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Evaluation of SYBR Green real time PCR for detecting SARS-CoV-2 from clinical samples

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ABSTRACT

The pandemic caused by SARS-CoV-2 has triggered an extraordinary collapse of healthcare systems and hundred thousand of deaths worldwide. Following the declaration of the outbreak as a Public Health Emergency of International Concern by the World Health Organization (WHO) on January 30th, 2020, it has become imperative to develop diagnostic tools to reliably detect the virus in infected patients. Several methods based on real time reverse transcription polymerase chain reaction (RT-qPCR) for the detection of SARS-CoV-2 genomic RNA have been developed. In addition, these methods have been recommended by the WHO for laboratory diagnosis. Since most of these protocols are based on the use of fluorogenic probes and one-step reagents (cDNA synthesis followed by PCR amplification in the same tube), these techniques can be difficult to perform given the limited supply of reagents in low- and middle-income countries. In order to develop an inexpensive SARS-CoV-2 detection protocol using available resources we evaluated the SYBR Green based detection of SARS-CoV-2 to establish a suitable assay. To do so, we adapted one of the WHO recommended TaqMan-based one-step real time PCR protocols (from the University of Hong Kong) to SYBR Green. Our results indicate that SYBR-Green detection of ORF1b-nsp14 target represents a reliable cost-effective alternative to increase the testing capacity.

1. Introduction

Ever since SARS-CoV-2 was identified as the etiological agent of a novel disease, COVID-19, at the beginning of the current year (Gorbalenya et al., 2020; Zhu et al., 2020a; Zhu et al., 2020b), the World Health Organization (WHO) has been following up on its spread. In addition, most of the scientific work has been mainly focused on three areas: i) the characterization of this virus and disease; ii) rapid development of diagnostic methods; and iii) patient treatments, antivirals and vaccines (Dennis Lo and Chiu, 2020).

The rapid spread of SARS-CoV-2 highlights the need for an effective surveillance method to be widely used in different laboratory settings (Thompson, 2020). This has prompted the development of a wide variety of molecular diagnostic methods based on the detection of viral genomic RNA. The vast majority rely on reverse transcription real time PCR (RT-qPCR), due to its high sensitivity and specificity (Chu et al., 2020; Corman et al., 2020; Huang et al., 2020; World Health Organization, 2020; Zhu et al., 2020a; Zhu et al., 2020b). This technique, either as a one-step or a two-step protocol, has accelerated PCR laboratory procedures and has had the strongest impact on virology as it is being applied for detection, quantification, differentiation and genotyping of animal and human viruses (Bankowski and Anderson, 2004; Kaltenboeck and Wang, 2005). Furthermore, it is regarded as a gold standard for analysis and quantification of pathogenic RNA viruses in clinical diagnosis (Espy et al., 2006). For instance, the WHO has recommended few molecular diagnoses for COVID-19 (World Health Organization, 2020). Since all these protocols are based on the use of fluorogenic probes and one-step reagents (cDNA synthesis followed by PCR amplification in the same tube), these techniques are limited to the use of more specific reagents and can be quite expensive. Moreover, these

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protocols involve the amplification of more than one gene, which implies different probes and fluorescent channels, adding to costs.

Therefore, several researchers have attempted to develop alternative SARS-CoV-2 detection methods that might be faster or cheaper to implement, such as loop-mediated isothermal amplification (LAMP) (Jiang et al., 2020; Park et al., 2020; Zhu et al., 2020a; Zhu et al., 2020b), droplet digital PCR (ddPCR) (Suo et al., 2020), multiplex PCR (Kudo et al., 2020) or even protocols based on CRISPR-Cas12 (Curti et al., 2020). Furthermore, considering the shortage in the supply of RNA extraction kits, others have evaluated alternative nucleic acids extraction methods (Bruce et al., 2020).

Quantitative PCR (qPCR) is a molecular technique widely used when detection and/or quantification of a specific DNA target is needed. qPCR is based on fluorescence to measure the amount of a DNA target present at each cycle of amplification during the PCR. The most common ways of generating a fluorescent signal are by using of specific hydrolysis probes (i.e. TaqMan® probes), or a double-stranded DNA binding dye (i.e SYBR® Green). SYBR-Green-based detection method presents several advantages over TaqMan chemistry ones, as being cheaper and not requiring the synthesis of specific probes. The main disadvantage is that any double stranded DNA including non-specific PCR products and primer-dimer can lead to false positive results if the diagnosis outcome is just based on the amplification data. For this reason, it is critical to control the specificity of the fluorescent signal observed at the end of PCR by melting curve analysis (Watzinger et al., 2006).

