Simultaneous Detection of 2019 Novel Coronavirus and Influenza Virus by Double Fluorescent RT-PCR

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Research Article

Keywords: 2019-nCoV, A/B influenza virus, nucleic acid detection, double Fluorescent RT-PCR

Posted Date: March 25th, 2020

DOI: https://doi.org/10.21203/rs.3.rs-18989/v1

Version of Record: A version of this preprint was published at American Journal of Laboratory Medicine on January 1st, 2020. See the published version at https://doi.org/10.11648/j.ajlm.20200501.16.

Abstract

this paper introduces a method for simultaneous detection of 2019 novel coronavirus(2019-nCoV) and influenza virus by dual fluorescent RT-PCR, providing some references for the current clinical first-line practice against the epidemic. More than 1500 samples of nasopharyngeal swabs, sputum and anal swabs were collected. Nucleic acid detection kits from two manufacturers of novel coronavirus and type A/B influenza virus were selected for the detection. Carboxyfluorescein (FAM) and green fluorescent protein (VIC) labeled probes were used to achieve simultaneous detection of the four gene targets using a double fluorescent RT-PCR reaction system. According to the analysis for the results of nucleic acid detection of existing samples, there is no cross infection between 2019 novel coronavirus and type A/B influenza virus. The Ct value of novel coronavirus nucleic acid in anal swab>Ct value of sputum > Ct value of nasopharyngeal swab in the same patient. Conclusion: A method for rapid and simultaneous detection of novel coronavirus and influenza virus by dual fluorescent RT-PCR was established to improve the detection efficiency and reduce the cost, which could be used for rapid and emergent detection of 2019 novel coronavirus and type A/B influenza virus.

1. Introduction

Corona Virus Disease 2019, referred to as " COVID-19", is a pneumonia caused by infection of a novel coronavirus.[1-2]Since December 2019, some hospitals in Wuhan, Hubei Province, have reported a number of pneumonia cases for unknown reasons due to exposure to the South China Seafood Market. So far, there have been more than 80,000 people infected by 2019 Novel Coronavirus in China, among whom more than 3,000 people died. Besides China, the epidemic situation in Italy, America、Spain、 Germany,and Iran is worsening rapidly. The Director General of WHO said that the COVID-19 outbreak was evolving into a global pandemic.[3-4] Quick and accurate diagnosis on infection of the novel coronavirus is very important for assessment and control of COVID-19 situation in the world. This paper is mainly to introduce simultaneous detection of novel coronavirus and influenza viruses (FluA and FluB) by dual fluorescence RT-PCR method.

2. Materials And Methods

2.1 Material

2.1.1 Experiment materials.

Source of samples: suspected clinical patients with fever from out-patient clinics of designated hospitals in this city, clinically diagnosed patients, close contacts, domestic and foreign people who returned to the city recently, and etc. Category of samples: nasopharyngeal swabs, sputum and anal swabs, total more than 3,000 samples.

2.1.2 Main reagent

The 2019 novel coronavirus nucleic acid detection kit and the FluA / FluB nucleic acid detection kit were purchased from Shanghai BioGerm Medical Biotechnology Co. Ltd and Jiangsu Shuoshi Biotechnology Co., Ltd. The viral nucleic acid extraction kit was purchased from Xi'an Tianlong Science and Technology Ltd. and Jiangsu Shuoshi Biotechnology Co., Ltd. RNeasy RNA purification kit was from QIAGEN GmbH, Germany.

2.1.3 Instrument

ABI Q7 Real-time Fluorescence Quantitative PCR Instrument; ABI 7500 Real-time Fluorescence Quantitative PCR Instrument; Roche 480 Real-time Fluorescence Quantitative PCR Instrument; Lawson DHB-200 Armor Bead Bath; Xiamen Zhiwei GI 100DX Autoclave sterilizer; AC2-5S1 A2 Biosafety Cabinet of Esco Micro Pte Ltd., NP968 Nucleic Acid Extraction Instrument of Xi'an Tianlong Science and Technology Ltd.; SSNP-2000A Nucleic Acid Extraction Instrument of Jiangsu Shuoshi Biotechnology Co., Ltd.; Thermo ST16R High Speed Refrigerated Centrifuge, and etc.

