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Nucleic acid-based molecular diagnostic testing of SARS-CoV-2 using self-collected saliva specimens

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Since the outbreak of coronavirus disease 2019 (COVID-2019), the infection has spread worldwide due to the highly contagious nature of severe acute syndrome coronavirus (SARS-CoV-2). To manage SARS-CoV-2, the development of diagnostic assays that can quickly and accurately identify the disease in patients is necessary. Currently, nucleic acid-based testing and serology-based testing are two widely used approaches. Of these, nucleic acid-based testing with quantitative reverse transcription-PCR (RT-qPCR) using nasopharyngeal (NP) and/or oropharyngeal (OP) swabs is considered to be the gold standard. Recently, the use of saliva samples has been considered as an alternative method of sample collection. Compared to the NP and OP swab methods, saliva specimens have several advantages. Saliva specimens are easier to collect. Self-collection of saliva specimens can reduce the risk of infection to healthcare providers and reduce sample collection time and cost. Until recently, the sensitivity and accuracy of the data obtained using saliva specimens for SARS-CoV-2 detection was controversial. However, recent clinical research has found that sensitive and reliable data can be obtained from saliva specimens using RT-qPCR, with approximately 81% to 95% correspondence with the data obtained from NP and OP swabs. These data suggest that self-collected saliva is an alternative option for the diagnosis of COVID-19.

Keywords: SARS-CoV-2 detection, Quantitative reverse transcription-PCR, Self-collected saliva

Introduction

Since World Health Organizations (WHO) was informed about a mysterious pneumonia in Wuhan, China, severe acute syndrome coronavirus 2 (SARS-CoV-2) was identified as the cause of the acute respiratory syndrome, coronavirus disease 2019 (COVID-19) [1]. Since then, the infection has rapidly spread worldwide due to high contagiousness of SARS CoV-2. The large number of patients with COVID-19 during outbreak is beyond the capacity of national health care systems. Hence the quick and accurate identification of the patients who need therapeutic treatment and isolation is important for the management of COVID-19.

There have been many diagnostic assays developed to cope with COVID-19 pandemic. Current testing approached are two types: nucleic acid (NA)-based and serological-based testing. NA-based testing types are molecular assays for the detection of SARS-CoV-2 viral RNA and known as the most sensitive detection for the presence of SARS-CoV-2 [2-4]. This category includes reverse transcription-polymerase chain reaction

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(RT-PCR). In addition to RT-PCR, other alternative methods such as isothermal nucleic acid amplification (LAMP) assays, transcription-mediated amplification and clustered regularly interspaced short palindromic repeats (CRISPR)-based methodologies are developed [4-8]. Serological-based testing types include serological and immunological testing. Serological assays rely on detection of the antibodies produced by infected patients. And immunological testing is based on detection of antigens in patients.

Patient antibody production against SARS-COV-2, known as sero-conversion, typically takes 5-10 days after the onset of initial symptoms [9]. NA-based testing directly detects RNA of SARS-CoV-2, therefore offers the earliest and most sensitive detection for the presence of the virus. Among these NA-based methodologies, the quantitative reverse transcription-PCR (RT-qPCR) for SARS-CoV-2 using nasopharyngeal (NP) and oropharyngeal (OP) swabs is recognized as the gold standard for COVID-19 diagnosis [2,7,10]. However, recently the use of saliva samples for RT-qPCR has been raised for its simplicity and convenience of the patients.

In this review, general workflow of RT-qPCR assay, genes used for RT-qPCR detection of SARS-CoV-2 detection and clinical usefulness of self-collected saliva samples for the diagnosis of COVID-19 are discussed.

General Workflow of RT-qPCR for SARS-CoV2 Detection

The principle of RNA-based SARS-CoV-2 testing is based on direct detection of viral RNA of swabs taken from a patient's nasal and/or throat passages. There are three main steps in the general workflow for RT-qPCR: sample collection and transport, lysis and RNA extraction, and reverse transcription and PCR amplification (Fig. 1). Almost all reagents required for the three main processes are commercially available. These reagents include patient's swabs, lysis buffer, RNA extraction kits, and RT-PCR kits [4,11].

Typically, a physician collects NP and/or OP swab samples from a patient and transfer the swab to a tube containing 2-3 mL of virus transport medium (VTM). Collected samples are moved to the laboratory for testing. In the laboratory, viral particles are lysed or inactivated by lysis buffer treatment or heating. A fraction of VTM samples is used for RNA preparation either by column type or magnetic bead type RNA purification kits using either manual methods or automatic NA extractors. One of the advantages of RNA purification is that the viral RNA present in swab collection sample can be concentrated and eluted in a buffer compatible with RT-PCR. The RNA in elution buffer is then reverse-transcribed and amplified using a one-step master mix containing reverse transcriptase and DNA polymerase enzymes in a real-time PCR instrument. The one-step master mix contains primer sets for specific regions of the viral genome. Primers targeting a human gene, such as RNase P, are included as an internal control for the three main steps described above; swab collection, RNA purification and RT-PCR amplification. Generally, fluorescent TaqMan probes are used to detect the amplified DNA and a threshold cycle of amplification is set to determine positive and negative results [2,4].

