion

The SARS-CoV-2 is still in a phase of evolution and currently, as it was for previous coronavirus pandemic infection, the COVID-19 tests available have shown few limitations. The main concerns are mainly related to the false negative/positive outcomes, risks that are linked to the low sensitivity of the screening procedures, the incongruous collecting specimen measures, the time of sampling and errors in the processing[18].

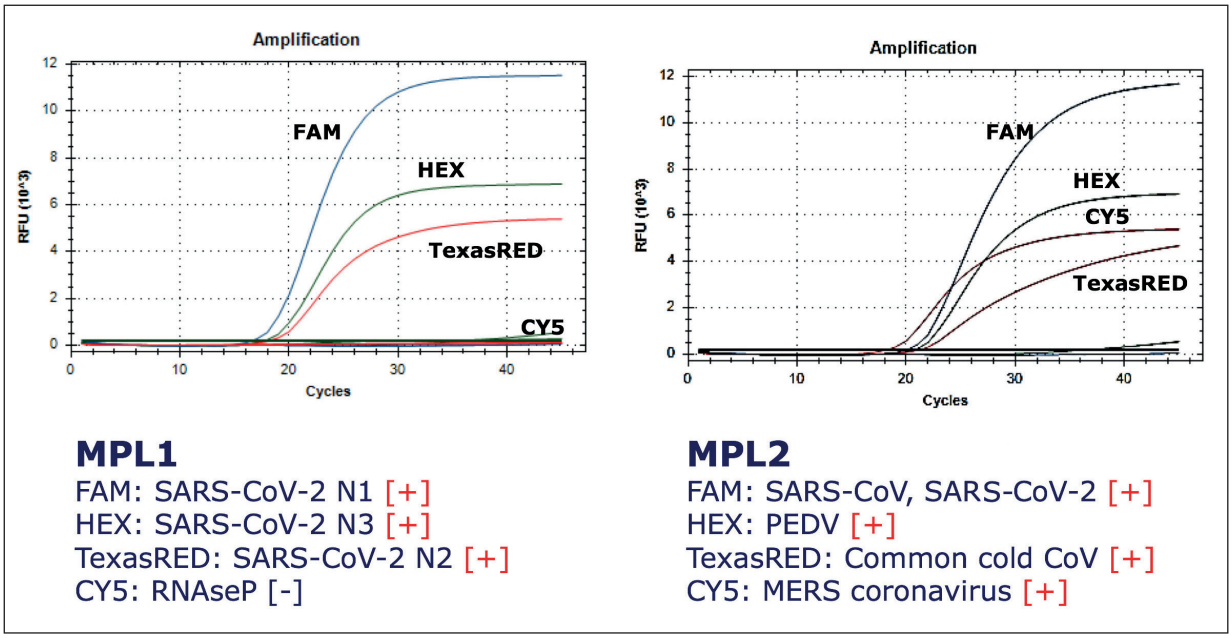


Figure 11. The positive control showed the amplification signal of FAM (N1), TexasRED (N2), HEX (N3) in MPL1 and MPL2; FAM: SARS-CoV, SARS-CoV-2 (E), HEX (PEDV), TexasRED (upE), CY5 (HCoV).