This technique has already been proposed and used for testing different pathogens, including viruses (Espy et al., 2006; Fernández et al., 2006; Gomes-Ruiz et al., 2006; Kumar et al., 2012), bacteria (Keerthirathne et al., 2016; Kositanont et al., 2007) and unicellular protozoan parasites (Espy et al., 2006; Haanshuus et al., 2019), among others. For SARS-CoV-2 detection, the WHO recommended routine confirmation of cases of COVID-19 based on detection of unique sequences of virus RNA by nucleic acid amplification test, such as RT-qPCR, with confirmation by nucleic acid sequencing when necessary (World Health Organization, 2020). Previous work have attempted to assess the analytical sensitivity and specificity of the different sets of primers and probes available (either commercially or in-house developed) (Barra et al., 2020; Nalla et al., 2020; Chu et al., 2020; Corman et al., 2020; Jung et al., 2020; Vogels et al., 2020) as well as adapted to SYBR Green (Dorlass et al., 2020; Meza-Robles et al., 2020; Won et al., 2020).

The aim of this study was to set up an alternative molecular protocol to detect SARS-CoV-2 from clinical samples, without the need of TaqMan probes or post-PCR steps (i.e. gel electrophoresis), which can be implemented in case of difficulties to get specific reagents or kits because of the current pandemic situation. Here we showed one TaqMan-based one-step real time PCR protocol recommended by the WHO (Chu et al., 2020) can be successfully adapted and alternatively used with SYBR Green-based two-step qPCR. Besides, performing a comparison of the different molecular techniques by their analytical sensitivity, we tested our assay with a panel of 53 clinical samples collected from negative or confirmed diagnosis for COVID-19. Our results showed that ORF1b-nsp4 primer set adapted to SYBR Green showed very low variation in the analytical sensitivity compared to the TaqMan based approach. Moreover, we found that SYBR Green technique was suitable for detecting SARS-CoV-2 in clinical samples and the Ct values observed in the TaqMan assay were positively correlated with those obtained with SYBR Green approach. Overall, our data indicates that this lower cost method is suitable to detect SARS-CoV-2 from clinical samples.

2. Materials and methods

2.1. Positive controls, clinical samples and ethical considerations

Positive controls were kindly provided by Dr. Leo Poon from the University of Hong Kong. A fragment containing a region of ORF1b-nsp14 or N targets of SARS-CoV-2 (genome sites 18849 to 18909 for ORF1bn-nsp14 and 29145 to 29254 for N, relative to reference genome NC_045512.2) were cloned into a standard plasmid.

Residual de-identified nasopharyngeal samples previously diagnosed (25 positive and 28 negative) were remitted to the Institut Pasteur de Montevideo. These were validated by the Ministry of Health of Uruguay as an approved center providing diagnostic testing for COVID-19. RNA extraction was performed using the QIAmp Viral RNA Mini Kit, QIAGEN following manufacturer instructions.

2.2. SARS-CoV-2 One Step RT-qPCR protocol with fluorogenic probes

The one-step RT-qPCR protocol evaluated in this study corresponded to the one developed by the University of Hong Kong (Chu et al., 2020), with modifications, which consists of two monoplex real-time RT-PCR probe-based assays targeting the ORF1b-nsp14 and N gene regions of SARS-CoV-2 (Supplementary data Table 1). Concentrations used were lowered to avoid non-specific amplification (data not shown). Briefly, a 20 μL monoplex reaction contained 5 μL of 4x TaqMan Fast Virus Master Mix (Thermo Fisher), 0.6 μL of each primer (0.3 μM final concentration each), 0.2 μL of the probe (0.1 μM final concentration), 9.6 μL of nuclease-free water and 4 μL of RNA. These monoplexes were performed for both N and ORF1b-nsp14 regions. Thermal cycling was run on a Step-One Plus RT-PCR thermal cycler (Applied Biosystems) with the following cycle parameters: 50 °C for 5 min for reverse transcription, inactivation of reverse transcriptase at 95 °C for 20 s and then 40 cycles of 95 °C for 5 s and 60 °C for 30 s. The expected amplicon sizes of ORF1b-nsp14 and N are 132bp and 110bp, respectively. This protocol was carried out with serial dilutions of plasmids containing N and ORF1b-nsp14 genes from SARS-CoV Urbani strain (Chu et al., 2020). We also performed this protocol with RNA standards for N and ORF1b-nsp14 targets from SARS-CoV-2 constructed in our laboratory. A non-template control (nuclease-free water) was included in every one-step RT-qPCR run. We manually set the threshold value in all assays to determine the threshold cycle (Ct). Titrations for the amplification of the controls were done to select the appropriate dilutions to test clinical samples in duplicates.

