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Article

Comparison of Four Real-time Polymerase Chain Reaction Assays for the Detection of SARS-CoV-2 on Respiratory Samples from Tunja, Boyacá - Colombia

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Abstract: Coronavirus disease (COVID-19) is an infectious disease caused by SARS-CoV-2. In Colombia, many commercial methods are now available to perform the RT-qPCR assays and laboratories must evaluate their diagnostic accuracy to ensure reliable results for patients suspected of being positive for COVID-19. The purpose of the study was to compare four commercial RT-qPCR assays for their ability to detect the SARS-CoV2 virus from nasopharyngeal swab samples referred to Laboratorio Carvajal IPS, SAS of Tunja, Boyacá - Colombia**.** This study utilized 152 respiratory tract samples (Nasopharyngeal Swabs) from patients suspected of having SARS-CoV-2. The diagnostic accuracy of GeneFinderTM COVID-19 Plus RealAmp (In Vitro diagnostic) (GF-TM), One-Step Real-Time RT-PCR (Vitro Master diagnostic) (O-S RT-qPCR), and the Berlin modified protocol (BM) were assessed using the gold standard Berlin protocol (Berlin Charite Probe One-Step RT-qPCR Kit, New England Biolabs) (BR) as a reference. Operational characteristics were estimated in terms of sensitivity, specificity, agreement, and predictive values. Using the gold standard BR as a reference, the sensitivity/specificity for the diagnostic tests were found to be 100%/92.7% for GF-TM, 92.75%/67.47% for O-S RT-qPCR, and 100%/96.39% for the BM protocol. Using BR as a reference, the sensitivity/specificity for the diagnostic tests were found to be 100%/92.7% for the GF-TM assay, 92.72%/67.47% for the O-S RT-qPCR, and 100%/96.39% for BM. With regard to the BR reference protocol, the GF-TM and BM RT-PCR assays had very similar results (k=0.92 and k=0.96 respectively) while the O-S-RT-qPCR was only moderately similar. We conclude that the GF-TM and BM protocols had the best sensitivity, specificity, and a very similar results in comparison to the gold standard BR protocol. We recommend evaluating the diagnostic accuracy of the OS-RT-qPCR protocol in future studies with a larger number of samples.

Keywords: Severe Acute Respiratory Syndrome-CoV-2 (SARS-CoV2); COVID-19; molecular diagnostics; real-time polymerase chain reaction (RT-qPCR)

1. Introduction

The outbreak of the coronavirus disease in 2019, also known as COVID-19, whose causative agent is novel severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2), was first detected in China on December 31st, 2019 [1]. It was quickly (January 30, 2020) declared a Pandemic by the World Health Organization (WHO), becoming the first global

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public health emergency of the 21st century [2]. COVID 19 disease is becoming more and more common in the population. Likewise, fear is generated due to the appearance of new variants, which is why vaccine-mediated immunity is needed to avoid the possible appearance of new variants capable of escaping the immune system [3]. Given the high speed of spread and the high cost in health services for this viral disease and the lack of effective treatment, diagnosis is essential with continuous evaluation of the kits offered on the market, as well as strategies for generate safe and effective vaccines for vulnerable and susceptible populations and improve response and coverage strategies in health systems [4].

Since the pandemic was declared, the National Institute of Health reported that at least 525,609,637 million people have been infected with 6,277,241 million deaths reported globally as of June 2022. In Colombia, 6,099,111 million cases have been reported with 4,214 active cases and 139,833 deaths. In the Department of Boyacá – Colombia where the study population is located, 125,328 cases were reported during the same period with a total of 2,785 patient deaths (National Institute of Health, 2021, https://www.ins.gov.co/Noticias/Paginas/Coronavirus.aspx). The viral genome of SARS-CoV-2 was sequenced on February 3, 2020 by the Shanghai Public Health Clinical Center, Fudan University, Shanghai, China. Thanks to the complete genomic sequencing of this new virus, the development of several vaccines and treatments against this viral disease were developed. Similarly, the complete sequence allowed for the advancement of different diagnostic protocols for the identification of specific sequences of the viral genome. Specificity was preferred using molecular techniques such as PCR and the implementation of duplex PCR. The latter would help decrease the time necessary to obtain test results and increase the processing capacity of laboratories worldwide [5].