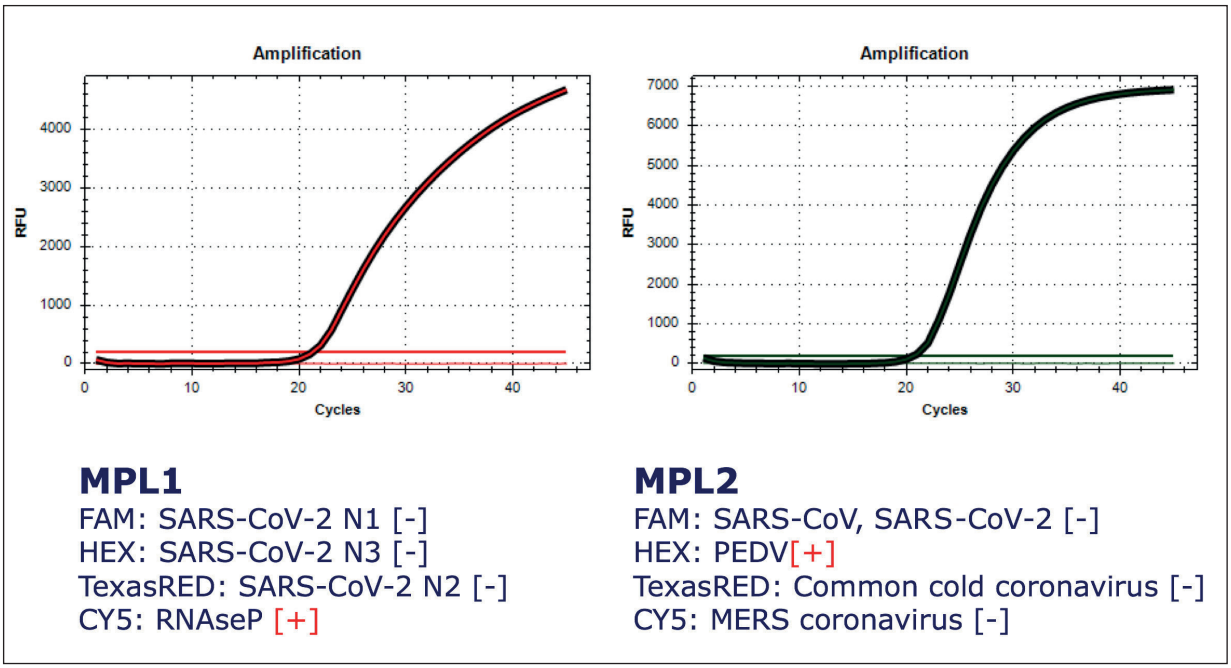


Figure 12. The negative samples with every coronavirus pathogens indicated no amplification of all the related target genes. This result is not false negative, the MLP1 must have the amplification of RP gene (CY5) of the host epithelial cell and the MLP2 must have the amplification of the internal control in the PEDV.