2.3. SARS-CoV-2 qPCR protocol with SYBR Green

First, cDNA of SARS-CoV-2 clinical samples was generated using SuperScript II Reverse Transcriptase (Invitrogen), 5 ng of random primers and 10 μL of RNA, according to the manufacturer's instructions. qPCR reactions were carried out using a Step-One Plus RT-PCR thermal cycler (Applied Biosystems), Luna Universal qPCR Master Mix (New England Biolabs), following manufacturer's instructions, and the same primers previously used in the One Step RT-qPCR TaqMan protocol. Each 20 μl reaction contained 10 μL of 2x Master Mix (NEB), 0.5 μL of each primer (0.25 μM final concentration each), 5 μL of nuclease-free water and 4 μL of cDNA. Again, non-template control (nuclease-free water) was included in every qPCR run as a negative control. We manually set the threshold value in all assays to determine the threshold cycle (Ct). As with the probe-based protocol, a test for the amplification of the control plasmids was done to select the appropriate dilution to use in test with clinical samples in duplicates. The cycling conditions were: initial denaturation at 95 °C for 20 s, 40 cycles of 95 °C for 5 s and 60 °C for 30 s, followed by a melting curve ranging from 60 °C to 95 °C (acquiring fluorescence data every 0.3 °C). With the aim of verifying specific amplification, in addition to the melting curve step during the run, we also confirmed the amplicon sizes by 2% agarose gel electrophoresis.

In order to prevent false positive results caused by contamination during RT and PCR, the laboratory is organized in two functional work areas: a pre-amplification area and a post-amplification area. These two areas are in separate rooms and all the supplies and equipment are dedicated to each work area and not interchanged between areas.

2.4. Molecular cloning of amplicons from clinical samples and Sanger sequencing

PCR products generated by the qPCR protocol with SYBR Green contain dA overhangs at the 3′ ends. Therefore, the fresh PCR products of the ORF1b-nsp14 and N target from clinical samples were directly cloned into pCR™2.1-TOPO® using the TOPO® TA Cloning® Kit (Invitrogen) following manufacturer's instructions. Next, cloning reactions were transformed in NEB® 5-alpha Competent E. coli (High Efficiency) by the heat shock method (42 °C, 30 s), plated in LB medium containing 50 μg/mL ampicillin (Amp), 40 μL X-Gal (40 mg/mL), 10 μL IPTG (100 mM) and incubated at 37 °C overnight. Three individual white colonies for each cloning reaction were isolated and overnight cultured in LB containing 50 μg/mL ampicillin. Plasmids were isolated using PureLink Quick Plasmid Miniprep Kit (Invitrogen) and Sanger sequenced with the universal primers M13Forward and M13Reverse.

2.5. Sequences analysis

Ab1 files from Sanger sequencing were analyzed using the Staden package v1.7.0 (http://staden.sourceforge.net). MEGAX (http://www.megasoftware.net) was used to perform sequence analysis. Primer specificity was addressed with the Primer-BLAST tool from the NCBI website (https://www.ncbi.nlm.nih.gov/tools/primer-blast/).

2.6. Construction of RNA for quantification standards

A fragment of 132 and 110 bp containing the ORF1b-nsp14 and N targets from SARS-CoV-2, respectively, were cloned into pCR™2.1-TOPO® using the TOPO® TA Cloning® Kit (Invitrogen) following manufacturer's instructions and transformed in NEB® 5-alpha Competent E. coli (High Efficiency) by the heat shock method (42 °C, 30 s). Plasmids were isolated using PureLink Quick Plasmid Miniprep Kit (Invitrogen) and quantified by spectrophotometric analysis (Biophotometer, Eppendorf). Then, 1 μg of each plasmid was linearized with SpeI and in vitro transcribed with T7 RNA Polymerase (Thermo Fisher) following the manufacturer's instructions. In vitro transcribed RNA was treated with DNase and purified with TURBO DNA-free™ Kit (Thermo Fisher). RNA purified was checked for size and integrity by gel electrophoresis and quantified by fluorometric analysis (Qubit 2.0, Thermo). The number of copies/μL was calculated as: $(NA \times C)/MW$, where, NA is the Avogadro constant expressed in mol^{-1}, C is the concentration expressed in g/μL, and MW is the molecular weight expressed in g/mol.

2.7. Determination of the analytical sensitivity of the assays by calibration curves

A stock containing around 2×10^{13} copies/μL of *in vitro* transcribed RNA (for both ORF1b-nsp14 and N) was used for standard curve and sensitivity determination of the qPCR assays. The calibration curve and sensitivity were determined by 10-fold serial dilutions of *in vitro*transcribed RNA stock. In the case of the one-step probe-based qPCR assays, 4 μL of the corresponding RNA 10-fold dilution was added to the mix and tested in triplicate. For the two-step SYBR Green-based qPCR 10 μL of the same 10-fold serial dilution of the in vitro transcribed RNA for each target were retrotranscribed and then 4 μL of the cDNA was used as template for SYBR Green qPCR. Each cDNA was tested in triplicate. Calibration curves were represented as Ct vs log copy number/reaction. Efficiency (E) was calculated as $E = 100 \times \left(10^{-1/s} - 1\right)$. Where s, is the slope of the calibration curve. The lower limit of detection was defined as the lowest copy number of target/qPCR, taking account for dilution, which amplified.