2.2 Method

The experimental operations were conducted strictly in accordance with the “Technical Guidelines for Laboratory Detection of Novel Coronavirus Infected Pneumonia (Version 2), the “Notice of the General Office of National Health Commission on the Issuance of Biosafety Guidelines for Novel Coronavirus Laboratory (Version 2) and the “Diagnostic Criteria for Influenza WS285-2008.[5][6][7]

2.2.1 Sample pretreatment: All test samples must be inactivated at 60℃ for 40 min.

2.2.2 RNA extraction: (1) manual extraction: RNeasy RNA purification kit was used according to the instructions for use.(2) Automatic extraction: NP968 Nucleic Acid Extraction Instrument or SSNP-2000A Nucleic Acid Extraction Instrument, add 20 L of protease K and 150 L of the sample to be tested to the instrument.

2.2.3 Detection of viral nucleic acid: ABI Q7, ABI 7500 and Roche 480 Real-Time Fluorescence Quantitative PCR Instruments were used for nucleic acid amplification AND detection. The reaction system of Shanghai BioGerm novel coronavirus and FluA/B virus detection kit: 12 μL of qRT-PCR reaction solution, 4 μL of qRT-PCR enzyme mixture, 4 μL of primer probe 2019-nCoV or FluA/B, total 20 μL. The reaction system of Shuoshi novel coronavirus and FluA/B virus kit: 7.5 μL of RT-PCR reaction solution, 5 μL of enzyme mixture, 4 μL of novel coronavirus reaction solution or FluA/B reaction solution, 3.5 μL of RNA enzyme water, total 20 μ.

2.2.4 Quality control: Negative control and positive control were used for each test run, and different targets were adjusted for baseline threshold according to corresponding negative control. The Ct value of negative control was higher than the value in specified the specification or was not detected. For the positive control, the amplification curve was S-shaped, with Ct value lower than the specified value. For the same test, if all of above conditions were met, the test was valid; otherwise, the test was invalid.

3. Results

3.1 General information of the four kits

All of the four kits for detection of novel coronavirus were of ORF1ab and N genes, FAM channel was of ORF1ab gene and VIC channel was of N gene. Flu A gene and Flu B gene were for detection of influenza A and influenza B. Flu A gene was detected through FAM channel, and Flu B gene was detected through VIC channel. A comparison of the four kits is shown in Table 1.

3.2 Analysis on the test results

More than 3000 nasopharyngeal swabs, sputum and anal swabs were tested simultaneously for novel coronavirus and Flu A/B virus, and for the same sample, the two novel coronavirus detection kits gave the same results. Synchronous detection of Flu A/B infection with novel coronavirus showed that no Flu A/B virus was detected in patients who were positive for the novel coronavirus, and no novel coronavirus was detected in patients who were positive for Flu A/B virus. Most of the submitted samples were negative for novel coronavirus and Flu A/B virus, as shown in Table 2 and Figure 1, and Figure 2. Nasopharyngeal swabs, sputum and anal swabs were collected at the same time for patients whose novel coronavirus was positive. Analysis of the test results revealed that for the same patient, the Ct value of amplification curve for anal swab > Ct value of the sputum >Ct value (see Figures 3 and 4).