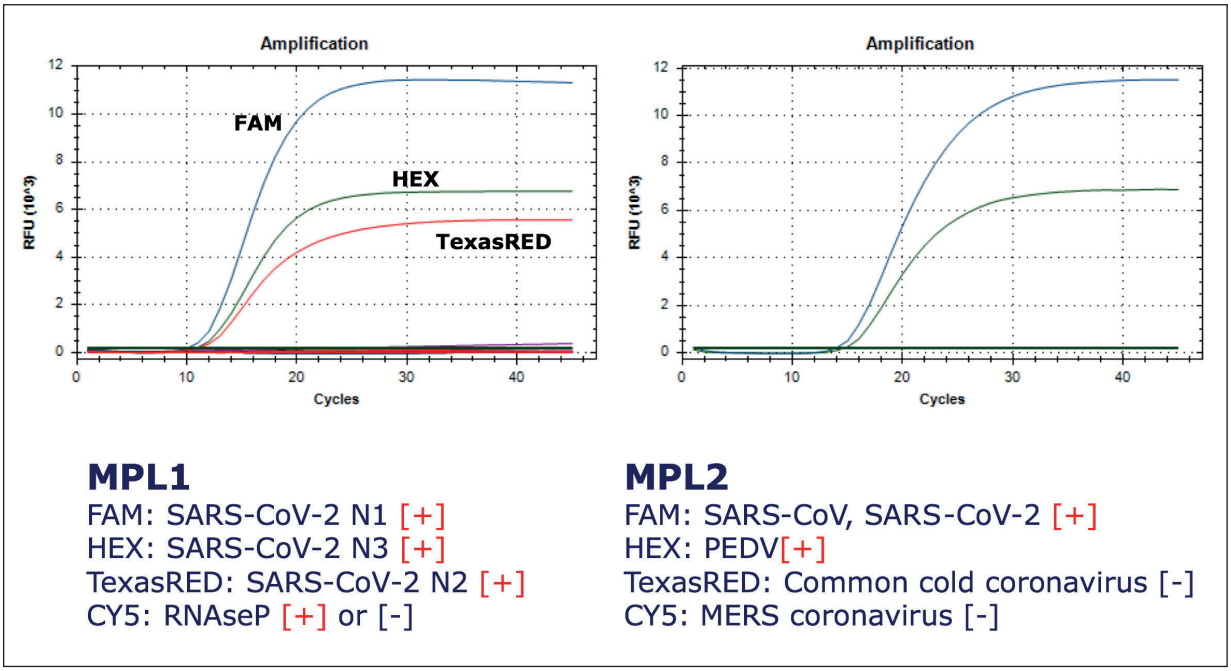


Figure 13. The samples positive for SARS-CoV-2: all target genes of the coronavirus pathogens including N1 (FAM), N2 (TexasRED), N3 (HEX) in MPL1 and E (FAM) were amplified in MPL1. In MPL2, the N gene (HEX) of PEDV was also amplified.

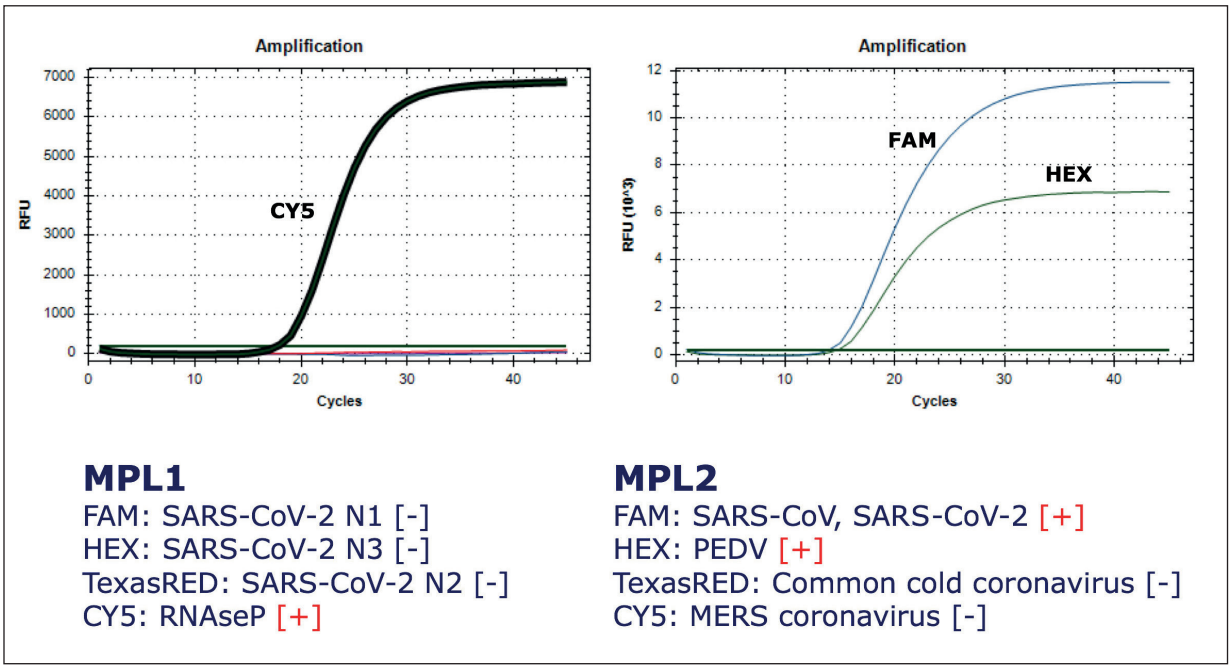


Figure 14. The positive SARS-CoV samples showed no amplification of SARS-CoV-2 targeted genes, N1 (FAM), N2 (TexasRED), N3 (HEX) in MPL1. The SARS-CoV E gene and SARS-CoV 2 (FAM) were amplified in MPL2. The N gene (HEX) of PEDV coronavirus in MPL2 and the RP gene (CY5) of host epithelial cell in MPL1 were also amplified.