Due to the new variants of SARS-CoV-2 that present specific mutations, the PCR assays could fail to detect some of the genes in the assay. In general, molecular kits should target conserved sites (e.g., genomic sequences that are the least likely to accumulate mutations over time). At this stage of the SARS-CoV-2 pandemic, an unprecedented number of genomes are available that can readily identify suitable candidates within conserved sites for diagnosis. These molecular assays involve protocols and procedures that allow SARS-CoV-2 to be identified using specific genes such as E, RdRp, S, and N, among others. Although the virus has had multiple mutations, several studies have shown that most of the mutations occurs in the region that encodes the Spike protein in specific sites of its genome. As a result, it has been recommend to develop tests against other viral proteins, because the use of S might decrease the specificity and cause false negative test results [6].

To address the diagnosis of this viral disease in the country, the head of the National Institute of Health had to face the challenge of implementing the diagnosis techniques based on World Health Organization (WHO) recommendations and the national guidelines for the laboratory surveillance of respiratory viruses. Therefore the diagnosis of SARS-Cov2 was based on the regulations stipulated by the WHO, that recommended the Charité Berlin protocol as the gold standard in diagnostic laboratories, which was implemented in Colombia and supervised by the Colombian National Institute of Health to collaborating laboratories (https://www.ins.gov.co/Pruebas_Rapidas/2.%20Protocolo%20Est%C3%A1ndar%20para%20validaci%C3%B3n%20de%20PR%20en%20Colombia.pdf). Currently, several commercial kits are offered for the molecular diagnosis and identification of SARS-CoV-2 in Colombia and laboratories must evaluate their diagnostic accuracy to ensure reliable results for suspected COVID-19 patients.

The objective of this study was to compare four commercial RT-qPCR assays for the detection of the SARS-CoV2 virus using nasopharyngeal swab samples referred to Laboratorio Carvajal IPS, SAS of Tunja, Boyacá - Colombia. This research was approved by the

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research ethics committee of the Universidad Pedagógica y Tecnológica de Colombia, which was given in the city of Tunja on the 18th of November of 2021.

2. Materials and Methods

2.1 Study design

A single-center prospective study was performed on 152 samples from female (70) and male (82) patients suspected of SARS-CoV2 infection in the department of Boyacá, Colombia. Samples with evidence of inadequate storage, presence of microbial, fungal, chemical contamination with solvents or reagents, or a volume less than 250 μL, as well as alterations or modifications in its labeling, were excluded.

2.2 Sample collection and preservation

152 samples from suspected COVID-19 patients were collected from the upper respiratory tract using nasopharyngeal swabs [7]. Samples were immediately placed into sterile tubes that had 3 mL of viral transport medium (VTM) [8]. Afterwards, the samples were kept between 2 °C and 8 °C and sent to the molecular biology laboratory of Carvajal Laboratorio IPS SAS to confirm the presence or absence of viral RNA.

The guidelines established by the National Institute of Health of Colombia (INS) (https://www.ins.gov.co/buscador-eventos/Informacin de laboratorio/Lineamientos para la vigilancia por Laboratorio de virus respiratorios.pdf), the Ministry of Health and Social Protection of Colombia (Minsalud) (https://www.minsalud.gov.co/sites/rid/Lists/BibliotecaDigital/RIDE/VS/ED/VSP/psps03-lineamiento-bioseguridad-red-nal-lab.pdf), and the provisional biosecurity guidelines of "Laboratory for the handling and transport of samples associated with the new coronavirus 2019 (2019-nCoV)" (https://www.minsalud.gov.co/sites/rid/Lists/BibliotecaDigital/RIDE/VS/ED/VSP/psps02-lineamientos-gmuestras-pandemia-sars-cov-2-col.pdf) for the reception of samples suspected of having SARS-CoV-2.

The samples were received along with the referral format "INS Basic Data Report Sheet 346", which included: names and surnames of each patient, date of first symptoms of the disease, date of sampling, type of sample (nasopharyngeal aspirate, bronchoalveolar lavage, necropsy etc.), as well as any additional epidemiological and clinical data (https://www.ins.gov.co/buscador-eventos/Lineamientos/345_ESI_Irag_2022.pdf). All samples met the criteria listed in the Guidelines for Laboratory Surveillance of Respiratory Viruses and the Manual of Sampling for Microbiological Analysis of the Ministry of Health of Bogotá [9].

2.3 RNA extraction

Samples were processed at the same time for the kits, so it was not necessary to thaw and freeze them again, thus avoiding RNA damage. The extraction of RNA from the virus was performed with the automated nucleic acid extraction system Nextractor® NX-48S (Genolutium) using the viral RNA extraction kit AN NX-48s (Genolutium) as a reagent which is compatible with the automation system, following the protocol established by the manufacturer. This system is automated which favors the reduction of handling errors, prevents cross-contamination, and significantly reduces processing times. The viral RNAs were stored at -80 °C for further analysis by RT-qPCR.