3. Results

3.1. Set up of SYBR Green and TaqMan based qPCR protocols with DNA controls for ORF1b-nsp14 and N targets

For the set up of SYBR Green and TaqMan based qPCR protocols we used plasmids containing N and ORF1b-nsp14 genes from SARS-CoV Urbani strain. In order to select an appropriate amount of control vector to use in the comparison between the two real time qPCR methods, we prepared plasmids dilutions (10^7, 10^6, 10^5 and 10^4 copies/μL) and assayed them following both protocols: the probe-based One Step RT-qPCR developed by the University of Hong Kong (Chu et al., 2020) and the in-house SYBR Green-based protocol adapted in this study. It is worth mentioning that previous results, from our laboratory, had indicated that a lower amount of primers and probes than initially suggested by Chu et al. (2020) rendered similar positive results, and diminished the amplification of primer dimers (data not shown). Real time PCR results, from SYBR and TaqMan chemistries, of different dilutions of the control vectors for the targeted regions (ORF1b-nsp14 and N) are shown in Table 1 and Fig. 1 (panels A, B, C and D). Since all dilutions amplified correctly and below a Ct of 37 (Fig. 1 and Table 1), we decided to use 10^6 copy number/μL as a positive control for subsequent assays (for both ORF1b-nsp14 and N genes).

Analyzing the specificity of the SYBR Green-based qPCR method (Fig. 1, panels C to H) from ORF1b-nsp14, we verified the presence of only one PCR product, corroborated by a unique melting peak (Tm = 81.55 °C) (Fig. 1E and Table 1). Agarose gel electrophoresis verified the expected product size (132bp) with no amplification in the negative control (Fig. 1G). In the case of the SYBR Green-based qPCR method for N gene amplification we observed for all N dilutions, a very clear peak at Tm = 81.70 °C, together with a non-symmetric melting temperature peak slightly skewed to a higher temperature, which might suggest the presence of two PCR products (Fig. 1F). However, when we separated the PCR products on an agarose gel only one product of the expected size (110bp) was observed (Fig. 1H), showing that the presence of non-symmetric peak was not indicative of non-specific amplification, at least not visualized on an agarose gel. For the non-template-control we observed a slight fluorescent signal (Ct = 37.76), although the melting curve showed a non-specific peak (Tm = 71.57 °C), which could be produced as consequence of primer dimer (Fig. 1F).

3.2. Comparison of the analytical sensitivity of SYBR Green and TaqMan based qPCR protocols

DNA standard controls used here to set up the qPCR protocols corresponded to the SARS-CoV Urbani isolate (Genbank Accession number MK062184). However, to validate this assay for SARS-CoV-2 detection, we constructed two *in vitro* transcribed RNAs containing the SARS-CoV-2 sequences for both ORF1b-nsp14 and N targets. Then, we measured the performance of both assays and targets by estimating the limit of detection and the qPCR efficiency (Stolovitzky and Cecchi, 1996).

The calibration curves showed that the Ct values of each reaction presented an inverse linear relationship with the log value of the RNA concentrations with a high correlation ($R^2 \geq 0.99$ except for N two step assay $R^2 = 0.9608$) (Fig. 2). The amplification efficiencies of the TaqMan RT-qPCR reference assays were 103.67 % and 100.99 % for ORF1b-nsp14 and N regions, respectively. Whereas the amplification efficiencies of the SYBR Green based qPCR assays were 99.77 % for ORF1b-nsp14 region and 102.56 % for N region. The limit of detection for ORF1b-nsp14 and N targets were 10 copies/reaction (2.5 copies/μL) for the probe-based qPCR (Fig. 2A and C). In the SYBR Green based assays, the limit of detection observed were 50 copies/reaction (12.5 copies/μL) for the ORF1b-nsp14 target and 250 copies/reaction (62.5 copies/μL) for N target (Fig. 2B and D).

Finally, the primer specificity was addressed *in silico* using the Primer-BLAST tool from the NCBI website. We found that ORF1b-nsp14