4. Discussion

The novel coronavirus continues to spread around the world, with confirmed cases on six continents except for Antarctica. According to the latest data released by WHO, new cases of COVID 19 have been reported in more than 170 countries and regions in the world, more than 380,000 people have been infected by novel coronavirus, and the number is still growing.[8-9]Due to the outbreak has developed rapidly, there should be a rapid and effective method for timely diagnosis among patients with different degrees of clinical symptoms and suspected patients. The novel coronavirus RNA detection can provide direct evidence for diagnosis. according to “Guidelines for Diagnosis and Treatment of COVID-19(Version 7)” of the National Health Commission, suspected cases should be confirmed for either etiology or serology.1. Real-time fluorescent RT-PCR was used for cases with positive results of novel coronavirus nucleic acid detection. 2. Virus gene sequencing is highly homologous with known novel coronavirus.3. If the serum novel coronavirus-specific IgM antibody and IgG antibody were positive, the serum novel coronavirus-specific IgG antibody was 4 times or more higher in the recovery period than in the acute phase.[10] Serological test was added to the new version of guidelines for diagnosis and treatment. IgM antibodies produced by the infected people were mostly positive in 3-5 days after the infection, and IgG antibodies were generally acquired by the body immunity after recovery. In the laboratory, IgM and IgG antibody colloidal gold strip was used to detect the serum of a positive patient after recovery and discharge from the hospital. IgM and IgG antibody were still detected after the serum sample was diluted by 16 times, indicating that the patient was likely to infect others and still need to be isolated and

observed for 14 days without interruption of detection. Real-time quantitative PCR is the best choice for detecting viral nucleic acid in the early stage of novel coronavirus infection (asymptomatic stage). Real-time fluorescent RT-PCR is for synchronous detection of novel coronavirus and influenza virus. The detection method has high sensitivity and specificity [11-12], and it can detect the same sample quickly and accurately at the same time, which may provide an important support for early clinical differentiation, early diagnosis and early treatment.

The laboratory tested more than 3000 samples for both novel coronavirus and Flu A/B virus, and it was found that no Flu A/B virus was detected in patients who were positive for the novel coronavirus, and no novel coronavirus was detected in patients who were positive for Flu A/B virus. This is not just a coincidence, but a reflection of clinical data. Based on current clinical data, there is no cross-infection between the novel coronavirus and Flue A/B. It has been verified in the laboratory that the novel coronavirus can be inactivated at 56℃ for 30 min. In this laboratory, all samples to be tested were inactivated at 60℃ for 40 min, so as to further protect the personnel for detection and avoid destructive effect on nucleic acid virus RNA. After the comparison on test results of nasopharyngeal swabs, sputum and anal swabs of positive patients, it was found that, for the same patient, the Ct value of the nucleic acid amplification curve of the anal swab was greater than the Ct value of the sputum, and the Ct value of the sputum was greater than the Ct value of the nasopharyngeal swab. It can be inferred that for an individual with samples of nasopharyngeal swab, sputum, and anal swab collected at the same time, the detection effect of anal swab was the best, followed by sputum, and then nasal and pharyngeal swab. However, clinically, the collection of anal swab is mostly limited by place. Although sputum samples are collected conveniently, many patients and the close contacts do not have sputum when they need to be collected, and they are often replaced by saliva, which affects the detection effect. The nose and pharynx samples were collected at the same time. Although the sampling was convenient, the virus bearing capacity of the nasopharynx was limited, the Ct value of the amplification curve was low, and omission was easy to occur. Therefore, it was necessary to adopt the plan of sampling and repeated detection on alternate days for suspected patients. At the same time, in the case of "negative converted to positive" after the patient was cured and discharged from the hospital, sampling and re-examination should be carried out evert two days until the nasopharyngeal swab, sputum and anal swab were completely negative for nucleic acid detection, and then regular monitoring should be conducted for a period of time.

We certify that this manuscript is original and has not been published and will not be submitted elsewhere for publication while being considered by Antonie van Leeuwenhoek. And the study is not split up into several parts to increase the quantity of submissions and submitted to various journals or to one journal over time. No data have been fabricated or manipulated (including images) to support my conclusions.

