Journal of Molecular Medicine (2020) 98:1727–1736
https://doi.org/10.1007/s00109-020-01992-x

JMolMed

ORIGINAL ARTICLE

Diagnosing the novel SARS-CoV-2 by quantitative RT-PCR: variations and opportunities

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Received: 9 July 2020 / Revised: 5 October 2020 / Accepted: 7 October 2020 / Published online: 17 October 2020

Abstract

The world is currently facing a novel viral pandemic (SARS-CoV-2), and large-scale testing is central to decision-making for the design of effective policies and control strategies to minimize its impact on the global population. However, testing for the presence of the virus is a major bottleneck in tracking the spreading of the disease. Given its adaptability regarding the nucleotide sequence of target regions, RT-qPCR is a strong ally to reveal the rapid geographical spreading of novel viruses. We assessed PCR variations in the SARS-CoV-2 diagnosis taking into account public genome sequences and diagnosis kits used by different countries. We analyzed 226 SARS-CoV-2 genome sequences from samples collected by March 22, 2020. Our work utilizes a phylogenetic approach that reveals the early evolution of the virus sequence as it spreads around the globe and informs the design of RT-qPCR primers and probes. The quick expansion of testing capabilities of a country during a pandemic is largely impaired by the availability of adequately trained personnel on RNA isolation and PCR analysis, as well as the availability of hardware (thermocyclers). We propose that rapid capacity development can circumvent these bottlenecks by training medical and non-medical personnel with some laboratory experience, such as biology-related graduate students. Furthermore, the use of thermocyclers available in academic and commercial labs can be promptly calibrated and certified to properly conduct testing during a pandemic. A decentralized, fast-acting training and testing certification pipeline will better prepare us to manage future pandemics.

Keywords COVID-19 · Coronavirus · Infrastructure · Polymerase chain reaction · Testing

Introduction

The World Health Organization (WHO) received notification on December 31, 2019 of pneumonia cases of unknown etiology in Wuhan, China. On January 11, 2020, the Chinese authorities identified a new type of coronavirus, which was isolated on January 7, 2020, which allowed its rapid sequencing and the public data deposition on January 12 to help with the development of diagnostic kits [1]. WHO declared on March 11, 2020, a pandemic the coronavirus disease 2019 (COVID-19), which is caused by SARS-CoV-2. In March 21, after nearly 3 months of the first notification, over 291,000 thousand cases were confirmed, and 12,776 deaths were reported worldwide [2]. As of May 1, 2020, over 3.2 million cases have been confirmed globally, with a death toll surpassing 234,000 people [3].

In addition to mitigation approaches (e.g., promotion of hygiene, social distancing, isolation of infected people, and restricting traveling), comprehensive testing of the infection in the population is central to track the disease spreading as well as inform public policies. It has been suggested that the demand for health services can only be maintained at manageable levels through a prompt adoption of public health measures to suppress virus spreading [4]. Indeed, countries that have adopted broad testing strategies early have better succeeded in limiting the spread of the disease, such as South Korea, Vietnam, and New Zealand [5].

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s00109-020-01992-x) contains supplementary material, which is available to authorized users.

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Ideally, tests should be easy to sample and analyze, quick to return results, accurate and precise, scalable, and inexpensive. Often, antibody-based point-of-care tests (POCT) fit this description. However, rapidly evolving epidemics due to novel viruses do not allow the timely development of antibody-based tests. Thus, viral load tests based on real-time, quantitative RT-PCR (referred herein as RT-qPCR) are an ideal platform for the rapid development of test kits due to the easy adaptability to the nucleotide sequence of the target.

Currently, RT-qPCR is a reliable test widely used for the detection of symptomatic and asymptomatic patients infected with SARS-CoV-2 [6, 7] with a technical limit of detection (LOD) <10 copies/reaction [8], and a detection threshold of 3.8 RNA molecules per reaction, depending on the amplified region and the primers and probes used in the analysis [9]. Indeed, although RT-qPCR requires special equipment, it allows for a relatively simple and rapid diagnosis by amplifying segments of the coronavirus genetic material with high specificity and reliability. Multiple research and clinical institutions around the world have developed molecular assays to diagnose SARS-CoV-2 and made the sets of RT-qPCR primers and probes publicly available (Table 1).