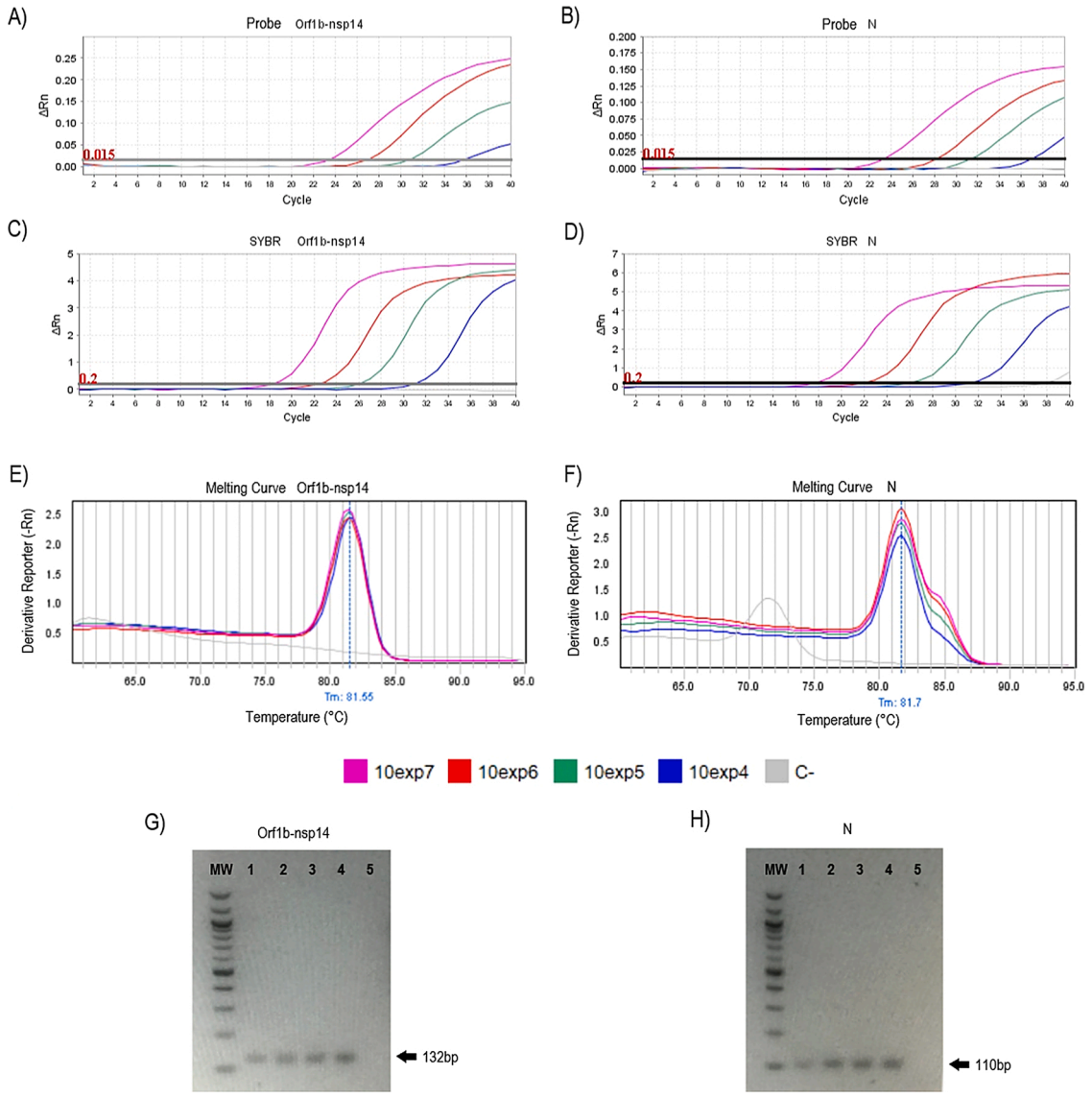

Fig. 1. Real time PCR results, from SYBR and TaqMan chemistries, of different dilutions of the control vectors for the targeted regions: ORF1b-nsp14 and N (left and right panels, respectively). A) and B) show the amplification plots for the RT-qPCR protocol employing fluorogenic probes. C) and D) show the amplification plots for the qPCR protocol developed in this study employing SYBR Green as a nucleic acid dye. E) and F) show the melting curves for the products amplified with the SYBR Green-based qPCR protocol. Below these panels are the references for each of the dilutions assayed expressed in plasmid copies (C-: non-template control). G) and H) show agarose gel electrophoresis of PCR products amplified with the SYBR Green-based qPCR protocol. MW: 100bp DNA Molecular Weight (New England Biolabs); lanes 1 to 4: control dilutions (10^4, 10^5, 10^6 and 10^7 copies/μL, respectively); lane 5: non-template-control.

primer set only matched with the expected target size and with SARS-CoV-1 and SARS-CoV-2 viruses. Additionally, the N primer showed the same matches than the ORF1b-nsp14 primer set plus bat SARS-like coronaviruses (data not shown). Overall, the results obtained indicate that the performance of the TaqMan probe assay as well as the ORF1b-nsp14 SYBR Green based are sensitive enough for SARS-CoV-2 detection.