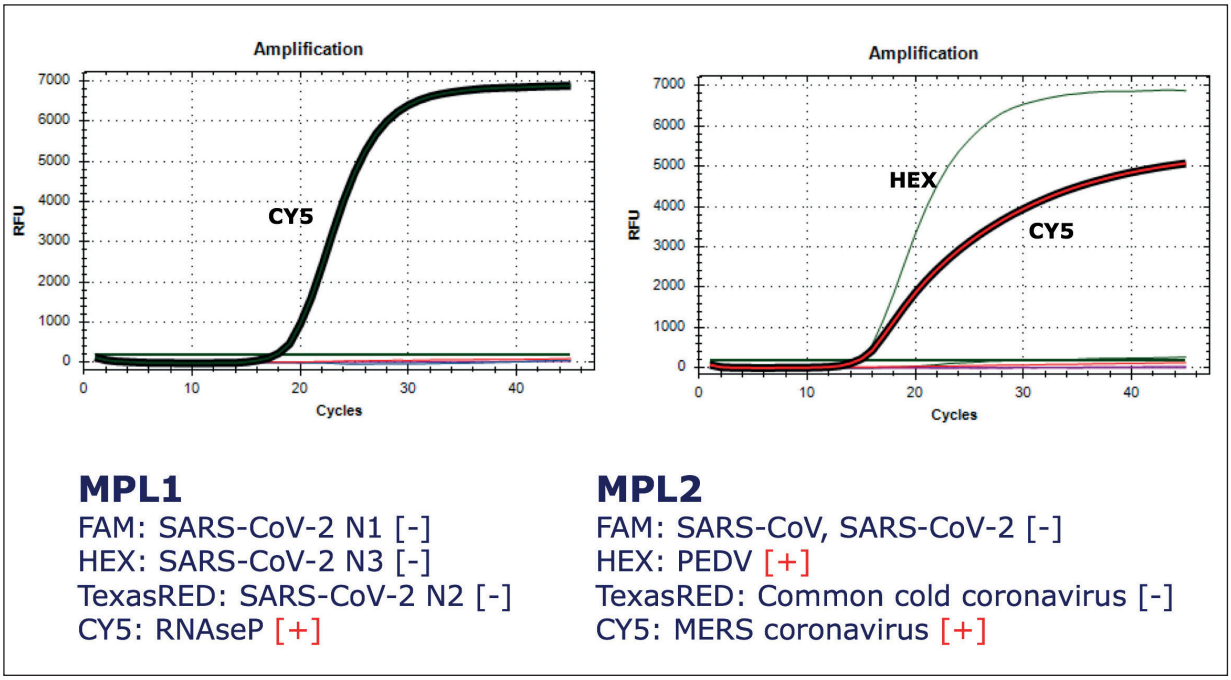


Figure 15. The samples positive to MERS-CoV are indicated by the lack of amplification of all target gene of the SARS-CoV-2 including N1 (FAM), N2 (TexasRED), N3 (HEX) in MPL1; the RP (CY5) of the host epithelial cell was amplified. In MPL2, there was no amplification for E gene (FAM) of SAR-CoV and SARS-CoV-2, as well as for replicase gene (CY5) of HCoV. However, the upE gene (TexasRED) of MERS-CoV and the N gene (HEX) of PEDV coronavirus were amplified.