Extensive testing of the population (and the subsequent isolation of those infected) is essential to containing a pandemic and avoiding a premature collapse of entire national health systems. For example, Li et al. [10] used infection data to model the spreading of the novel coronavirus in China before travel restrictions were imposed. They concluded that undiagnosed SARS-CoV-2 carriers accounted for 79% of the cases and the primary source of infection, which rapidly spread the disease across the globe [10]. However, as the global demand for RT-qPCR testing kits surges abruptly during a fast-spreading pandemic, commercial suppliers are unable to deliver kit components in a timely manner, which prevents the effective assessment of the disease spreading in the population in order to identify and isolate those infected and avoid further spreading.

The present work aimed to contribute to the study of SARS-CoV-2 diagnosis through the analysis of the virus genome sequences that were deposited at the NCBI GenBank as it spread throughout the world as well as to study the various testing approaches recommended by official government authorities from several countries in order to assess methodological testing differences and to indicate prospects of effective diagnosis via RT-PCR. Our approach underscores the importance of analyzing the evolution of genome sequences of novel viruses as it spreads around the globe, allows for the revision of adopted testing reagents, and contributes to avoiding the development of imprecise molecular tests based on mutated regions, as already observed [11, 12].

Methods

Phylogenetic analysis

The keyword "SARS-CoV-2" was used to search for complete genomes of the novel coronavirus deposited to the National Center for Biotechnology (NCBI) nucleotide database (https://www.ncbi.nlm.nih.gov/genbank/sars-cov-2-seqs/). We analyzed 226 SARS-CoV-2 genome sequences deposited at the NCBI GenBank from samples collected by March 22, 2020 (Online Resource 1). Following, we selected the complete genome sequences and sorted them according to the collection dates (Table 2). We only considered the first complete genome deposited from each country for further analyses, since this study did not intend to propose new molecular assays for the diagnosis of SARS-CoV-2 but rather demonstrate the importance of genomic analysis of the virus, especially regarding disease dissemination and validation of assays already developed via RT-PCR.

Nucleotide sequences were utilized to calculate the phylogenetic distances through the tool *ClustalW* [13] using default parameters. The tree was generated through the *MEGA 7.0* software [14], with branches inferred using the Maximum Likelihood method based on the Hasegawa-Kishino-Yano model [15].

Table 1 Summary of available protocols (adapted from WHO–House Assays, 2020)

Country	Institute	Target genes
China	Center for Disease Control and Prevention (CDC)	ORF1ab, N
France	Institut Pasteur	RdRP (2 targets)
Germany	Charité - Universitätsmedizin Berlin	RdRP, E, N
Hong Kong SAR	The University of Hong Kong (HKU)	ORF1b-nsp14, N
Japan	National Institute of Infectious Diseases (NIID)	Pancorona, multiple targets, spike protein
Thailand	National Institute of Health (Thai NIH)	N
USA	Centers for Disease Control and Prevention (CDC)	N (3 target regions)

Table 2 Complete genome sequences of the novel coronavirus (SARS-CoV-2) deposited at the NCBI GenBank by March 22, 2020

GenBank ID	Collection date	Location	Genome size (bp)
MN908947	2019-12	China: Wuhan	29,903
LC529905	2020-01	Japan	29,903
MT039890	2020-01	South Korea	29,903
MT072688	2020-01-13	Nepal	29,811
MN985325	2020-01-19	USA: WA	29,882
MT192772	2020-01-22	Vietnam	29,891
MT007544	2020-01-25	Australia: Victoria	29,893
MT192759	2020-01-25	Taiwan	29,862
MT066156	2020-01-30	Italy	29,867
MT050493	2020-01-31	India: Kerala State	29,851
MT093571	2020-02-07	Sweden	29,886
MT233519	2020-02-27	Spain: Valencia	29,782
MT126808	2020-02-28	Brazil	29,876
MT240479	2020-03-04	Pakistan: Gilgit	29,836

Virus mutations and molecular assays developed in-house

For the analysis of amplified regions used in diagnostics, we used the tool *ClustalW* [13] to align all regions of the 14 SARS-CoV-2 complete genome sequences against the primer and probe sequences recommended for diagnosis by various research and clinical institutions around the world (Table 1). The images displaying nucleotide sequences alignments were generated by *GeneDoc* (http://www.nrbsc.org/gfx/genedoc/; Online Resource 2). Additionally, we searched the *Web of Science* database for studies on PCR variations used to diagnose viruses and compared the methods.