The submission has been received explicitly from all co-authors. And authors whose names appear on the submission have contributed sufficiently to the scientific work and therefore share collective

responsibility and accountability for the results.

Conflict of Interest: The authors declare that they have no conflict of interest.

The manuscript does not contain experiments using animals and human performed by any of the authors.

Informed consent was obtained from all individual participants included in the study.

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Tables

Table 1 comparison of general information on the four kits

The reagent manufacturer		Bojie□Double Fluorescent RT-PCR□		Shuoshi□Double Fluorescent RT-PCR□	
items		2019-nCoV	FluA /B	2019-nCoV	FluA /B
et gene		ORF1ab, N	Flu A, Flu B	ORF1ab, N	Flu A, Flu B
eic acid extraction od		Automatic extraction of magnetic beads□ Manual column extraction		Automatic extraction of magnetic beads□ Manual column extraction	
rescence detection amplification cycle ber		55℃□40 cycles		55℃□45cycles	
ılt rmination	□	ORF1ab, N gene Ct≤35 S curve		Ct≤35and S curve growth□both of ORF1ab and N meet the above conditions	
	—	Ct□38 or not detected		Ct□38 or not detected	
	dubious	S curve□and 35□Ct≤38 Need to review□If the review results are consistent, the result is positive		One of the channel Ct≤35□another channel is 35□Ct≤38□need to repeat testing again□If the repeated testing show Ct is still 35-38 and S curve growth, it is positive, otherwise it is negative.	
st detection Limit		1×10^3copies/mL		1×10^3copies/mL	
ar detection range		2×10^3-1×10^8copies/mL		2×10^3-1×10^8copies/mL	

Table 2: Test results of some samples

	The reagent manufacturer	Novel coronavirus detection kit		FluA /B detection kit	
		Bojie	Shuoshi	Bojie	Shuoshi
Sample ID	CBYJG20200001	—	—	—	—
	CBYJG20200002	—	—	—	—
	CBYJG20200003	—	—	—	—
	CBYJG20200004	—	—	□(FluA)	□(FluA)
	CBYJG20200005	—	—	—	—
	CBYJG20200006	—	—	—	—
	CBYJG20200007	—	—	—	—
	CBYJG20200008	—	—	—	—
	CBYJG20200009	—	—	—	—
	CBYJG202000010	—	—	—	—
	CBYJG202000011	—	—	—	—
	CBYJG202000012	□	□	—	—
	CBYJG202000013	—	—	—	—
	CBYJG202000014	—	—	—	—
	CBYJG202000015	—	—	—	—
	CBYJG202000016	—	—	—	—
	CBYJG202000017	—	—	—	—
	CBYJG202000018	—	—	—	—
	CBYJG202000019	□	□	—	—
	CBYJG202000020	—	—	□(FluA)	□(FluA)

