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REVIEW

True or false: what are the factors that influence COVID-19 diagnosis by RT-qPCR?

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ABSTRACT

Introduction: The Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) disease has had a catastrophic impact on the world resulting in several deaths. Since World Health Organization declared the pandemic status of the disease, several molecular diagnostic kits have been developed to help the tracking of viruses spread.

Areas Covered: This review aims to describe and evaluate the currently reverse transcriptase-quantitative polymerase chain reaction (RT-qPCR) diagnosis kit. Several processes used in COVID-19 diagnostic procedures are detailed in further depth to demonstrate optimal practices. Therefore, we debate the main factors that influence the viral detection of SARS-COV-2 and how they can affect the diagnosis of patients.

Expert Opinion: Here is highlighted and discussed several factors that can interfere in the RT-PCR analysis, such as the viral load of the sample, collection site, collection methodology, sample storage, transport, primer, and probe mismatch/dimerization in different brand kits. This is a pioneer study to discuss the factor that could lead to the wrong interpretation of RT-qPCR diagnosis of SARS-CoV-2. This study aimed to help the readers to understand what very likely is behind a bad result of SARS-CoV-2 detection by RT-PCR and what could be done to reach a reliable diagnosis.

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Coronavirus; diagnosis; COVID-19; RT-PCR; sensitivity

1. Introduction

The Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) belongs to the *Coronaviridae* family and contains a positive sense nonsegmental single-stranded RNA with around 30 Kb length. In December 2019, it was discovered that infections by SARS-CoV-2 led to coronavirus disease, later named COVID-19 [1]. COVID-19 has spread rapidly worldwide and became pandemic in March 2020 [2]. According to WHO, until august 2021, approximately 207,784,507 cases of COVID-19 were reported worldwide with 4,370,424 deaths.

The gold standard for SARS-CoV-2 infection diagnosis is Reverse Transcription-quantitative Polymerase Chain Reaction (RT-qPCR), also named real-time PCR, which is based on the use of probes and primers to specifically amplify a targeted region of viral genetic material [3]. Despite the high specificity and sensitivity of RT-qPCR, the method may be affected by several factors, such as the diagnostic kit used, viral loads, site of collection, and time of infection [4]. Indeed, false results from RT-qPCR analyses were reported from Wuhan hospitals which several factors can influence during the sample collection and processing [5,6].

Thus, the objective of this work was to list all factors that can contribute to false-negative or false-positive results in the RT-qPCR test for SARS-COV-2. However, before going more in-depth in the discussion, a brief background on coronaviruses and RT-qPCR is required.

2. Coronaviruses

The Coronaviridae family is divided into four main genera: alpha, beta, gamma, and delta-CoVs [7,8]. Before the ongoing SARS-CoV-2 outbreak in December 2019, SARS-CoV (2002–2003) and MERS-CoV (2012) smaller outbreaks lead to severe respiratory illnesses [7,9,10]. SARS-CoV and MERS-CoV reached several countries, infecting and killing thousands of individuals by causing respiratory and neurological diseases with a high case fatality rate [9]. Coronaviruses share similar structures with genomes enclosed in a lipid envelope (Figure 1). Regarding the proteome, they have a nucleocapsid protein (N) physically attached to RNA. Additionally, the viral gene encodes a small membrane envelope protein (E), spike protein (S), and a membrane protein (M) (Figure 1) [8].

Among those proteins, given its importance to viruses entering the cell, spike protein has become a target for drug development and antibody neutralization. However, spike protein is the most variable protein in coronaviruses making it

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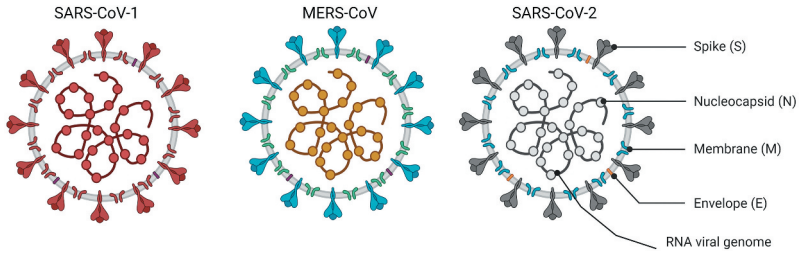

Figure 1. Morphology of the three relevant coronaviruses with epidemic/pandemic impact on human health. SARS-CoV-1, MERS-CoV and SARS-CoV-2. The Structural proteins are shown in the figure, such as spike (S), membrane (M), nucleocapsid (N) and envelope (E). Their genes are commonly used as target for real time PCR detection. Created in BioRender.com.

a bad target for diagnosis [11,12]. Despite S protein that present high mutational rate, the other structural proteins in which are more conserved among the human coronaviruses including SARS-CoV, MERS-CoV, and SARS-CoV-2 were used to develop or adapt the existing diagnosis technologies for be able to detect COVID-19.