3.3. Validation of SYBR Green and TaqMan based qPCR methods with clinical samples

To validate the SYBR Green qPCR protocol, we evaluated the assay specificity, of both qPCR methods using the primer set ORF1b-nsp14 and testing a set of 53 previously diagnosed clinical samples (n = 25 positive and n = 28 negative). Importantly, at this point we continue working only with the ORF1b-nsp14 gene because N target showed a non-acceptable performance in terms of analytical sensitivity with the SYBR green assay (Fig. 2D). The results show that by using the method developed by Chu et al. (2020) it was possible to consistently differentiate samples previously diagnosed as positive or negative for COVID-19 (Table 2). When the same approach was performed with the SYBR Green-based qPCR protocol, we failed to detect a previously diagnosed positive sample (Table 2). However, in this sample, the melt curve analysis showed a Tm peak that matched the SARS-CoV-2 positive control, indicating that the outcome of the tested samples using the SYBR Green method should be addressed taking into account the Tm peak (Fig. 3A) (Table 2). This sample, which showed a Ct value of 40 using the SYBR-Green method, had Ct value of 34.08 by the probe-based reference RT-qPCR.

Then, we compared the Ct values obtained for both methods using the positive clinical samples. For ORF1b-nsp14 detection, the Ct values observed for probe based (Ct average = 28.27 ± 1.33) and SYBR Green (Ct average = 29.66 ± 1.64) based qPCR were not significantly different (two tailed Wilcoxon matched-pairs signed rank test, P = 0.0953)

Table 1
Ct values and melting temperatures (Tm) of the amplified control dilutions according to the probe-based RT-qPCR protocol versus the SYBR Green-based qPCR protocol developed in this work.

Target	Copy number	Probe-based	SYBR Green-based	
		Ct (Threshold 0.015)	Ct (Threshold 0.2)	Tm (°C)
ORF1b-nsp14	10^4	35.79	31.08	81.55
	10^5	30.78	26.11	81.40
	10^6	26.93	22.54	81.40
	10^7	23.38	18.40	81.40
	C-	40.00	40.00	
N	10^4	37.07	31.27	81.70
	10^5	31.52	26.03	81.70
	10^6	28.19	21.93	81.70
	10^7	23.47	17.61	81.70
	C-	40.00	37.76	71.57*

C-: non-template-control.
* Non-specific signal.

(Fig. 3B). Regarding the association between the amplification data from TaqMan and SYBR Green assays, Spearman coefficient analysis showed a significant and positive correlation between both methods using the ORF1b-nsp14 primer set (Spearman, $r = 0.837$, $P < 0.0001$) (Fig. 3B). All results generated by RT-qPCR are shown in supplementary data (Table 2).

4. Discussion

The qPCR technique is widely used in clinical virology diagnostic laboratories because of its high sensitivity, specificity, reproducibility and no need of post PCR steps (Josko, 2010). SYBR-Green based qPCR has relatively lower cost, whereas TaqMan-based qPCR is more expensive. In addition, the specificity of the qPCR is mainly provided by the use of specific primers, although TaqMan probes increase the specificity because only sequence-specific fluorescent signals are measured (Tajadini et al., 2014).

Furthermore, qPCR allows for high throughput testing and reliable target quantification over a broad dynamic range with detection limit to single copy numbers when the assay is well optimized (Bustin, 2000; Valasek and Repa, 2005). In addition, monitoring viral load in patients may have relevance for the course of disease, clinical outcome and management of COVID-19 outbreaks (Pujadas et al., 2020). However, this issue remains unclear. Previous works have found that a positive qPCR result does not necessarily means SARS-CoV-2 transmission (Cevik et al., 2020; La Scola et al., 2020; Shrestha et al., 2020). Therefore, the Ct value observed for the clinical sample could help to determine the isolation measures on the basis of viral transmission and/or infectivity.

Here we not only reproduced the analytical sensitivity of the probe-based protocol designed by Chu et al. (2020) and also reported by others (Vogels et al., 2020) (Fig. 2A, C) but we determined its suitability by employing a SYBR Green-based assay (Fig. 2B,D). As expected, the probe-based method showed a higher sensibility than the SYBR-Green

Table 2
Outcome evaluation of clinical samples with both methods.

Outcome	Method[a]	Cut-off < 40 Ct*	Cut-off Tm peak*,[b]
Positive	Probe-based	25/53 (47.17 %)	NA
Negative		28/53 (52.83%)	NA
Positive	SYBR Green-based	24/53 (45.28%)	25/53 (47.17 %)
Negative		29/53 (54.72%)	28/53 (52.83 %)

* Using ORF1b-nsp14 target.
[a] The probe based method was previously described by Chu et al. (2020).
[b] NA: not applicable.