Results

Phylogenetics

Through the analysis of the 14 complete genomes available (Fig. 1) available during the early stages of the pandemic, we could trace the geographical spreading of SARS-CoV-2 since the beginning of the pandemic [1]. Accordingly, the phylogenetic classification of complete genome sequences can be used to trace sources of infection and inform clinical and outcomes of novel viruses and should be considered during the design of treatments and, eventually, vaccines [12]. While several therapeutic approaches to treat COVID-19 are being developed in the course of the pandemic [16], tracking the evolution of the virus may also be helpful to determine the impact on the host, including high-risk populations and genetic variations associated with different responses, as well as to inform vaccine development and interventions (cf. [17]).

Our phylogenetic analysis (Fig. 2) shows the formation of three distinct clades. In line with our findings, a recent study that analyzed 160 complete SARS-CoV-2 genomes also found three central variants named A, B, and C. The A variant was the ancestral type and closest to a coronavirus from bats and pangolins. The B variant had a higher incidence in East Asia, while the C variant was found first in patients from France, Italy, and Sweden and is the prevalent version in Europe [12]. This analysis also revealed the main origins of the virus in other countries, such as Brazil, which reported its first sequenced genome from a patient who had returned from Italy. Indeed, in our phylogenetic reconstruction, Brazil and Italy fell in the same clade (variant C), alongside Australia, Sweden, and South Korea, which in line with the report by Forster et al. [12]. In a recent study, 54.8% of the SARV-CoV-2 cases in Brazil by March 5, 2020, were estimated to be

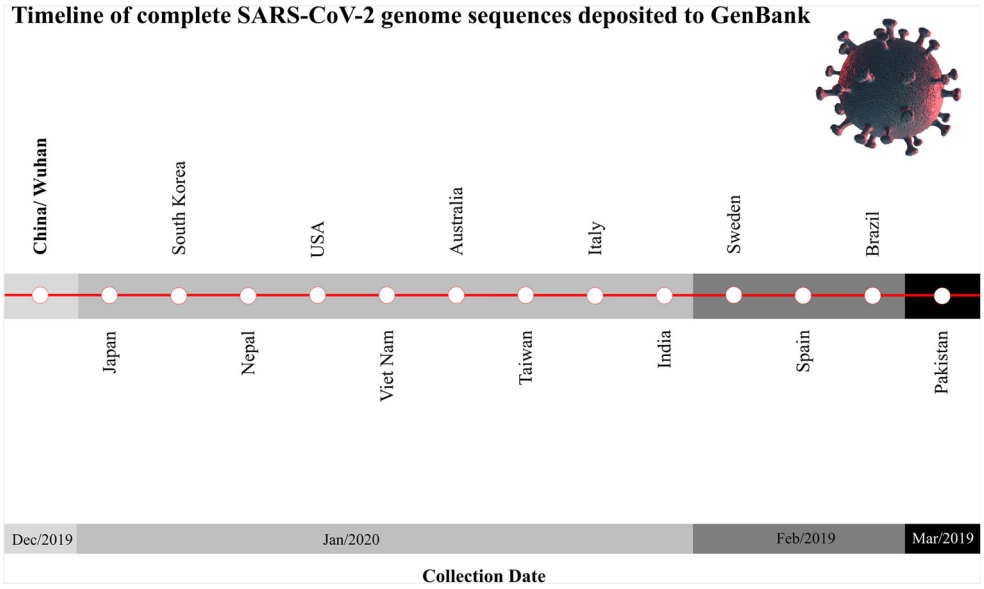

Fig. 1 Timeline of complete SARS-CoV-2 genome sequences deposited to GenBank, according to their collection dates. The viral structure image was designed using resources at Freepik.com