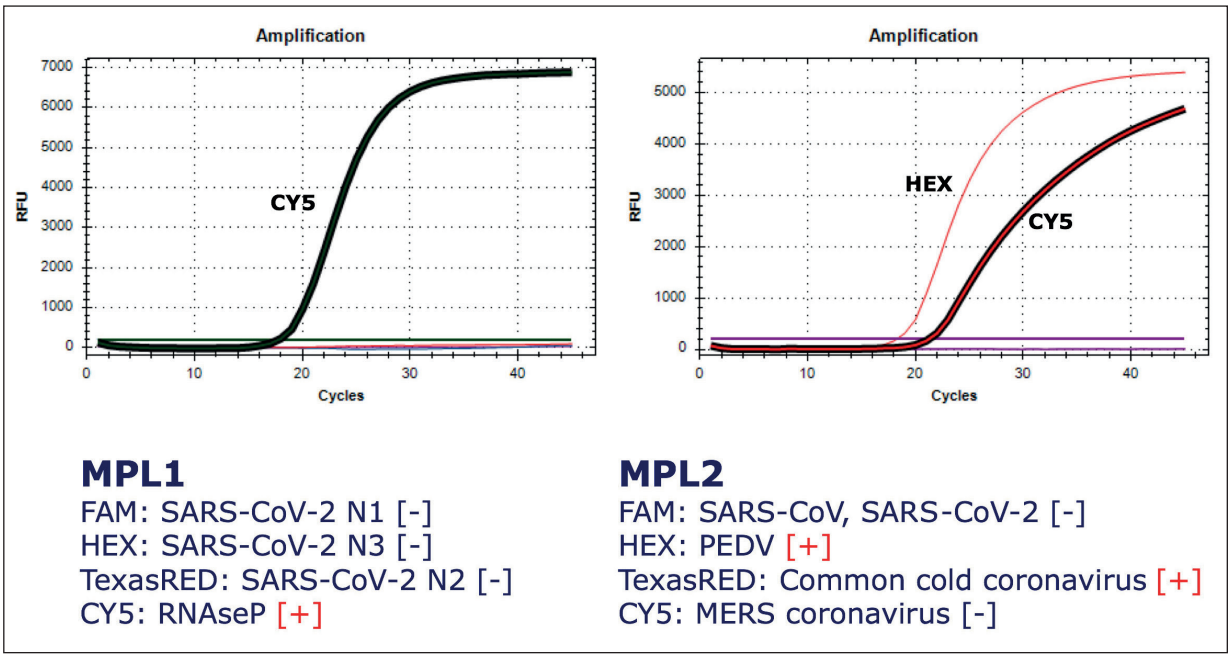


Figure 16. The positive samples with HCoV are indicated without the amplification of all target gene of the SARS-CoV-2 including N1 (FAM), N2 (TexasRED), N3 (HEX) in MPL1, except for the RP (CY5) of the host epithelial cell that was amplified. In MPL2, the E gene (FAM) of SAR-CoV and SARS-CoV-2 and the upE gene (TexasRED) of MERS-CoV were not amplified. However, replicase gene (CY5) of HCoV and the N gene (HEX) of PEDV were amplified.