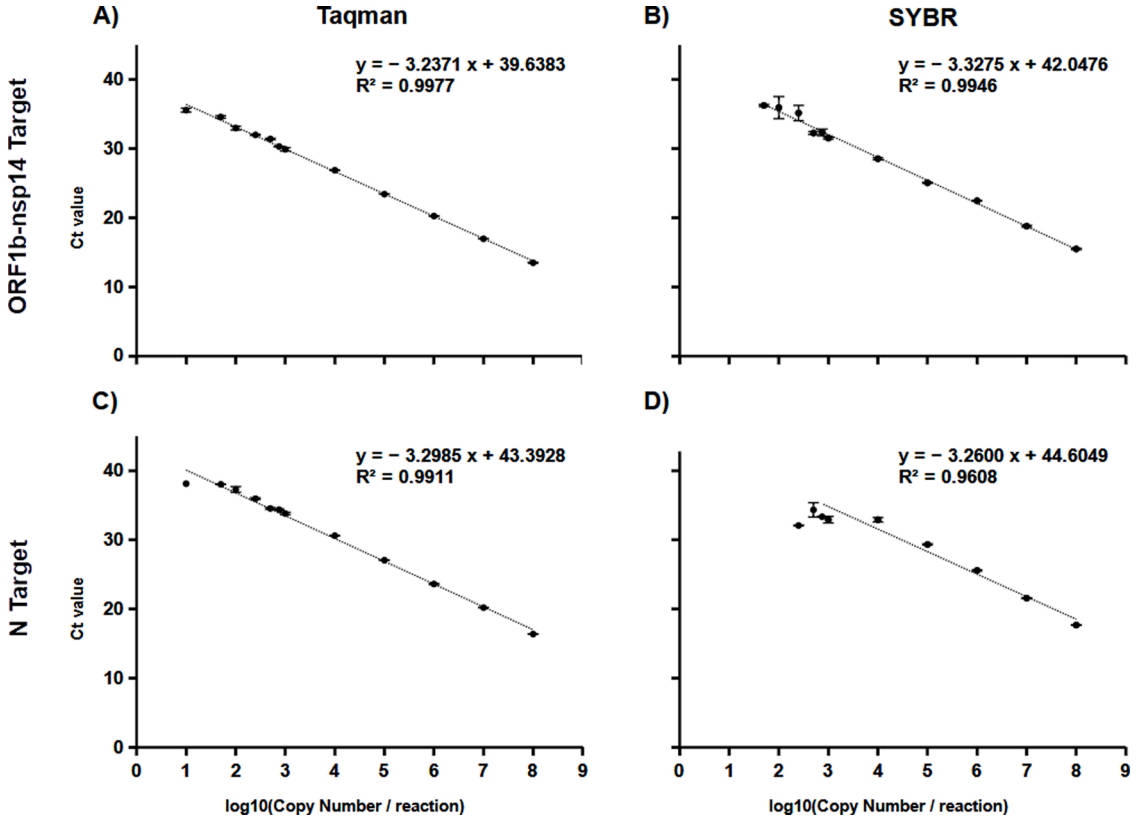

Fig. 2. Calibration curves of TaqMan and SYBR Green based qPCR for targets ORF1b-nsp14 and N. Serially diluted RNA containing ORF1b-nsp14 (A, B) or N (C, D) targets were amplified and analyzed in both Taqman (A, C) and SYBR (B, D) qPCR protocols. The threshold cycle (Ct) mean values were plotted against copy number of RNA standards/reaction. The coefficient of determination (R^2) and the lineal regression curve (y) were determined. Each dilution was assayed in triplicate.

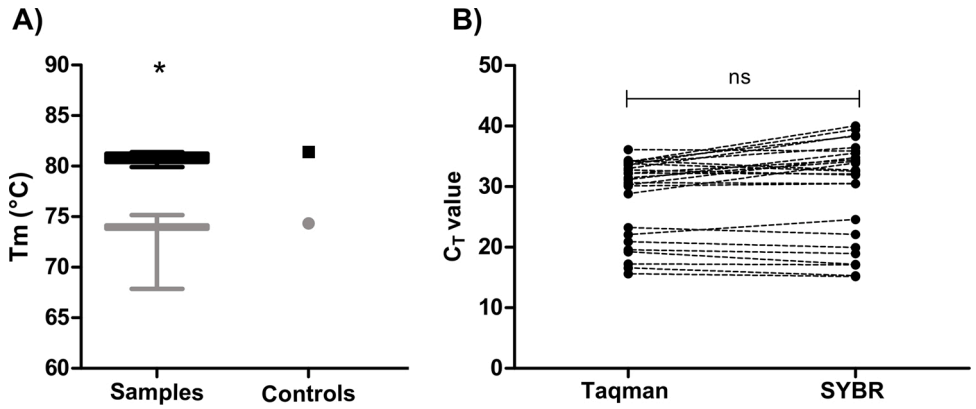

Fig. 3. Comparison of the RT-qPCR protocol employing TaqMan probe and SYBR Green assays to detect SARS-CoV-2 in clinical samples. Adapted method previously described by Chu et al. (2020). Each clinical sample was assayed in duplicate. A) Melting temperature. Asterisk indicate Tm average values significantly different (Mann Whitney test, $P < 0.0001$). Controls refers to the positive (black) and negative controls (gray) B) Ct values for ORF1b-nsp14 region ($n = 25$).