Figures

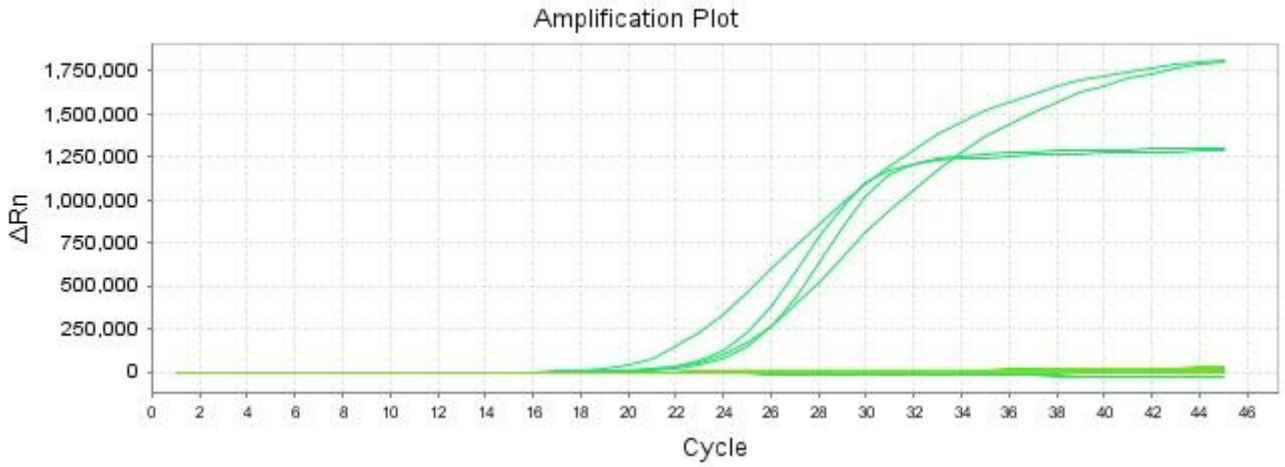

Figure 1

Curve for results from simultaneous detection of novel coronavirus and Flu A/b virus by BioGerm kit

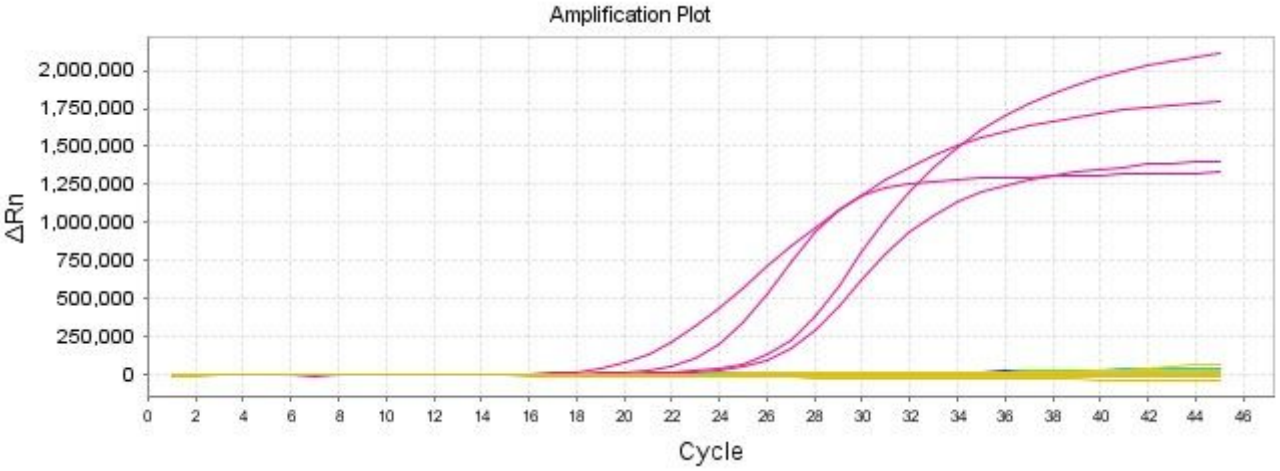

Figure 2

Curve for results from simultaneous detection of novel coronavirus and Flu A/b virus by Shuoshi kit