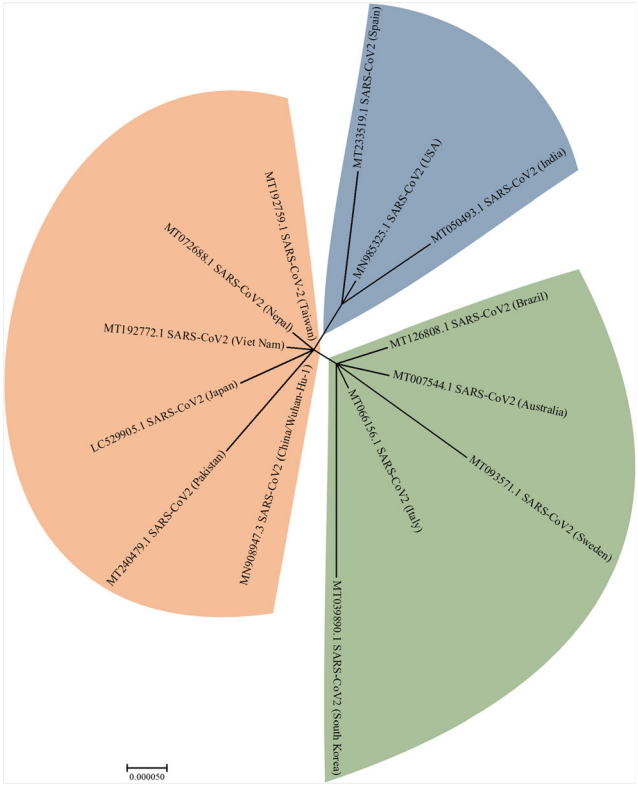

Fig. 2 Phylogenetic analysis of 14 complete SARS-CoV-2 genome sequences published by several countries. The molecular phylogenetic analysis was conducted in MEGA7 and inferred by the maximum likelihood method on the Hasegawa-Kishino-Yano model. For more complete studies on the evolution of SARS-CoV-2 during the pandemic and follow-up discussions on proper methodologies of phylogenetic analyses, please refer to [18–21]

originated from contaminated travelers arriving from Italy [22].

Phylogenetic analyses assessing the origin of the novel coronavirus have already been published. These studies revealed that bats might be the primary reservoir of SARS-CoV-2 [23–25]. Therefore, phylogeny is an essential tool to monitor the evolution of pandemic pathogens and can help, considering its limitations, in tracing the spreading of viruses. However, such studies must utilize representative datasets and the correct analysis methods to produce results with the necessary robustness for monitoring lethal outbreaks, such as for the novel coronavirus [18–21].

Mutations and molecular assays developed in-house

The study of genome mutations of pathogens as epidemics spread can help us better understand emerging outbreaks [26]. This type of data is essential, as it can indicate the frequency and extent of the genetic variation of novel viruses. The alignment of complete SARS-CoV-2 genomes revealed that the new coronavirus had undergone several mutations (Online Resource 2). Korber et al. [27] developed an analysis pipeline to facilitate real-time mutation tracking of SARS-CoV-2. This analysis initially focused on the spike (S) protein because it mediates cell infection, and it is the focus of most vaccine strategies and antibody-based therapeutics. Using this method, the authors identified fourteen mutations that are accumulating in S [27]. In the host, a major focus of study is the gene ACE, which is directly involved with SARS-CoV-2 infection and which genetic variations may be involved with the severity of the disease and may help explain why the virus is hitting southern Europe so hard [28]. In our study, however, we focused our analysis on the regions used for diagnosis via PCR.

We examined the regions used for the design of SARS-CoV-2 primer and probe sets by leading official institutions around the world (Fig. 3). We noticed that the most common genomic region of the virus used for diagnosis comprises the gene “N”, followed by ORF1b, ORF1a, and the gene “E” (Fig. 3). Five institutions from four countries (Thailand, China (The University of Hong Kong (HKU) and Center for Disease Control and Prevention (CDC), the United States, and Japan) recommended the “N” gene region for diagnosis, with at least one set of primers and probe. The US Centers for Disease Control and Prevention (CDC) developed a SARS-CoV-2 Detection Panel composed of three sets of primers and probes for that region, which comprise a universal set for SARS beta-coronaviruses, and two other sets specific for SARS-CoV-2 [29]. Chu et al. [8] developed two quantitative assays via RT-qPCR to detect two different regions of the viral genome (ORF1b and gene ‘N’—HKU-Hong Kong/China) and demonstrated that could detect SARS-CoV-2 < 10 copies/reaction [8]. On the other hand, Corman et al. [9] developed an assay targeting the genes N, E and ORF1b (RNA-dependent RNA polymerase/RdRP) (Charité/Germany) and obtained the best sensitivity with the respective limits of detection (LOD): 8.3, 5.2 and 3.8 copies/reaction, at the 95% detection probability [9].