3. RT-qPCR for SARS-CoV-2 detection

RT-qPCR is the most employed technique to identify the presence or absence of SARS-CoV-2, including for early diagnosis of COVID-19 disease (Figure 2) [13–16]. SARS-CoV-2 detection is similar to that employed for other acute respiratory

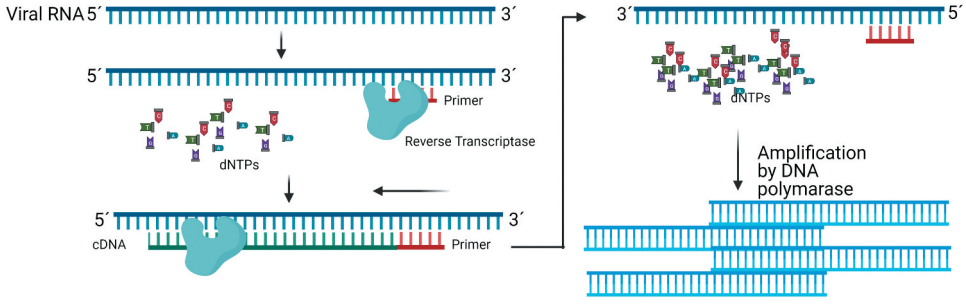

Figure 2. Scheme showing gene amplification in RT-qPCR process. In the RT-qPCR the first step is the construction of complementary DNA (cDNA) using RNA as model by reverse transcriptase. Thereafter, the stable double-strand DNA is used as template for the exponentially amplification of the product. Created in BioRender.com.

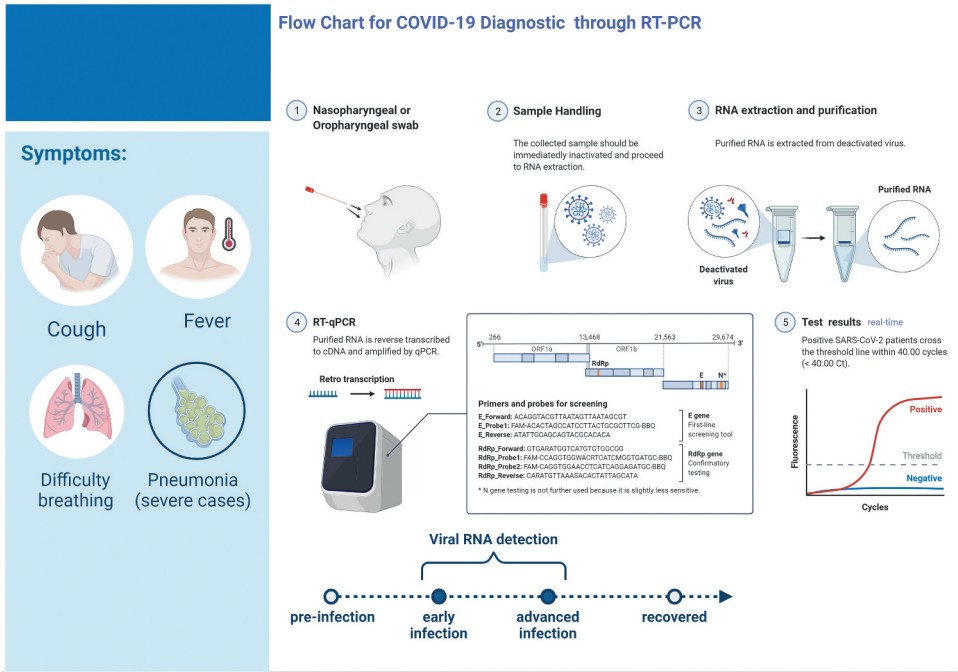

Figure 3. Flow Chart for COVID-19 diagnostic by RT-qPCR. Patients presenting symptoms of COVID-19 are subjected to the test. (1) occurs the sample collection by either naso- or oropharyngeal. (2) The collected sample is immediately processed. (3) RNA extraction. (4) RT-qPCR process and (5) Data analysis. Created in BioRender.com.

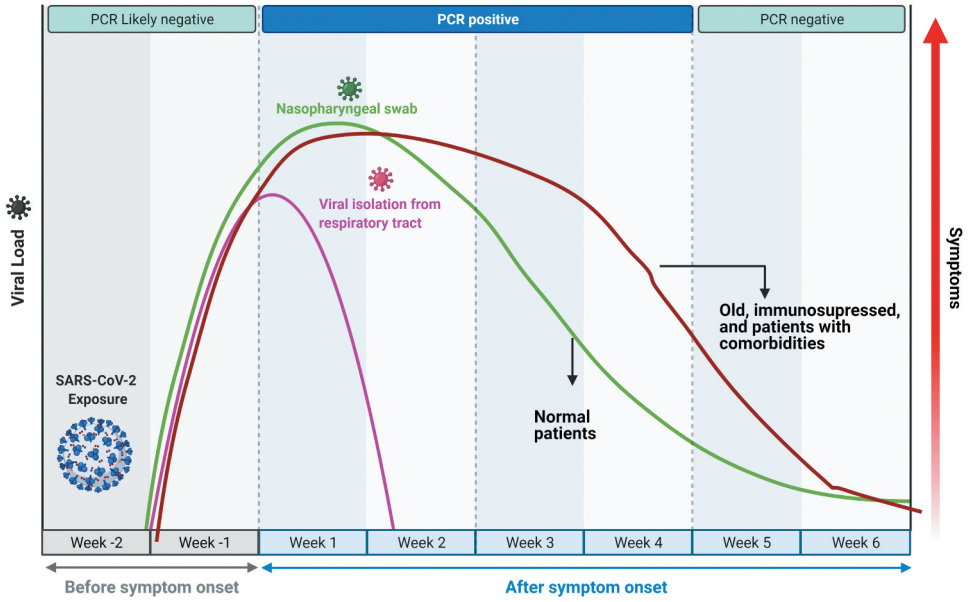

Figure 4. Flow Chart suggesting the best time to perform COVID-19 diagnosis. After exposition to SARS-CoV-2, symptomatic stage is going until 2 weeks after contact, which is the best time to SARS-CoV-2 detection. In normal patients and compromised patients' low viral loads could still be detected in 3–4 weeks. Created in BioRender.com.