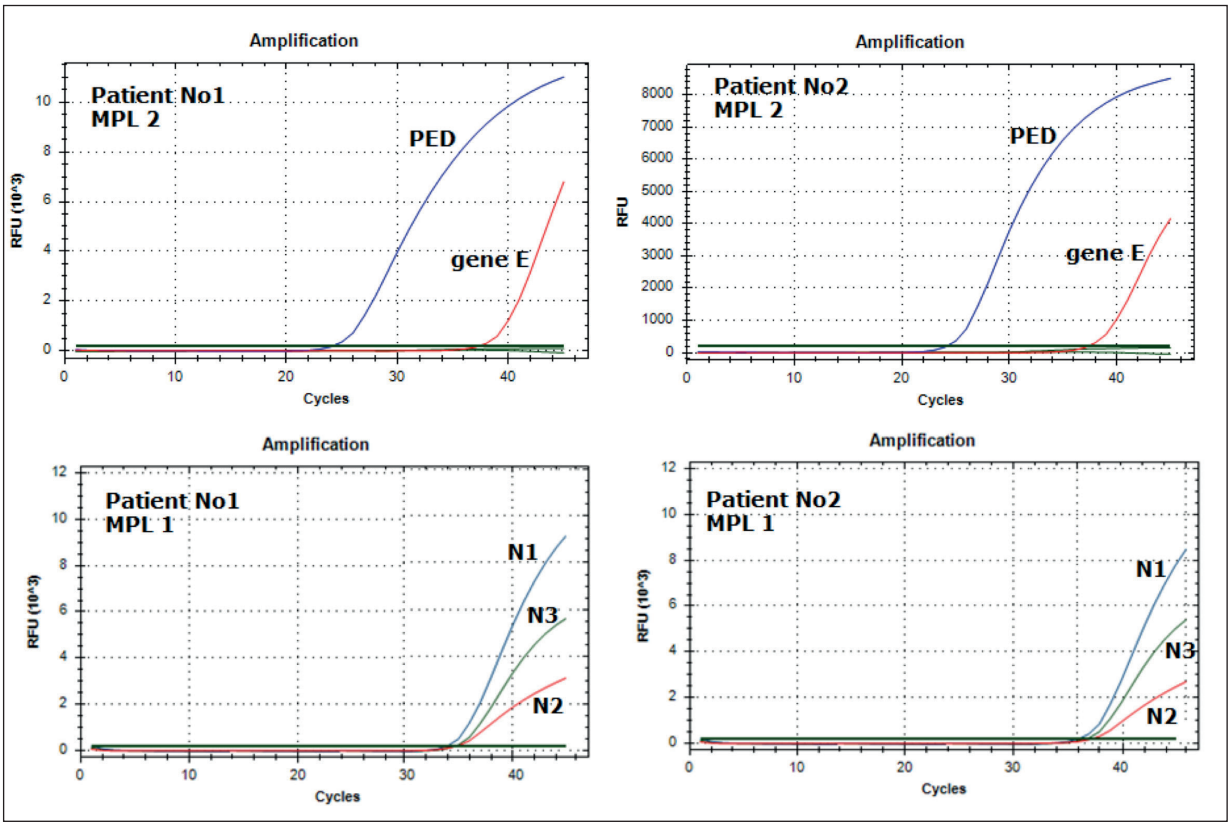


Figure 17. Two extracted RNA from 2 patients who were positive for SARS-CoV-2 (COVID-19). The PEDV and E genes were amplified in each MPL2 of patients (up left and right), the MPL1 of patient 1 and 2 showed the amplification of N1, N2 and N3 gene specific targets of COVID-19. The *in vivo* results determined a strong positive outcomes related to the SARS-CoV-2 presence.