Our SARS-CoV-2 genome alignment also revealed that the mutations (Online Resource 2) were outside of the annealing sequences designed for primers and probes (Fig. 3), that is except for the virus circulating in South Korea, which holds a mutation in the region of a probe recommended by two European organizations, the Charité - Universitätsmedizin Berlin and the Pasteur Institute of France. Therefore, the tests developed by these institutions may be ineffective to accurately detect that virus version. Indeed, mutations lying within primer annealing sites have already been reported for SARS-CoV-2 [30].

Variations of PCR techniques and diagnosis methods

Since the genetic code of coronaviruses consists of RNA, a critical step of RT-qPCR protocols is RNA purification from

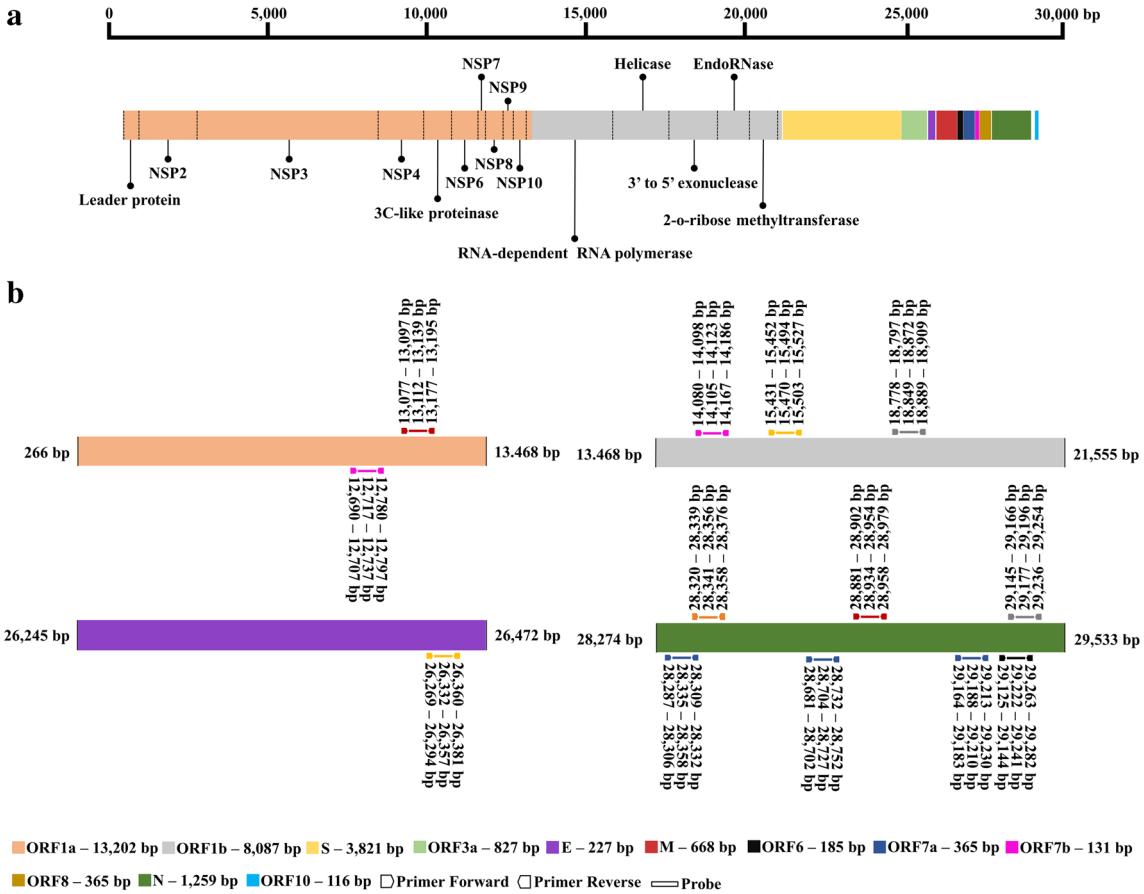

Fig. 3 **a** Representation of the SARS-CoV-2 genome sequence depicting annotated genes. This scheme uses the nucleotide coordinates of a virus sequenced in China (NC_045512). **b** Positions of the primer/probe targets used for detection by institutions in six countries. Colors of primers and probes correspond to countries: China, CDC (red); USA, CDC (blue); Germany (yellow); China/Hong Kong (gray); France (pink); Japan (black); and Thailand (orange). The numbers above each primer and probe indicate nucleotide position (coordinate) in the genome. Important details: France developed two assays for the RdRP gene (identified in the figure as ORF1a and ORF1b, according to the reference sequence [NC_045512] used). The assay developed by Germany for gene E was also tested in France. Germany developed two probes for the same RdRP gene region indicated in the image, one being specific for SARS-CoV-2 and another common for SARS-CoV-2, SARS-CoV, and bat SARS-related coronavirus, but only one assay is indicated in the image. Japan also indicated assays for nested RT-PCR, but the image above only represents RT-qPCR assays. An important aspect of RT-qPCR assay development for viral detection is confirming that primers and probes are specific to the virus of interest and do not detect viruses from the same family. This is particularly important for coronaviruses, since members of this group already circulate in the human population

the testing samples. Specific RNA isolation kits for SARS-CoV-2 detection were officially recommended by institutions in six countries (Table 3). All the protocols endorsed included RNA extraction by affinity columns that allow automation to optimize time and minimize errors.