infections caused by viruses [14,16]. The sample collection for diagnostics can be performed from several points from human body, such as nasopharyngeal and oropharyngeal swabs, human fluids such as blood, blood serum, saliva, urine, and anal (Figure 3) [17,18]. Furthermore, SARS-CoV-2 could be found in peripheral blood specimens, although variable results have been reported [19].

Overall, PCR reactions are applied to samples composed of DNA, allowing direct amplification by Taq polymerase activity and detection by the machine. However, to detect RNA viruses, like SARS-CoV-2, the process is a bit different (Figure 2). In this context, a previous step for viral mRNA conversion to DNA is required. Then, the RT-qPCR detection for RNA viruses occurs in two steps: 1) a reverse transcription reaction to produced complementary DNA (cDNA) using copies of mRNA as primer catalyzed by an RNA-dependent DNA polymerase (reverse-transcriptase) Taq polymerase is applied to amplify the specific segment of genome which provide result about virus presence (Figure 2) [20]. Most RT-qPCR tests for SARS-CoV-2 are quantitative by using fluorescence measurements that are sometimes referred to as RT-qPCR. Briefly, cDNA polymerizes with a probe targeted with both fluorescent and quencher labels. After polymerization into double-stranded DNA (ds-DNA), the quencher and fluorescent probes are separated and light emission from the fluorophore is observed upon light excitation [20].

SARS-CoV-2 detection by RT-qPCR is quite simple, as summarized in Figure 3. Patients are eligible to be tested once they present symptoms. The most common symptoms are cough, dyspnea, chest pain, myalgia/arthralgia, diarrhea, nausea, vomiting, and common systemic symptoms observed: fever, chills, and fatigue [21]. First, the health professional performs the sample collection from patients primarily by nasopharyngeal or oropharyngeal swab method. Second, after the collection, sample handling is involved in virus transport, which should occur at a controlled temperature (2–8°C) to a specialized laboratory, followed by virus inactivation which could be by physical (heat and exposure to UV light) or chemical (chlorinated disinfectants). The third step is regarding the RNA extraction and purification, and then the fourth step is the RT-qPCR itself. At this point, the RNA purified is first converted cDNA, and the amplification starts (Figure 2 and 3) [22].

For the amplification process, the common targets employed are E, ORF1ab, and N genes, which are considered stable genes. In this step, two primers and one probe are used for each gene. The probe is involved in fluorescence releasing, used for reverse transcriptase quantitative polymerase chain reaction (RT-qPCR) detection (Figure 3). The fifth is probably the most crucial step in data analysis. At this point, the data analysis will reveal if the patient is positive or negative for SARS-CoV-2. Many factors could affect this process, including poor sample collection and handling, RNA extraction, and RT-qPCR runs [13,20].

Another important point that could affect the results is the time of sample collection (Figure 3). If the sample is collected too early, in a time called pre-infection, the results could be a false-negative because the amount of the virus is too low and is not detected yet. At this point, the virus is still in the replication process (Figure 4). Likewise, if the collection is made too late, the patient could present a negative result because now, it is in the recovery process, where the body already eliminates the virus. The optimal time for collection comprehends three days after the first symptoms come up until the fourteen-day [23]. This timeline provides a more reliable true negative or positive result. In this manuscript, we discussed the factors that may interfere in SARS-CoV-2 detection by RT-qPCR tests.

4. Influencing Factors on SARS-CoV-2 Detection

4.1. Disease staging

Several data reported the positive correlation between viral loads and disease staging [4]. Thus, if the patient decides to perform an RT-qPCR test to diagnose COVID-19 on the first day of symptoms, the result probably would be a false negative because the viral load is still extremely low [24]. In contrast, in normal conditions and in patients with no comorbidities, if the test is taken at 13–14 days of the first symptoms, it can also lead to a false negative result (Figure 4). However, in old or immunosuppressed patients with comorbidities, the infection and positive RT-qPCR results could still be positive even after 3–5 weeks later [25].

Here, we reinforce the diagnostic window importance on SARS-CoV-2 detection, to ensure a correct diagnostic. Overall, 5 to 6 days after symptoms come up, high loads of SARS-CoV-2 in their upper and lower respiratory tracts are detected (Figure 4) [26–28].