2.4 Real-time polymerase chain reaction (RT-qPCR)

In this study, the diagnostic accuracy of GeneFinderTM COVID-19 Plus RealAmp (In Vitro diagnostic) (GF-TM), One-Step Real-Time RT-PCR (Vitro Master diagnostica) (O-S RT-qPCR), and the Berlin modified protocol (BM) were assessed. The gold standard Berlin protocol (Berlin Charite Probe One-Step RT-qPCR Kit, New England Biolabs) (BR) was used as a reference. Table 1 shows the characteristics of each commercial kit evaluated during the study.

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The positive confirmation of SARS-CoV-2 was performed by RT-qPCR using the E, N, and RdRp genes in the GF-TM, O-S RT-qPCR, and BM protocols. In contrast, the BR reference protocol diagnoses SARS-CoV-2 by the amplification and detection of a region of the E gene which is shared by different betacoronaviruses of the Sarbecovirus subgenus. In these samples, a positive PCR test was performed in order to detect a specific region of SARS-CoV-2 located in the RdRp gene. For the purpose of reducing these limitations, a duplex PCR test for the detection of the E and RNase P genes called the gold standard, was validated. The performance panel was performed with positive and negative samples for SARS-CoV-2 viral RNA. Additionally, the RNase P gene was included as a control to identify the viability of the samples and to rule out the presence of PCR inhibitors or poor extraction of viral RNA, PCR grade water was used as negative control for RT-PCR.

For each amplification event, a reaction was performed using a total volume of 25μL which contained 5 μL of RNA extracted in the previous step, 12.5 μL of 2 X reaction buffer provided with the Superscript III one-step RT-PCR amplification system with Taq Platinum Polymerase (Invitrogen; containing 0.4 mM of each dNTP and 3.2 mM of Magnesium Sulfate), 1 μL of reverse transcriptase, 0.4 μL of 50 mM of Magnesium Sulfate solution (not provided with the kit), 1 μg of non-acetylated bovine serum albumin, and 1.5 μL of each primer which was added from a stock solution of 10 μM. RT-qPCR was performed using the CFX-96 for 10 min at 55°C, 3 min at 95°C, and 45 cycles of 15 s at 95°C and 30 s at 58°C [10].The data was analyzed using the Bio-Rad CFX Manager software (version 3.1.3090.1022; Applied Biosystems). It should be noted that the primers and probe sequences of primers were established by each of the commercial firms based on "Diagnostic detection of 201-nCoV by real-time RT-PCR protocol – Berlin 2020" (https://www.who.int/docs/default-source/coronaviruse/protocol-v2-1.pdf).

The GF-TM and O-S RT-qPCR were performed according to the manufacturer's recommendations. The BM protocol was supplied by the Laboratory of Virology at the Universidad del Bosque, which was modified from the Charité-Berlin protocol (https://www.who.int/docs/default-source/coronaviruse/protocol-v2-1.pdf), by including a single multiplex PCR reaction for the identification of the E and N genes. All assays used the RNA genomic SARS-CoV2 which was provided by the INS or reference laboratories indicated as a positive control (Table 1).

2.5 Statistical analysis

The distribution of the variables was assessed with Kolmogorov-Smirnov test. Categorical data was summarized in absolute frequencies and percentages while categorical variables were summarized by relative and absolute frequencies.

Sensitivity and specificity were calculated by 2 x 2 tables at each level. The sensitivity (95% CI), specificity (95% CI), and positive and negative predictive values were calculated using BR as the gold standard. Matched pairs of recorded cycle threshold values (Ct values) were compared by the Spearman correlation coefficient. Indeterminate results were excluded from the data analysis.

Diagnostic similarities among GF-TM, O-S RT-qPCR, BM, and the gold standard BR were calculated using accordance analysis with the Fleiss' Cohen's kappa (κ) test, in which $\kappa > 0.80$ signifies a high similarity between the methods. A value of $p < 0.05$ was considered statistically significant. Data obtained were systematized in Microsoft Excel v15.0 and all statistical analyses were performed with IBM® SPSS® 22.0 software (IBM, Armonk, NY, USA).

Table 1. Characteristics of the commercial kits assessed for the detection of SARS-CoV-2.