All official institutions (Table 3) recommended using one-step RT-qPCR (the reverse transcriptase reaction automatically precedes the PCR phase in the same tube), which is regarded as the gold standard for viral detection and diagnosis (Fig. 4). Most of the endorsed assays were designed to detect two regions of the SARS-CoV-2 genome. However, the diagnosis of SARS-CoV-2 can also be performed by conventional PCR reaction after reverse transcription (RT-PCR), nested RT-PCR reaction (i.e., two successive PCRs with the second reaction using primers that anneal internally to the first amplicon) or via two-step RT-qPCR reaction (two successive reactions with the cDNA synthesis via reverse transcription preceding the PCR).

Thus, given the scarcity of detection kits officially recommended by each institution, analyzing the effectiveness of technique variations could help diagnosis, since the isolation of infected patients and epidemiological models depend on the availability of such data.

Much of the PCR work published on the development of detecting other viruses can be adapted for the diagnosis of SARS-CoV2. These studies include the use or comparison between PCR variations for the detection of viruses [31, 32], the use of PCR and the ELISA serological method [33, 34], commercial kits for virus detection [35, 36], and the chemistry (e.g., the general double-strand DNA intercalating dye SYBR

Table 3 Kits for viral RNA extraction and detection used by institutions from six countries listed by WHO

Kits utilized (company)		Country
Virus RNA extraction	MagNA Pure 96 System (Roche)	Germany
	QIAamp Viral RNA Mini Kit (Qiagen)	Hong Kong, Japan, USA
	NucleoSpin RNA Virus - Macherey (Nagel)	France, Thailand
	QIAamp DSP Viral RNA Mini Kit (Qiagen)	USA*
Virus detection: RT reaction and qPCR platform	SuperScript III One-Step RT-PCR System with Platinum Taq DNA Polymerase (ThermoFisher)	Germany
	TaqMan Fast Virus 1-Step Master Mix (ThermoFisher)	Hong Kong
	SuperScript III Platinum One-Step qRT-PCR Kit (ThermoFisher)	France, Thailand
	QuantiTect Probe RT-PCR Kit (Qiagen)	Japan**
	TaqPath 1-Step RT-qPCR Master Mix, CG (ThermoFisher)	USA

*The US CDC indicates the possibility of using several RNA extraction kits. This table only lists the first kit indicated by each country

**The Japan NIID also indicates the reagents for nested RT-PCR

Green, or post-amplification exonuclease-based probes, such as the TaqMan system) used for detecting and quantifying DNA amplification [37, 38]. Moreover, these studies aimed to determine simple or multiplex reaction assays for virus detection and serotyping [39–42] and the development of tests for simultaneous detection of viruses [43–46].