4.2. Sample collection methodology and sample storage

According to WHO (2021), laboratory tests in COVID-19-suspected patients should be performed using samples collected from the upper respiratory tract, such as nasopharyngeal and oropharyngeal swab (Figure 3) as well as lower respiratory specimens [29]. However, depending on the sample collection site, different viral loads are recovered [4]. Also, the effect of specimen collection time on the detection rate of novel coronavirus is important for the diagnosis success. Liu et al. (2020) demonstrated the nasopharyngeal swab detection rate, nasal swab, and oropharyngeal swab are higher before washing in the morning and lower after washing morning and during the afternoon. The study suggests that this is probably due to the human body resting during the night, increasing virus propagation. During the day, the activity state of the human body might affect the virus accumulation [30].

Some collection sites provide different features. For instance, saliva samples have been reported as a low cost-effective and noninvasive alternative, since it has been used to detect other respiratory viruses [31]. The collection made by swabs taken from the throat immediately upon symptom onset is 6.4% less effective than nasal swabs to yield positive results in nasal swabs [4].

A nasopharyngeal and oropharyngeal swab are often recommended for screening or diagnosis and provide a great sensitivity to early infection [16,28,32]. A single nasopharyngeal swab has become preferred because it is well accepted by the patient and is safer for the operator. Nasopharyngeal swabs have an inherent quality control reached by the correct area to be tested in the nasal cavity. W. Wang et al. (2020b) have just reported that oropharyngeal swabs in China are frequently employed (n = 398) than nasal swabs (n = 8) to diagnose COVID-19 outbreak; however, the SARS-CoV-2 was only detected in 32% of oropharyngeal swabs. That result is 50% lower than those for nasal swabs (63%) [33].

The collection by both nasopharyngeal and oropharyngeal swabs, either as an independent sample or a single aliquot of viral transport medium, is an attractive option in normal circumstances. As such, institutions should consider the potential outbreak effect on national/international supply chains. Despite that, there are not only nasopharyngeal and oropharyngeal swabs to collect and diagnose. It was reported that sputum specimens or bronchoalveolar lavage fluid specimens have a remarkably high detection rate for novel coronavirus [34]. However, it is not possible to perform these kinds of collection samples in some patients. Other sample collection sites such as anal swabs, serum, stool, urine, feces, or ocular secretions have also shown to be viable sources of SARS-COV-2 detection [35,36]. Although the sample size has a high influence on the viral load recovered, it directly influences test sensitivity. For example, according to Mattioli et al (2020), SARS-CoV-2 could be found in 78% of serum but only in 50% of plasma samples. Therefore, it is clear that the collection site is determinant to viral load measurements.

The standardization of the collection method is one of the most important steps in diagnosing infectious diseases. Pondaven-Letourmy et al (2020) described two techniques for evolving the nasopharyngeal region: nasopharyngeal swab or nasopharyngeal wash/aspiration. The chosen technique could influence the viral load recovered, as well as the sample collection correct execution. Well-trained testing teams should also help increase the sensitivity of the test, which would avoid false-negative results [37].

On 8 May 2020, the salivary test was approved by the Food and Drug Administration, but we also found that it can be done in different ways: collecting through the spit, expectorated saliva, saliva collected directly from the salivary gland. Auto sampling methodology, where the patient collects himself, usually is not standardized, which can generate more bias for studies [38,39].

Those different methods may also interfere with the viral load recovered from the patient and, subsequently, on its detection accuracy (Table 1). Indeed, there is not a consensus yet if saliva samples are accurate as a nasopharyngeal sample for SARS-CoV-2 detection. Landry et al (2020) have shown that saliva samples are more sensitive than nasopharyngeal samples. Wyllie et al (2020) revealed that saliva samples allowed higher detection of SARS-CoV-2 RNA copies than samples collected by nasopharyngeal swab from the same patients at the same time. Interestingly, the authors discuss that saliva samples presented more positive

Table 1. Differences in sample collection sites for SARS-CoV-2 detection.

Sample collection site	Characteristics (advantages and disadvantages)	Reference
Nasopharyngeal swab	Great sensitivity to early infection. Gold standard on SARS-CoV-2 detection. Although, this method depend on the collector expertise.	29, 37
Oropharyngeal swab	Noninvasive alternative. Lower detection rate when compared to nasopharyngeal samples	33
Sputum specimens bronchoalveolar lavage fluid	High detection rate. Although, it is not possible to collect from this site in some patients	29, 34
Saliva	Low cost-effective, noninvasive alternative, and not dependent on the expertise of the collector.	31, 38, 39, 40
Serum, plasma	Low detection rate. These sample sites have a low detection rate and are not recommended.	6
Anal swabs, urine, feces, ocular secretions or semen	These sample sites have a low detection rate and are not recommended.	29, 33, 35, 36

results than nasopharyngeal samples up to 10 after COVID-19. One to 5 days after COVID-19 diagnosis, from 70 patients, 81% of the saliva samples were positive compared to 71% positive for nasopharyngeal samples [40].

This mentioned study with 13 health care workers who are completely asymptomatic was carried out by testing samples from saliva and nasopharyngeal swabs. All 13 have SARS-CoV-2 detected on saliva samples. When tested by nasopharyngeal samples, only 6 had SARS-CoV-2 detected. In conclusion, nasopharyngeal samples provide more false negative results when compared to saliva samples. This could be explained by the variation in nasopharyngeal sampling. It is known nasopharyngeal sampling is harder than saliva and this could be responsible for the variations and false results provided by nasopharyngeal samples. In contrast, saliva sampling is too easy and can be done by the patient providing solid results [40]. Recently, a study validated a safe and sensitive SARS-CoV-2 detection by RT-qPCR using saliva, which they called DRUL saliva test, with limit of detection similar to nasopharyngeal swab samples in order to implement the method for "back to work/school [41].