assay (Tajadini et al., 2014). Despite we found a 5-fold reduction in the analytical sensitivity for ORF1b-nsp14 target detection using SYBR Green. We were able to detect ~10 copies/μL (Fig. 2B), which is comparable to the TaqMan based method. This small difference in the detection limit indicates that the specificity of the assay is mainly determined by the ORF1b-nsp14 primer set. Other groups proposed other assays for detecting SARS-CoV-2 based on SYBR Green with similar (Dorlass et al., 2020; Won et al., 2020) or even higher detection limit than us (Meza-Robles et al., 2020). In contrast, the SYBR Green assay for N region seems to be not suitable for SARS-CoV-2 diagnosis, at least, in our experimental conditions. However, we cannot rule out that a better SYBR Green qPCR performance could be achieved by further optimization of the N target primer-set.

In addition to the slight decrease in sensitivity due to the lack of use of a probe, SYBR Green-based qPCR approach needs a previous step of cDNA synthesis. In order to increase the specificity of the SYBR Green-based qPCR for ORF1-nsp14 assayed here, it may be worth evaluating the use of specific primers instead of random hexamers during the reverse-transcription step. Another disadvantage of the SYBR Green vs probe-based qPCR is that any non-specific product including primer-dimer can produce a fluorescent signal that can lead to "false positives" if results are not well interpreted. For this reason, the melting curve analysis must be performed to confirm that only specific amplification was obtained. One advantage of the melt curve analysis is that it can help to identify mutations occurring in the PCR target region to improve other molecular methods where mismatched probes or primers may lead to false negative results.

We also evaluated the specificity of the assay *in silico* using the Primer-BLAST since we did not obtain clinical samples from patients with other respiratory viral infections. We found no amplification of other human coronaviruses currently circulating. This is in agreement with the results obtained by Chu et al. (2020) using: i) RNA extracted from cultured human coronaviruses as well as a camel coronavirus, several human and avian influenza virus and adenovirus; ii) human respiratory samples retrospectively tested for human coronaviruses, influenza, adenovirus, adenovirus, rhinovirus and respiratory syncytial virus; and iii) RNA extracted from sputum samples with no respiratory viral infections. They did not find any non-specific amplification using the same primer-sets supporting that our SYBR Green-based assay is highly specific for detecting SARS-CoV-2.

Finally, we validated the SYBR-Green assays by testing a set of 53 previously diagnosed samples ($n = 28$ negative and $n = 25$ positive) for COVID-19. Despite one positive clinical sample showing a Ct value of 40 with the SYBR Green RT-qPCR method, our results were consistent and correlated positively with the reference probe-based protocol using the same primer set. Importantly, this clinical sample showed a specific peak in the melting curve remarking its importance for the diagnostic analysis.

Altogether, both SYBR Green-based qPCR and TaqMan probe-based qPCR assays for detecting SARS-CoV-2 were set up in our laboratory conditions and their consistencies, as well as their advantages and disadvantages, were analyzed. This work could help to increase the testing capacity of some places in the world with limited access to TaqMan specific reagents and help disease management.

5. Conclusions

The performance of SYBR Green and TaqMan-based qPCR methods using the reference assay reported by Chu et al., 2020 has been evaluated by analytical sensitivity and specificity. The analytical sensitivity of the SYBR Green assay with the ORF1b-nsp14 target was slightly lower (5-fold) than the TaqMan reference assay but with a comparable performance. In contrast the analytical sensitivity of the N primer-set SYBR Green assay was lower (25-fold) than the reference probe assay. The evaluation of the SYBR Green-based qPCR on a set of clinical samples previously diagnosed as positive or negative for COVID-19 was consistent and correlated positively with the reference probe-based protocol showing comparable sensitivity and specificity to detect SARS-CoV-2. Therefore, SYBR Green-based qPCR assay with ORF1b-nsp14 primer set can be performed for specific and reliable diagnosis of SARS-CoV-2 infection.

Author's statement

On behalf of all authors, I declare that this manuscript is original, is not being currently considered for publication in any other journal and we do not have any conflict of interest

Authors' contributions

PM and GM conceptualized the study design; AF, FL-T, FA, PP, MP-G and AC performed the laboratory tests; NE, MP-G and AC made the tables and figures; AF, FL-T, FA, PP, NE, MP-G, AC, PM and GM analyzed the data and interpreted the results; NE, MP-G, PM and GM wrote the manuscript. All authors read and approved the manuscript.

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Declaration of Competing Interest

The authors report no declarations of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jviromet.2020.114035.

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