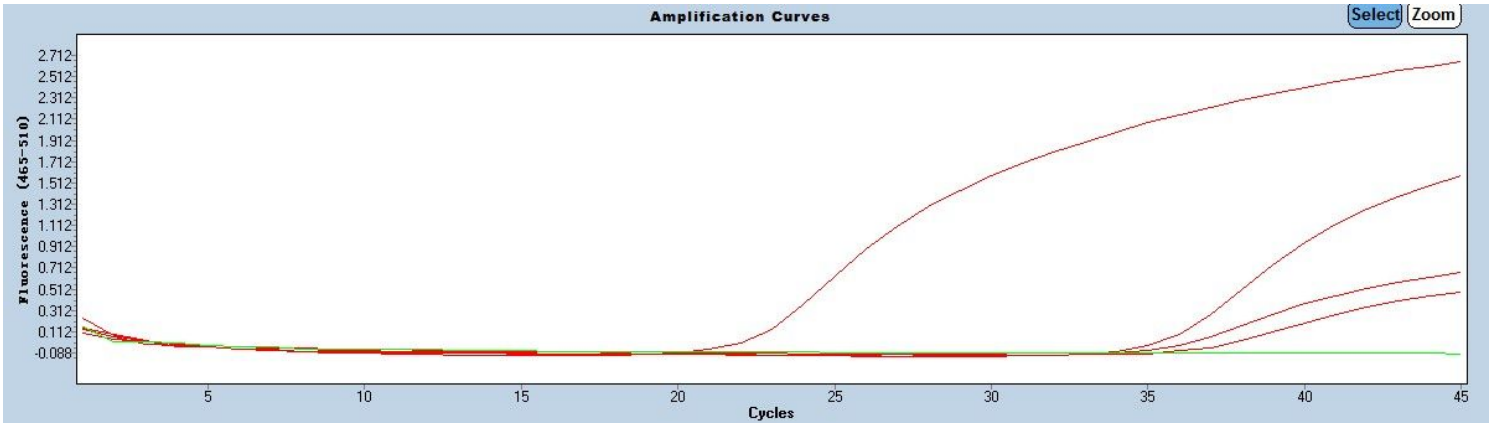

Figure 3

Curve for ORF1ab target gene results for a patient with positive results (positive control, anal swab, sputum, nasopharyngeal swab, negative control)

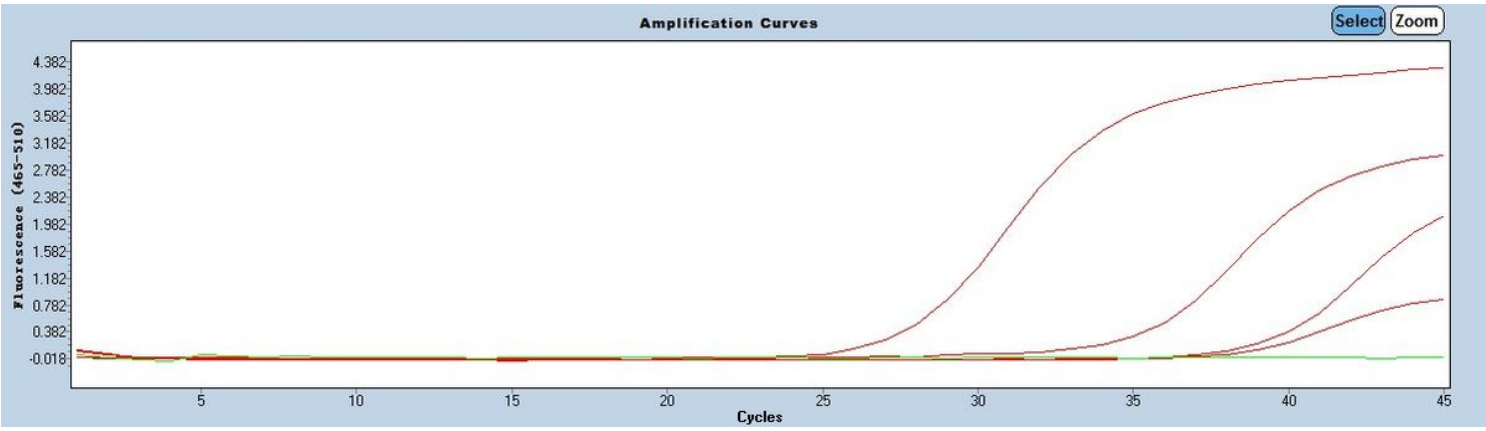

Figure 4

Curve for N target gene results for a patient with positive results (positive control, anal swab, sputum, nasopharyngeal swab, negative control)