WHO (2021) has recommended that after collection, the samples must be stored at 2–8°C for no longer than 72 hours. Samples with a delay in testing or shipping must be stored at −70°C or below. Yet, due to the high demand for tests in labs during the most critical periods of the SARS-CoV-2 outbreak, there was a delay in the deadline to process samples and release results. That scenario required sample storage for a time higher than 72 hours at 2–8°C. A failure in storage can lead to RNA degradation contributing to a false-negative test [42]. Furthermore, if sample transportation is required, the material collected should be transported in an ideal transport medium under cold conditions in a triple-layered packaging which consists of a leak-proof receptacle [43].

Until now, none of the variants has posed any difficult on the identification by either nasopharyngeal or salivary samples. However, the newest variant Omicron require some adjustments during collection to provide a reliable diagnostic. In case of Omicron, the best site of collection is by saliva swabs than nasal swabs. Marais et al. (2021) revealed that saliva samples present a positivity of 100% (95% CI: 90–100%). In contrast, saliva sample presented a positivity of 86% (95% CI: 71–94%) to Omicron. This fact is explained because Omicron is more adapted to accumulate in saliva than in nasopharyngeal samples [44].

4.3. RNA extraction methodologies

Several RNA extraction strategies and kits have emerged or been adapted as alternatives to accelerate the sample processing step and increase the viral load recovered after extraction (Table 2). A study showed the combination of heat treatment and proteinase K to improve the RNA yield after extraction and SARS-CoV-2 detection by RT-qPCR (Table 2) [45]. The authors found that proteinase K and heat pre-treatment combination led to a higher yield of RNA collected and the obtention of lower cycles quantification in RT-qPCR reaction for SARS-CoV-2 compared with heat only or no pre-treatment.

Table 2. RNA extraction methods.

RNA extraction method	Characteristics	Reference
EXTRAzol	Less efficient when compared to column methods	44, 45
Column-based methods	Great efficiency	44, 45
Magnetic bead extraction	Great efficiency	46
Proteinase K and heat pre-treatment combination + any extraction method	Promotes a higher yield of RNA collected	43

Other studies performed a comparative analysis of different RNA extraction methods, including Qiamp DSP Virus Spin Kit (Qiagen, Hilden, Germany, Cat.61704), Total RNA Purification Kit (Norgen, Rome, Italy, Cat. # 17200), Viral Nucleic Acid (DNA/RNA) Extraction Kit I (Fisher Molecular Biology, Rome, Italy, Cat. DR-003), BSA-based method, acid pH-based-method, High temperature-based method, TRIzol (Ambion da Life Technologies®) and EXTRAzol (BLIRT S.A., Gdańsk, Poland, Cat. EM30-100) [46,47]. The results were pertinent in demonstrating the RNA isolation efficiency by EXTRAzol was lower than that extracted by column-based methods and this low yield is likely to affect the RT-qPCR performance. Moreover, the acid- pH-based method was considered an excellent alternative to commercial systems.

Other extraction upgrades or methods are constantly mentioned in literature to improve the test sensitivity. Klein et al (2020) provide an alternative method for RNA extraction based on magnetic bead extraction [48]. Another study proposed workable COVID-19 testing which might be implemented by sampling directly into a lysis buffer and RT-qPCR master mix without intermediate steps such as extraction processing [49].

4.4. SARS-CoV-2 Detection by RT-qPCR

When it comes to performing the maximum SARS-CoV-2 detection tests in a minimum period of time, there are some methods we can rapidly think of, like reverse transcription loop-mediated isothermal amplification (RT-LAMP) and RT-qPCR [48]. Although there are other SARS-CoV-2 detection protocols, the real-time qPCR is the gold-standard method recommended by the WHO [50]. Nowadays, the challenge is the detection specificity and sensitivity, which are variable and occasionally low [33]. Collection site, period of collection, sample conservation and transportation, low patient viral load, sporadic shedding, and variation in detection kits from different manufacturers contribute to the low sensitivity of SARS-CoV-2 detection [51].

In RT-qPCR, primers/probes act as biorecognition elements for different target genes, such as ORF1ab, N, and E gene [6]. Different diagnostic kits may use different SARS-CoV-2 targets to identify the virus presence/absence on the sample. Different laboratories around the world have developed several modifications of these assays.

CDC designed FDA EUA 2019-nCoV CDC kit (IDT, USA) searches for N1 and N2 targets, two regions on virus nucleocapsid gene (N) [52]. In contrast, the kit developed by Fiocruz (SARS-Cov2 (E) – Bio-Manguinhos (according to the Berlin protocol) searches for one region on the E gene [14,53].

Some other protocols also may use the RNA polymerase gene (RdRp/Helicase) or even the Spike gene (S) [14].

All possible chosen targets are susceptible to nucleotide substitution in SARS-CoV-2, affecting the oligonucleotide hybridization efficiency if mutation occurs in the primer on probes annealing regions. Yet, it is known that some regions of the SARS-CoV-2 genome are more likely to undergo mutations than others [54,55].