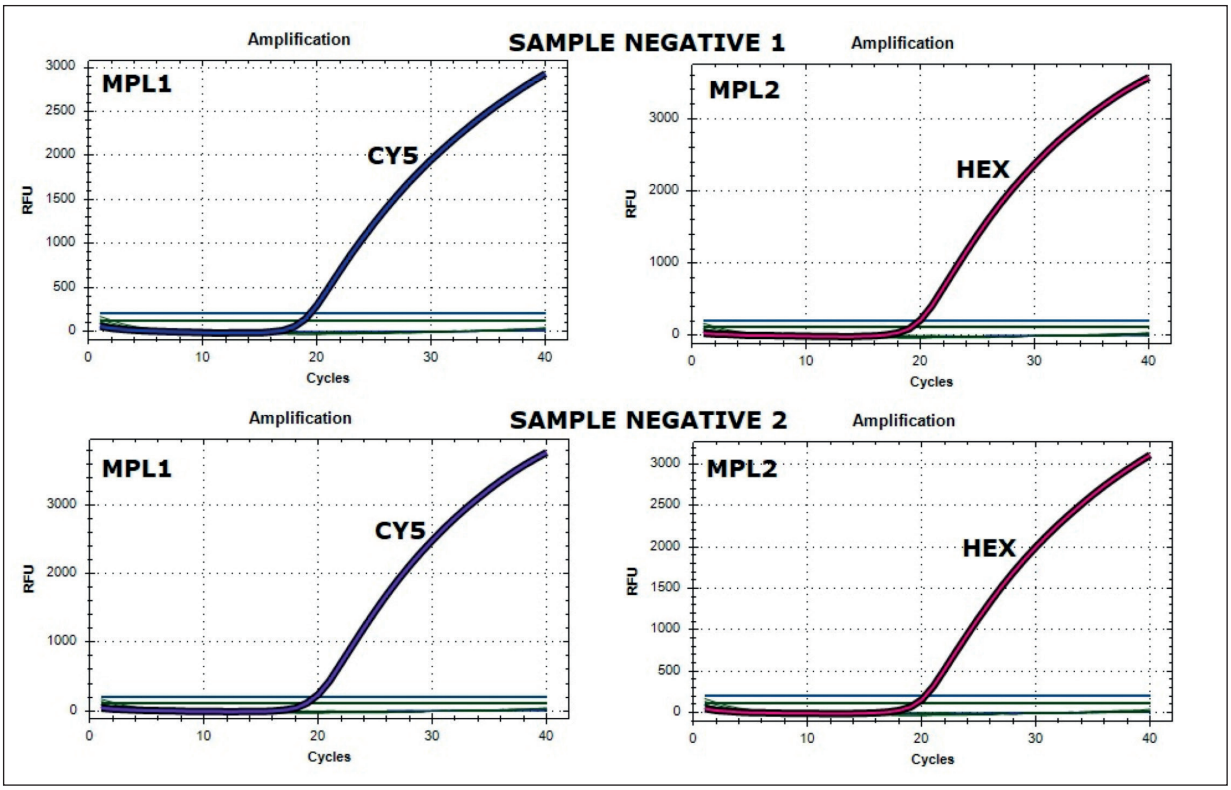


Figure 18. Two extracted RNA from 2 patients who were negative for SARS-CoV-2 (COVID-19). MPL1 detected only the presence of host epithelial cells, MPL2 detected only the PEDV. This indicated these were real, not false negative results.

The WHO recommendation follows a similar procedure adopted during the SARS-CoV pandemic two decades ago. Sequential samples from suspected patients should be kept for future use, in addition the health authorities should collect and store data on the clinical and contact history, to generate a clear logarithm that shows the virus-specific traits and patterns and its way of transmission[19]. Patients' samples should be available for rRT-PCR analysis, virus culture and antigen detection, serological antibody assays. The WHO warmly supports local Governments to create a capillary net of designate health task forces which include centers for prevention and treatment and laboratories for investigation and/or referral of specimens from possible COVID-19 patients[20].

For the current translational study, researchers at University of Medicine Phan Chau Trinh of Danang City Vietnam, the International Institute of Genetics and Immunology of Ho Chi Minh City Vietnam and at the Bio-Medical Team of University of Bari "Aldo Moro" in Italy set up a task force, based on the recent Vietnamese positive outcomes against COVID-19, in order to produce a reliable rapid diagnostic tool based on one-step rRT-PCR assay for the SARS-CoV-2 detection in humans.

The outcomes of current rRT-PCR assays look promising, this multiplex real-time PCR test could reveal few advantages: (1) combine rRT-PCR in just one step procedure; (2) with this based rRT-PCR innovative methodology, we have used the multiplex procedure to detect 4 virus targets in just one reaction. The current test allows the user to detect all coronavirus that have been known as harmful pathogens to humans including HCoV causing common cold, SARS-CoV causing SARS, SARS-CoV-2 causing COVID-19, and MERS-CoV causing MERS; (3) we set up a control procedure to detect the presence of possible external and internal contamination to validate the presence of negative outcomes, as well as the high level of the sensitivity with amplification phase through the DNA positive control; (4) a check-step was set up to avoid the false negatives by using the PEDV-CoV and the RP gene as the internal control; (5) the total time procedure including the RNA extraction and rRT-PCR takes less than 3 hours and it can be applied in any laboratory with real-time PCR facilities.