Currently, several integrated, random-access, point-of-care molecular devices are under development for the diagnosis of SARS-CoV-2 infections. These assays are expected to be simple, fast, accurate, safe, and amenable to be used in local hospital and clinic settings bearing the burden of testing and treating patients [47]. Recent studies evaluated different tests available and explored the possibility of improving SARS-CoV-2 diagnosis [47–49]. Tests based on biomarkers (e.g., serum porphobilinogen and aminolaevulinic acid) can be sensitive, specific, and low cost, and could even be used to monitor the response to treatments [50]. The international, non-profit organization Foundation for Innovative New Diagnostics (FIND) has identified almost 800 testing pipelines proposed to detect SARV-CoV-2 (for updates, cf.: https://www.finddx.org/covid-19/pipeline). As of September 1, 2020, these included 403 immunoassays, 362 molecular assays, 17 sample collections/inactivation, and 7 digital solutions.

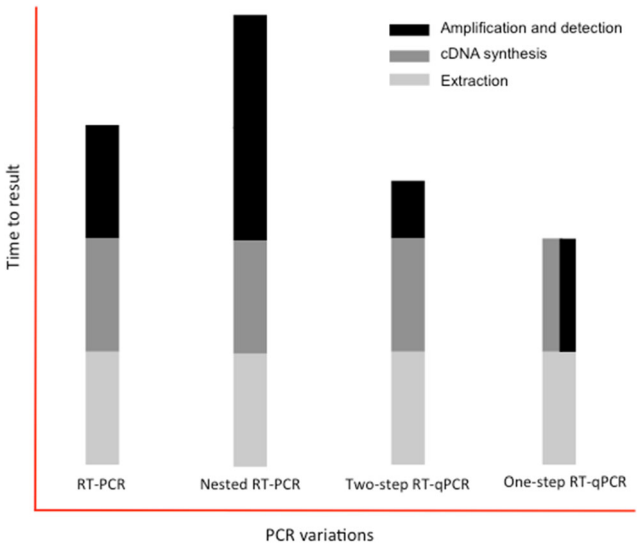

Fig. 4 Variations of the polymerase chain reaction (PCR) used for virus diagnoses. In conventional PCR after reverse transcription (RT-PCR), the target DNA is detected at the end of PCR amplification, requiring a post-PCR process for visualization (e.g., DNA electrophoresis). The nested RT-PCR significantly enhances the sensitivity of conventional PCR, but it nearly doubles the amplification time. All qPCR protocols detect and measure target DNA after each polymerization (extension) cycle during the exponential amplification through fluorescence and do not require post-PCR processing. While two-step RT-qPCR involves two distinct stages of reverse transcription followed by qPCR, one-step RT- involves a single reaction in which cDNA synthesis and amplification occur successively

Discussion

Interpretation

Most of the internal diagnostic assays developed by several groups around the globe were designed to detect two regions of the SARS-CoV-2 genome. Here we present a workflow to validate the internal tests indicated for the novel coronavirus through a phylogenetic approach. It becomes evident that monitoring the evolution of the virus is crucial for the validation of internal tests and even for the development of new protocols.

Furthermore, we suggest there is viability in using PCR variations (RT-PCR, nested RT-PCR, and two-step RT-qPCR) for the diagnosing of SARS-CoV-2 as a way to face one of the major bottlenecks that emerged with the new pandemic, namely the availability of inputs to carry out one-step RT-qPCR, which is the gold standard of virus diagnosis. Moreover, the investment to utilize the infrastructure already

existent in many university campuses around the world, and especially in developing countries, of laboratories fully equipped to carry out RT-PCR or nested RT-PCR analyses to diagnose SARS-CoV-2 is much lower and amenable than creating at speed new labs with qPCR equipment while many academic labs are at a standstill situation during the adoption of quarantine restrictions. Express laboratory certifications could be issued by regulatory agencies by using resources in local offices, and personnel teams could be trained for virus diagnosis to enable comprehensive testing of the population.

All in all, the validation of other PCR methods, mainly regarding sensitivity and specificity for the diagnosis of SAR-CoV-2, could help countries handle higher volumes of diagnosis by using alternative supplies and amplify the laboratory network for the diagnosis, thus also reducing the test turnaround time.