As observed by several studies which critically compared the efficiency and sensitivity of widely used RT-qPCR kits, the primer-probe set, and variability of SARS-CoV-2 genome have a clear participation on the reaction limit of detection. One of the key factors for detection sensitivity is the primer/probe efficiency in binding target [56]. Therefore, the accumulated mutations in SARS-CoV-2 genome during its pandemic outbreak, if it occurs in the primer region of the target DNA, implies that mismatches may affect the detection of the target [57].

Table 3 shows the results from a few studies which demonstrated the mismatch frequency of four RT-qPCR kits for different targets that had occurred in a specific number of analyzed SARS-CoV-2 genomes. The most problematic mismatch is on China CDC kit (targeting N gene), with a frequency between 12.7% and 85.3% [58,59], and Charité (targeting ORF1b) with a frequency reaching 100% [60]. As explained by Corman and Drosten (2020), a plausible reason for mismatch presence observed in some detection kits was the incomplete genomic information available at the point of designing [61].

According to Corman et al. (2020) data, PCR assays using the N gene were slightly less sensitive than assays using E and RdRp genes. Indeed, ORF1ab and N genes were not recommended for RT-qPCR testing by institutions worldwide [62] and the S gene is more susceptible to mutations, which could affect diagnosis. Ramírez et al. (2021) affirmed that sensibility on detection lineage B.1.1.7 could be affected if PCR kit is directed to the Spike (S) gene [63]. Considering that mutations in the S gene are present in different lineages, this is not a good target for diagnosis assays.

Table 3. The real-time qPCR kit for SARS-CoV-2 detection and their mismatch frequency.

		Mismatch analysis		
Source	Target gene	Frequency*	Total Samples	Refs
CDC (China)	ORF1ab	0.4%	992	56, 57, 58, 78
		0.05–0.39%	~16,000	
		1.1%	177	
		0.03%	2,569	
	N	12.7%	992	56, 57, 58, 78
		18.8%	16,662	
		85.3%	177	
		13.9%	2,569	
Charité	E	0.4%	992	56, 57, 58, 78
		0.03%–	~16,000	
		0.14%	177	
		1.1%	2,569	
		0%		
	ORF1b	99.8%	992	57, 58, 78
		100%	17,004	
		0.03%	2,569	
HKU	N	0.5%	992	56, 57, 58, 78
		0.3%	16,667	
		58.2%	177	
		0.07%	2,569	
	ORF1b	0.2%	16,932	58, 78
		0%	2,569	
CDC (US)	N	0.2–3.9%	992	56, 57, 58, 78
		1.6%	16,920	
		1.7%	177	
		0.3%	2,569	

*frequency of mismatch in forward/reverse primer or probe

Also, Buchta et al. (2021) showed that the same patient sample could alter Ct values if run with different diagnostic PCR kits with different targeting genes. This is critical, once Ct values are used as a reference to define clinical decisions and to guide patient care. Therefore, the choice of a SARS-CoV-2 gene target on diagnosing and patient monitoring is crucial [64].

Another important aspect of SARS-CoV-2 detection by RT-qPCR is the endogenous internal control. To guarantee uniformity, reproducibility, and the extraction process quality, the 'Minimum Information for publication of Quantitative real time PCR Experiments' guideline recommend that the choice of reference endogenous genes, also called endogenous housekeeping gene, should be essential part of RT-qPCR experiments [13]. FDA and other authors have reported the optimal human endogenous genes in the SARS-CoV-2 RT-qPCR detection. The RNAse P and Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH) are among the genes that act as an excellent internal control by excluding the possibility of false results due to the presence of low quality and integrity of RNA samples [65–67].

4.5. Data analysis

The result interpretation depends on detection kit guides that recommend a maximum Cycle Threshold (Ct) to be considered a positive result. The Ct represents the amplification cycle that trespasses the specific fluorescence intensity (named threshold line) programmed automatically or manually on equipment. The variation of Ct values reported in few studies was critically discussed [13]. In diagnostic laboratories day-to-day, some situations can impact the result analysis. For example, suppose a patient has a Ct strictly within the limit recommended by the kit guide. In that case, some analysts may consider this sample as positive, while others may consider automatically or manually as negative.

It has been reported that Ct values of 25–28 are considered appropriate as an indicator of SARS-CoV-2 positivity, and higher values (>28) could be due to the inactivation of Taq-polymerase [59]. Generally, the Ct value below 40 is recommended as a SARS-CoV-2 positivity in different protocol RT-qPCR kits. However, some conditions mentioned before, such as collection site, period of collection, sample conservation, and transportation, might affect the sensitivity of the method. Vogels et al. (2020) highlighted the possibility of Ct values >35 could be considered negative. Therefore, it is required from the analyst's experience to interpret false-positive and -negative samples.

Also, inconclusive results are also quite common. It can be obtained, for example, in diagnostic kits with two gene targets, where only one target amplifies. This common situation reinforces the influencing factors on SARS-CoV-2 diagnostic here discussed, once the same methodology for genetic material assessment was performed, including collecting site,

extraction and RT-qPCR methodologies, sample storage and manipulation. Besides, it elucidates the concern on a reliable result in diagnostic kits that use only one gene target.