Feature	GF-TM	O-S RT-qPCR	BM	BR

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Manufacturer	In Vitro diagnostic	Vitro master diagnostics	Forest University	New England Biolabs
Sample types	Bronchoalveolar lavage fluid, nasopharyngeal swabs, oropharyngeal swabs, nasal swabs, mid-turbinate nasal swabs or sputum specimens	Bronchoalveolar lavage fluid, nasopharyngeal swabs	Nasopharyngeal swabs, oropharyngeal swabs	Bronchoalveolar lavage fluid, nasopharyngeal swabs, oropharyngeal swabs
Sample vol required	5 µL	8 µL	5 µL	5 µL
Extraction required	Yes	Yes	Yes	Yes
Target gene of SARS-CoV-2	E, N, RdRp	E and N	E and N	And
Internal quality control	RNAse P	RNAse P	RNAse P	RNAse P
Analytical sensitivity	RdRp: 10 copies/test	Gen N: 10 copies/test	Gen N: 10 copies/test	Gen N: 10 copies/test
	N: 10 copies/test	Gen E: 10 copies/test	Gen E: 10 copies/test	Gen E: 10 copies/test
	E: 10 copies/test			
Analytical specificity	1	1	1	1
Maximum performance of each kit	100 samples	100 samples	Not specified	100 samples
Test run time	1 h 35'	1 h 2'	1 h 5'	43'
Recommended platform	Biosystems® 7500 Real-Time PCR Instrument (ABI 7500). StepOneTM Real-Time PCR System (Applied Biosystems). CFX96TM Real-Time PCR Detection System (Bio-Rad).	QuantStudioTM 3 Real-Time PCR System (Applied Biosystems). QuantStudioTM 5 Real-Time PCR System (Applied Biosystems). Biosystems® 7500 Real-Time PCR Instrument (ABI 7500). StepOne PlusTM Real-Time PCR System (Applied Biosystems). StepOneTM Real-Time PCR System (Applied Biosystems). CFX96TM Real-Time PCR Detection System (Bio-Rad). Rotor – Gene – Q (Qiagen).	CFX96TM Real-Time PCR Detection System (Bio-Rad).	CFX96TM Real-Time PCR Detection System (Bio-Rad). QuantStudioTM 5 Real-Time PCR System (Applied Biosystems).

Abbreviations: GeneFinderTM COVID-19 Plus RealAmp (In Vitro diagnostic): **GF-TM**, One-Step Real-Time RT-PCR (Vitro Master diagnostics): **O-S RT-qPCR**, Berlin modified protocol: **BM** and

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gold standard Berlin protocol (Berlin Charite Probe One-Step RT-qPCR Kit, New England Biolabs): **BR.**

3. Results

3.1. Comparison of the results between the four RT-qPCR assays

A total of 152 samples from patients suspected of having COVID-19 ranging in age between 1-81 years were included in our analysis. There were 82 samples from men and 70 from women. Most of the samples belonged to adults between the ages of 25 and 64 years old which is the working age population (57.9%), followed by adolescents between the ages of 15 and 24 years old (21.1%), elderly 65 years old and older (14.5%), children between 5 and 14 years old (5.3%), and children under 5 years of age (1.3%). The supplementary Table S1 details the primary data obtained from this study, data were analyzed using the OpenEpi ® software.

Table 1 shows the comparative results between the four RT-qPCR assays used. Using BR as reference, a total of 152 samples were tested (62 positive and 83 negative), the sensitivity/specificity for the diagnostic tests were found to be 100%/92.7% for the GF-TM assay, 92.72%/67.47% for the O-S RT-qPCR assay, and 100%/96.39% for the BM assay. Taking into consideration the BR reference protocol, the GF-TM and BM RT-PCR assays had very similar results (k=0.92 and k=0.96 respectively) while the O-S-RT-qPCR was less similar (Table 2). The Supplementary Table S2 details the concordant and discordant results found in 152 samples analyzed.

Table 1. Comparison of the results between the four molecular assays for the detection of SARS-CoV-2 using Berlin protocol (BR) as a reference (n=152, from which 62 were positive and 83 negatives).