Limitations

Studies on the use of RT-qPCR have sought to optimize the assays, aiming mainly at increasing the efficiency of viral detection [51]. However, due to the short time in which the virus appeared and spread, few studies aimed to analyze the assay variables, such as the PCR variations for viral load detection, and the comparison between the molecular genetic methods and serological tests. Some studies aimed at evaluating and comparing already established RT-qPCR tests [52] or molecular point-of-care tests (e.g., RT-LAMP—reverse transcriptase loop-mediated isothermal amplification) with RT-qPCR [53]. Notwithstanding, the broad spectrum of PCR inputs and methodologies potentially available to efficiently detect SARS-CoV2 still warranted further analysis. Consequently, studies developed for other viruses show some avenues that can be adapted to diagnosing SARS-CoV-2.

Therefore, it is clear that we can still improve the RT-qPCR technique, although it is currently considered the gold standard for diagnosing patients with an ongoing viral infection. Another PCR variant called digital droplet PCR (ddPCR) has great potential in this area, especially to detect low viral loads in samples, which is a major limitation of the RT-qPCR [54]. Recently, Yu et al. [55] reported a study in which they analyzed viral loads of SARS-CoV-2 in infected patients and concluded that although the RT-qPCR is sensitive and reliable, ddPCR was better to detect samples with low viral loads [55]. Falzone et al. [56] also evaluated the ddPCR sensitivity and specificity for detecting SARS-CoV-2 comparatively to RT-qPCR and reported that ddPCR was superior in both parameters. They noticed that ddPCR could also be used to detect SARS-CoV-2 in blood and saliva samples, which have not been optimally established for RT-qPCR. ddPCR could indeed improve the diagnostic procedures since rhino-pharyngeal swabs are often not practical or even possible in some patients [56]. Suo et al. [57] pointed to the superiority of ddPCR for clinical diagnosis of SARS-CoV-2 to reduce false-negative results [57]. However, we argue that the limited availability of ddPCR instrumentation and expertise worldwide, as well as the higher analysis costs, still makes RT-PCR as the gold standard for diagnosing and monitoring viral pandemics, at least for the time being.

Another technique based on nucleic acid detection that has been successfully used to detect SARS-CoV-2 [58–63] but does not depend on sophisticated equipment is the reverse transcription-loop-mediated isothermal amplification (RT-LAMP) [64, 65]. Indeed, quantitative RT-LAMP has been utilized for diagnosing and the surveillance of viruses because it is a highly sensitive and specific method allied to being simple (one-step, single tube), fast, and low-cost [66]. Commercial RT-LAMP kits have already been developed for SARS-CoV-2 during the course of the pandemic (cf. https://www.finddx.org/covid-19/pipeline). We speculate that RT-LAMP may soon become standard in comprehensive point-of-care testing and surveillance of ongoing pandemics.

Conclusion

An efficient strategy to enable and increase the efficiency of molecular testing via PCR for viral detection is the examination of the protocols endorsed by WHO collaborating institutions and the technique variations. Since many tests have been developed or are under development, the range of options will eventually increase and can alleviate the scarcity of diagnostic kits, thus quickly relieving the demand for testing kits. The present study underscores the importance of using phylogenetic approaches not only to understand the evolution of the virus but also to designing and reviewing primers and probes used for SARS-CoV-2 diagnosis via PCR. We also show the versatility of RT-qPCR for the viral diagnosis during a pandemic and identified testing alternatives that can be used in the SARS-CoV-2 diagnosis.

Acknowledgments The authors thank their respective institutions for time and scientific resources available to conduct this work.

Authors' contributions All authors contributed to the study conception and design. Material preparation, data collection, and analysis were performed by Horllys Gomes Barreto, Matheus Martins Daúde, and Vagner Augusto Benedito. Flávio Augusto de Pádua Milagres and Gessi Carvalho de Araújo Santos contributed with critical methodological concepts in the development of the study. The first draft of the manuscript was written by Horllys Gomes Barreto and all authors contributed with editing. All authors read and approved the final manuscript.

Data availability All data used in this study is available to the scientific community upon request.

Compliance with ethical standards

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

Consent to participate Not applicable.

Consent for publication Not applicable.

Ethical approval This manuscript does not contain clinical studies or patient data.

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