4.6. Primer-probe dimerization occurrence

Currently, many companies have developed diagnostic RT-qPCR kits without proper validation testing. One of the influencing parameters of sensitivity and specificity that might alter the data analysis is the primers/probe's dimer occurrence. Won et al. (2020) and Jaeger et al. (2021) showed that the US-CDC kit (N gene) had unexpected amplification during RT-qPCR in negative samples and no-template controls [68,69]. This was also demonstrated by another study with Charité RT-qPCR kit (E gene) that found high amounts of unspecific signals in late cycles in no-template control [70]. These examples are categorical in describing the primer-dimer as a cause of the unspecific amplification.

In this study, we provide an in-silico analysis to confirm the dimer possibility of primer-probe sets. Self- and heterodimer formation was performed using OligoAnalyzer v3.1 (Integrated DNA Technologies®). To determine the likelihood of a primer/probe binding to each other, the software calculates the Gibbs free energy (ΔG) of nucleic acid hybridization as an indicator of dimer formation [71]. Our analysis displayed the binding energy variation of self and hetero-dimer among RT-qPCR kits for SARS-CoV-2 detection.

It has been established as the optimal standard properties for a primer set, including primer size, product size, melting temperature, GC content, and binding energy [72]. The most important property is the thermodynamic parameter that guarantees the nonoccurrence of primer/probe dimerization for the designer. In other words, larger negative values of free energy binding value imply a higher probability of self and hetero hybridization [73]. The optimal free binding energy required to break the dimerization well tolerated is usually ≥ – 9 kcal/mole [74–76]. Among of primer/probe set analyzed, three primer/probe with < – 9 kcal/mole binding energy in the self-dimer analysis were detected: China CDC (ORF1ab target), Charité (E target), and HKU (N target) (Table 4). Moreover, the probe of RT-qPCR kit HKU for N target detection had the lowest self-dimer energy (−14.35). In the hetero-dimer analysis, the probe-reverse primer dimerization was detected with < −9 kcal/mole for China CDC (N target), Charité (E target), and US CDC (N2 and N3 target (Table 4).

Jaeger et al. (2021) and Park et al. (2020) proposed several points to avoid primer/probe dimer formation and to optimize qPCR performance [69,77]. Among them, the reduction of primer set concentration, probe concentration, $MgSO_4$ concentration, annealing and extension temperature, and reverse transcription time. Therefore, it is well known the importance of primer design and optimization of qPCR reaction for proper validation and commercial distribution of diagnostic kits [78].

4.7. Other influencing factors

Besides the factors mentioned above, Bentivegna et al. (2021) discussed the possibility of false negative results in RT-qPCR in patients who have tested negative between two positive exams [79]. According to the authors, the negative result could be obtained due to a prolonged viral clearance, which raises another point of discussion to patients who have been qualified as reinfection ones.

After all the factors mentioned, it is also important to highlight the patients with pulmonary CT finding but who tested negative for SARS-CoV-2 (Table 5). These patients usually stay

Table 4. Binding energy of self- and hetero dimer occurrence for different RT-qPCR kit for SARS-CoV-2 detection.

Source	Target gene	Sequence (5`- 3`)	Self-dimer Binding Energy (kcal/mole)	Hetero dimer Binding Energy (kcal/mole)
CDC (China)	ORF1ab	F – CCCTGTGGGTTTTACACTTAA	−6.14	Probe/Reverse
		P – CCGTCTGCGGTATGTGGAAAGGTTATGG	−6.68	-5.09
		R – ACGATTGTGCATCAGCTGA	-13.39	
	N	F – GGGGAACTTCTCCTGCTAGAAT	−5.12	Probe/Reverse
		P – TTGCTGCTGCTTGACAGATT	−3.55	-10.21
		R – CAGACATTTTGCTCTCAAGCTG	-6.34	
Charité	E	F – ACAGGTACGTTAATAGTTAATAGCGT	−6.3	Probe/Reverse
		P – ACACTAGCCATCCTTACTGCGCTTCG	−9.89	-9
		R – ATATTGCAGCAGTACGCACACA	-7.05	
	ORF1b	F – GTGAAATGGTCATGTGTGGCGG	−5.38	Probe/Reverse
		P – CAGGTGGAACCTCATCAGGAGATGC	−6.01	-6.57
		R – CAAATGTTAAAAACACTATTAGCATA	-5.24	
HKU	N	F – TAATCAGACAAGGAACTGATTA	−9.51	Forward/Reverse
		P – GCAAATTGTGCAATTTGCGG	−14.35	-6.59
		R – CGAAGGTGTGACTTCCATG	-5.38	
	ORF1b	F – TGGGGTTTTACAGGTAACCT	−6.36	Probe/Reverse
		P – TAGTTGTGATGCAATCATGACTAG	−8.53	-5.24
		R – AACACGCTTAACAAAGCACTC	-6.68	
CDC (US)	N1	F – GACCCCAAAATCAGCGAAAT	−3.61	Forward/Probe
		P – ACCCCGCATTACGTTTGGTGGACC	−6.3	-8.91
		R – TCTGGTTACTGCCAGTTGAATCTG	-6.62	
	N2	F – TTACAAACATTGGCCGCAAA	−9.28	Probe/Reverse
		P – ACAATTTGCCCCCAGCGCTTCAG	−13.09	-9.89
		R – GCGCGACATTCCGAAGAA	-10.36	
	N3	F – GGGAGCCTTGAATACACCAAAA	−3.9	Probe/Reverse
		P – AYCACATTGGCACCCGCAATCCTG	−5.37	-10.09
		R – TGTAGCACGATTGCAGCATTG	-7.05	

Table 5. Factors that has a influence on SARS-CoV-2 diagnose and recommendations to minimize false results.