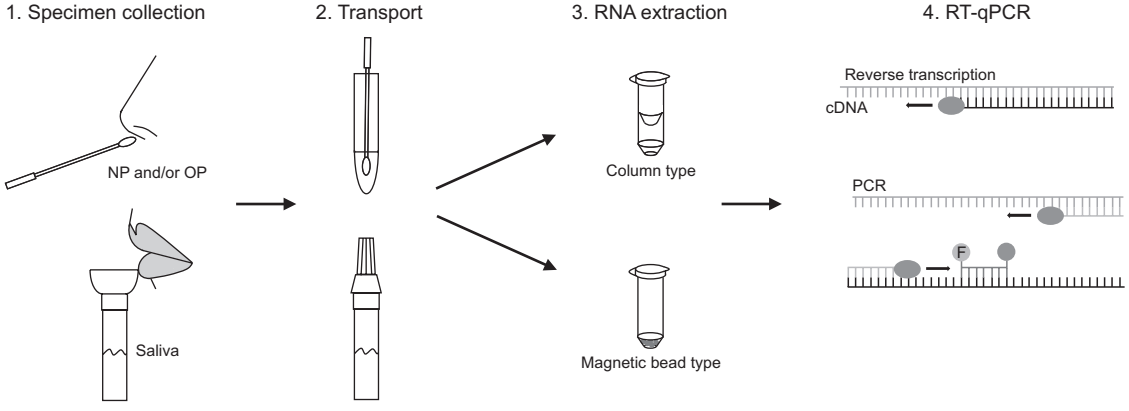

Fig. 1. Workflow of severe acute syndrome coronavirus 2 (SARS-CoV-2) detection using quantitative reverse transcription-polymerase chain reaction (RT-qPCR). Specimen collection via nasopharyngeal (NP) and/or oropharyngeal (OP), or self-collected saliva, transportation in virus transport media (VTM), lysis and RNA extraction either via column type or magnetic bead type extraction methods, and reverse transcription and polymerase chain reaction process are depicted.

Genes Used for SARS-CoV-2 Detection

Generally, corona virus genomes are approximately 30 kB in length. The first genome sequence of SARS-CoV-2 was reported by Wu et al. [1]. It contains 29,903 nucleotides RNA genome [1]. The order of gene was replicase *ORF1ab*, *spike (S)*, *envelope (E)*, *membrane (M)*, and *nucleocapsid (N)* from 5' to 3 (Fig. 2).

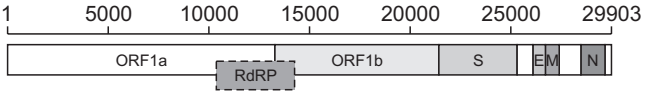

Fig. 2. Genome organization of severe acute syndrome coronavirus 2. ORF, open reading frame; RdRP, RNA-dependent RNA polymerase; S, spike protein; E, envelope protein; M, membrane protein; N, nucleocapsid protein.

Many research articles regarding the development and application of RT-qPCR assays for detection of SARS-CoV-2 are published [4,9,11-14]. Among the genes described above, E gene, N gene, RdRP gene, and ORF1ab region are most frequently used to design SARS-Cov-2 primer set. In Table 1, primer and probe sequences, and the representative institution names including Charité Germany, China Centers for Disease Control and Prevention (CDC) and US CDC are listed. Many commercial assay kits for COVID-19 diagnostic kits are developed based on the sequences announced by representative institutions.

Table 1. Primer sets used for SARS-CoV-2 detection using RT-qPCR testing

Gene	Primers and probes (5'→3')	Institution
E and RdRp	E gene Forward: ACAGGTACGTTAATAGTTAATAGCGT Reverse: ATATTGCAGCAGTACGCACACA Probe: FAM-ACACTAGCCATCCTTACTGCGCTTCG-BBQ RdPR gene Forward: GTGARATGGTCATGTGTGGCGG Reverse: CARATGTTAAASACACTATTAGCATA Probe 1: FAM-CAGGTGGAACCTCATCAGGAGATGC-BBQ Probe 2: FAM-CCAGGTGGWACRTCATCMGGTGATGC-BBQ	Charite, Germany
ORF1ab and N	ORF1ab gene Forward: CCCTGTGGGTTTTACACTTAA Reverse: ACGATTGTGCATCAGCTGA Probe: FAM-CCGTCTGCGGTATGTGGAAAGGTTATGG-BHQ1 N gene Forward: GGGGAACTTCTCCTGCTAGAAT Reverse: CAGACATTTTGCTCTCAAGCTG Probe: FAM-TTGCTGCTGCTTGACAGATT-TAMRA	China CDC
N	N1 gene Forward: GACCCCAAAATCAGCGAAAT Reverse: TCTGGTTACTGCCAGTTGAATCTG Probe: FAM-ACCCCGCATTACGTTTGGTGGACC-BHQ1 N2 gene Forward: TTACAAACATTGGCCGCAAA Reverse: GCGCGACATTCCGAAGAA Probe: FAM-ACAATTTGCCCCCAGCGCTTCAG-BHQ1 N3 gene Forward: GGGAGCCTTGAATACACCAAAA Reverse: TGTAGCACGATTGCAGCATTG Probe: FAM-AYCACATTGGCACCCGCAATCCTG-BHQ1 RP-F RNAse Forward: AGATTTGGACCTGCGAGCG Reverse: GAGCGGCTGTCTCCACAAGT Probe: FAM-TTCTGACCTGAAGGCTCTGCGCG-BHQ-1	US CDC