The rRT-PCR still remains a solid and reliable method to ensure the presence of COVID-19

infection especially if this could be used in support of objective clinical screens and diagnostic imaging procedures. Furthermore, this current kit-diagnostic system could be of great support in reaching the highest grade of predictability and accurateness in uncovering the 4 coronavirus different strains as SARS-CoV, SARS-CoV-2, HCoV and MERS-CoV due to high grade of sensitivity and target specificity adopted in the analyzing procedure.

The intent to include also different *Coronaviridae* members such as SARS-CoV, HCoV and MERS-CoV in this diagnostic procedure was mainly due to the atypical behavior of the COVID-19. In Italy we have been experiencing since the ending of 2019 a very bizarre form of lung-flu disease, an infection that showed many common traits with the current COVID-19 disease. The clinical manifestation included dry cough, dyspnea, fever, multi-organ/system collapse, body aching, gastro-intestinal tract disorders, sepsis, idiopathic skin rushes and several deaths as well. The early hematic, chest-X Ray and clinical biochemistry and molecular clinical biology reports revealed that those patients mainly located in the north of Italy (Lombardy) suffered from an abnormal form of interstitial lung disease, characterized by diffuse cellular infiltrates in a periacinar location, lymphopenia, extremely high level of C-reactive Protein and pro-inflammatory cytokine such as TNF-a and IL-6 associated to a secondary form of bacterial infection due to *Pneumococci* and *Staphylococcus aureus*. As a matter of fact, medical conditions also included a wide range from self-limited inflammatory processes to severe debilitating fibrosis of the lungs.

In addition, local Italian Ministry of Health sentinel departments confirmed the presence of an allied to COVID-19, in Lombardy as second pathogens with a 2-3 times capacity of increasing the disease effects like the Coronavirus from Middle East MERS-like, or HCoV-HKU1, or from domestic animals as pig, bovine, rats, mice[21].

To worsen an already complicated situation there is the huge variety of coronavirus species and sub-species and the diversity related the full taxonomy structure of the different *Coronaviridae* members. However, despite this crucial matter, they still all potentially cause severe respiratory disease to humans[22-29].

Therefore, following the recent years pandemic outbreaks, it is rational to adopt wider diagnostic approaches that specifically target the natural diversity of the *Coronaviridae* species involved in the rise of planetary infection. It follows that major efforts and further researches are constantly needed to be updated on genomic and genetic potential mutations.

The solutions are already available especially if we consider the field of multiplex rRT-PCR-based methology. The idea, as strongly suggested by some authors, is to improve and apply multispecific combined diagnostic tests that allow scientists in short time to target bacteria, fungal or virus-specific pathogens to generate a world-scale qualitatively strategic therapeutic actions. Simultaneously, the further reduction of time will also help to limit both social and economic consequences of pandemic outberaks[30-35].

Conclusions

The existing "pandemic" scenario requires solid and reliable diagnostic tests in order to proceed with the required decision making. A reliable, sensitive test will ease to organize and settle the whole countermeasures to face up to a pandemic outbreak and its consequences. Under these dramatic circumstances we hope to be of help and support to governmental organizations, health centers and hospitals in this tragic public health situation. We are dedicating all our available resources to acquiesce the outcomes of this novel diagnostic procedure with the precise intent to contribute in preventing and limiting the spread of this COVID-19 outbreak.

Author Contributions

"Conceptualization, V.H.P. and C.G.I; methodology, K.CD.N.; D.K.T. and Q.V.N.; editing, G.D.; validation, A.B. and S.C.; formal analysis, H.T.P. and S.T.P.; investigation, S.A. and A.B.; supervision A.B. and F.I. All authors have read and agreed to the published version of the manuscript."

Patent

The following is a patent resulting from the work reported in this manuscript: 102020000011701.
Name: Università Degli Studi di Bari Aldo Moro (80%) Phan Chau Trinh University (20%). Inventors names: Andrea Ballini, Francesco Inchingolo, et al. Title: "Metodo e kit diagnostico per l´individuazione multipla di virus della famiglia *Coronaviridae*: SARS-CoV2, SARS-CoV, HCoV e MERS-CoV".

Conflict of Interests

The Authors declare that they have no conflict of interests.

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