Factor	Authors recomendation
Disease staging	Collection centers should give orientation about the correct collection time (3–10 days after first symptoms). Samples outside this window should not be accepted.
Sample collection methodology	Ideally, the collection should always be performed by a trained team. Self-collection might impact the viral load recovered.
Storage sample	Samples should be stored at 2–8°C/3 days at maximum. If diagnostic centers cannot keep the samples in these conditions, the samples should be discharged and collected again.
RNA extraction methodologies	Column-based methods and extraction methods upgrades are recommended to improve the test sensitivity.
RT-qPCR detection kit	The same kit must be used to a patient if there's an intention to compare or monitoring the infection. Among the possible targets, E and RdRp genes has shown to has a higher sensibility.
Data analysis	We recommend the use of kits with two gene targets, if possible.
Primer-probe dimerization occurrence	Primers testing should be performed again, if any bias occurs on reactions.

in isolation for long periods, considering the clinical condition, even with a negative molecular test. Many of the factors here cited may be the main cause for the negative result, or even more than one factor, combined. The point is, after more than one year that COVID-19 became pandemic, there is still much to learn about the immunity response to the virus, and its fast mutation rate, which has a direct impact on molecular tests and disease control.

5. Conclusion

The target gene standardization used in SARS-CoV-2 detection, as well as the method and collection site would be the ideal measure to enable comparison between patients results and CTs. However, considering the diversity in fabricants kits worldwide, it is impracticable to standardize all these features. Therefore, we reinforce the importance of COVID-19 diagnosis being concluded based on combined tests, so that the correct diagnosis can be reached, even with the occurrence of false-negative or false-positive molecular tests. Additionally, the association of clinical-epidemiological information and complementary exams would help to avoid false-positive or false-negative results.

6. Expert opinion

COVID-19 massive diagnosis is a critical method for effectively monitoring and controlling its spread. Furthermore, in the absence of a fully vaccinated population, increasing COVID-19 monitoring capability with trustworthy results for large-scale sampling seems to be the most promising option to understand, contain, and defeat this epidemic. The COVID-19 pandemic emphasizes the importance of establishing a robust and long-term mechanism for the accelerated growth, dissemination, and implementation of adapted diagnostic tests against the virus.

The rapid and intensive manufacture of molecular kits by numerous laboratories have significantly assisted countries' testing. A large number of kits are now commercially available while others are still being developed. The kits that have gained federal agency of the Department of Health and Human Services approvals are the most preferred to use or proceed with when implementing the massive diagnosis. However, several findings, even some related in this study, revealed the limitations and weak points of the available COVID-19 diagnostic kits. As mentioned in this study, multiple influence factors in sample selection, nucleic acid extraction, and RT-qPCR, may be decisive for a successful diagnosis. Therefore, the knowledge about these factors is essential to provide a reliable diagnostic and even understand what could led to a wrong diagnosis.

It is clear in our review that the sample collection methodology and storage can directly influence in the viral loads recovered. This is a quite important point because a bad storage sample could lead to false-negative results, which is a results of virus degradation and not essentially the absence of the virus. Nasopharyngeal swabs are still a good alternative for almost all SARS-CoV-2 variants (Alpha, Beta, Gamma and Delta), with exception for the Omicron variant that is preferable detected by the oropharyngeal swab. In case of Omicron, this happens because Omicron replicates more efficiently in oropharyngeal region. Therefore, we claim attention to the need of best practices in this context.

In addition, the RNA extraction of sample possible infected with SARS-COV-2 is a sensible step with important reflection in the result and diagnostic. Based on that, the professionals must be informed the possible variation of RNA viral yield due to the RNA extraction methodology. The best extraction method identified was the acid-Ph-based method.

The interpretation of molecular result can be a postanalytical issue and must be relevant during the training sections of the professionals. For example, cycle threshold (ct) line when incorrect determined could represent an inconclusive result that can contribute for the clinical misconduct. Another issue identified in this review was the mismatches and primer/probe dimerization that, for example, can threaten the precision of COVID-19 diagnosis. Diagnostic kit vendors, as well as diagnostic laboratories, must be aware of these concerns in order to avoid more consequences for the public. As a result, additional tests and studies are urgently needed to improve the production of a SARS-CoV-2 detection kit with more sensitivity and specificity. Since there are several emergent SARS-CoV-2 variants, it is necessary the appropriate supervision and regulation to avoid the inaccuracy issues in the diagnosis kits due to the high range of genetic variability of SARS-CoV -2 variants.

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Declaration of interest

The authors have no relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript. This includes employment, consultancies, honoraria, stock ownership or options, expert testimony, grants or patents received or pending, or royalties.

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