SARS-CoV-2, severe acute syndrome coronavirus 2; RT-qPCR, quantitative reverse transcription-polymerase chain reaction; E, envelope protein; RdRP, RNA-dependent RNA polymerase; ORF, open reading frame; N, nucleocapsid protein; CDC, Centers for Disease Control and Prevention.

Use of Self-collected Saliva for SARS-CoV-2 Detection

RT-qPCR assays with specimens collected from NP and OP regions provided sensitive and accurate COVID-19 diagnosis [3,15,16]. Saliva droplets are recognized as the main cause of human-to-human transmission of SARS-CoV-2. This implies that saliva droplets contain certain level of the viruses, therefore it can be considered as sample specimen for SARS-CoV-2 detection [17-22]. Oral saliva and posterior OP saliva should be distinguished from each other. The former is produced by the salivary glands which are separated from the respiratory tract. The latter contains pharyngeal secretions which are part of respiratory tract. In this study we reviewed the reports used oral saliva as specimen.

There are several advantages of using saliva as specimen for SARS-CoV-2 detection. Firstly, saliva specimens are easily collected by the patients [17,22,23]. Thus, using saliva as specimen can release the burden from a patient. Secondly, using saliva as a specimen can reduce the risk of the healthcare providers from being exposed to viral droplets. Collecting NP and OP swabs require a close contact of the patients and medical workers, which increase the risk of virus transmission to healthcare providers. Such close contact can be avoided when self-collected saliva specimen is used. Thirdly, sample collection time and cost can be lowered [18,20,23,24]. The disadvantage of saliva samples is the possibility of the lower viral doses than in NP samples [25]. Until recently, the sensitivity and accuracy issues of saliva specimen for SARS-CoV-2 detection remained controversial. The reported data suggested that diagnostic sensitivity is rather broader than those obtained with NP and OP swabs.

Recently, Azzi et al. [17] reported that all of the salivary samples of 25 COVID-19 patients were positive for the presence of SARS-CoV-2. They suggested that saliva is a reliable specimen to detect SARS-CoV-2.

A Japanese team evaluated the usefulness of saliva samples in detection of SARS-CoV-2 with several detection methods including RT-qPCR [20]. Among 103 COVID-19 confirmed patients 84 patients showed positive with saliva specimen when used with laboratory developed RT-qPCR test, which is about 81.6% correlation. However, when they used a rapid antigen test, only 11.7% of the specimen were SARS-CoV-2 positive with saliva specimen. It was suggested that self-collected saliva can be an alternative specimen option for SARS-CoV-2 detection, but antigen test alone is not recommended for an initial COVID-19 diagnosis.

A research group at Yale school of medicine compared self-collected saliva specimens and NP specimens from 70 hospitalized patients with COVID-19 [26]. They found that SARS-CoV-2 RNA copies were higher in saliva specimens than NP specimens (5.58 vs. 4.93 mean log copies/mL). In addition, at 1–5 days after COVID-19 diagnosis, saliva specimens showed higher percentage of positive than NP specimens (81% vs. 71%).

Migueres et al. [25] compared the sensitivity of saliva and NP specimens for COVID-19 diagnosis for total of 123 inpatients and ambulatory patients. Among them 44 individuals were with at least one positive specimen. Asymptomatic and early detected symptomatic patients showed 88.2% and 94.7% SARS-CoV-2 detection with saliva specimen using RT-qPCR assay, respectively. They concluded that saliva specimens provided relevant and reliable data.

Considering the convenience of patients and the benefits for the health care providers, self-collected saliva can be a good candidate specimen for SARS-CoV-2 detection. In addition, the clinical data obtained suggested that self-collected saliva is an alternative option for COVID-19 diagnosis. In conjunction with NA-based RT-qPCR assays the sensitivity is sufficiently higher for clinical use in clinical settings and facilities.

Conflicts of Interest

No potential conflict of interest relevant to this article was reported.

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