Assay		BR Positive	BR Negative	Kappa (k) (±95%cl)	Sensitivity	Specificity	PPV	PNV	GIVES
GF-TM	**Positive**	69	6	0.92	100%	92.70%	92%	100%	96.05%
	Negative	0	77						
O-S RT-qPCR	**Positive**	64	27	0.58	92.75%	67.47%	70.33%	91.48%	78.95%
	Negative	5	56						
BM	**Positive**	69	3	0.96	100%	96.39%	100%	95.87%	98.30%
	Negative	0	80						

Abbreviations: GeneFinderTM COVID-19 Plus RealAmp (In Vitro diagnostic): **GF-TM**, One-Step Real-Time RT-PCR (Vitro Master diagnostica): **O-S RT-qPCR**, Berlin modified protocol: **BM** and gold standard Berlin protocol (Berlin Charite Probe One-Step RT-qPCR Kit, New England Biolabs): **BR,** predictive positive value: **PPV**, predictive negative value: **PNV**, Diagnostic accuracy: **DA.**

Figure 1 shows the correlation between Ct cycle threshold values between the RT-qPCR assays for the detection of the SARS-CoV2 virus. There was a statistically significant strong positive correlation between BM versus BR protocols (r=0.746, p<0.0001) and GF-

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TM versus BR (r=0.622, p<0.001) protocols. Likewise, there was a significant moderate positive correlation between O-S RT-qPCR versus BR (r=0.482, p<0.001) protocols.

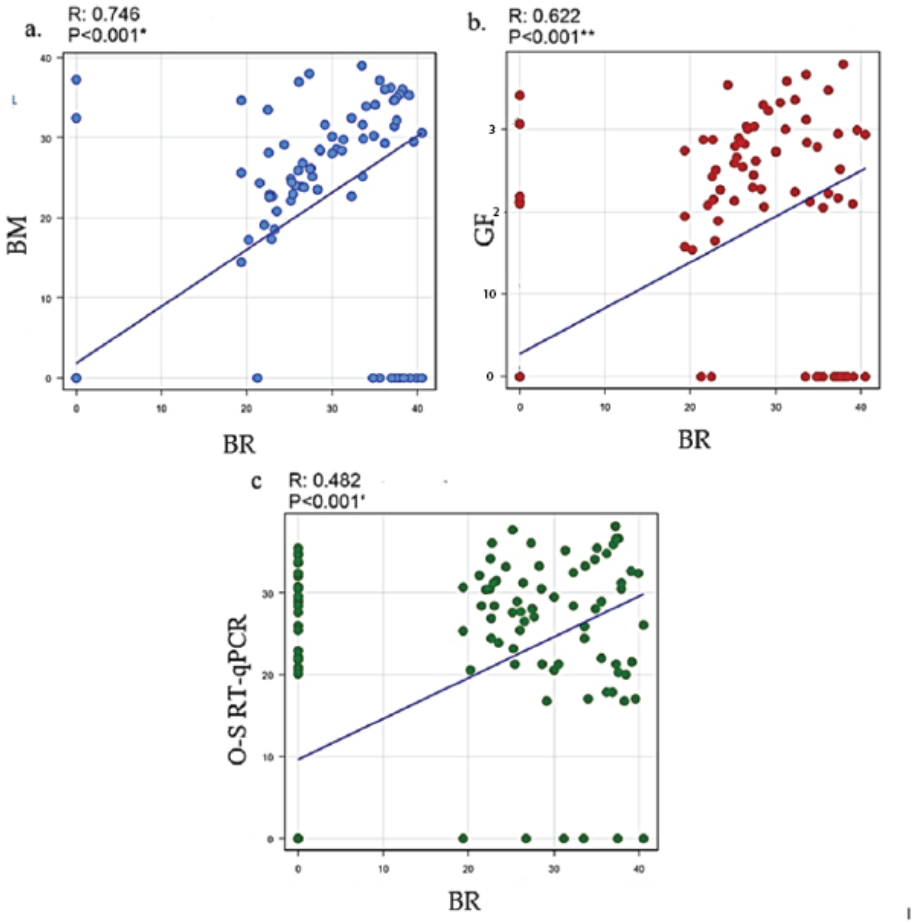

Figure 1. Correlation between Ct values between the RT-qPCR assays for the detection of the SARS-CoV2 virus**.** (a). Correlation the Ct values of E gene between BM vs BR protocols (b). Correlation the Ct values of E gene between GF-TM vs BR protocols (c). Correlation the Ct values of E gene between O-S RT-qPCR vs BR protocols. **Abbreviations:** GeneFinderTM COVID-19 Plus RealAmp (In Vitro diagnostic): GF-TM, One-Step Real-Time RT-PCR (Vitro Master diagnostics): O-S RT-qPCR, Berlin modified protocol: BM and gold standard Berlin protocol (Berlin Charite Probe One-Step RT-qPCR Kit, New England Biolabs): BR.

The ROC curve analysis indicated that the best diagnostic kit was the BM with a predictive capacity of 93%, followed by the GF-TM Kit with 87%; and the O-S RT-qPCR Kit with a 79.7% predictive capacity (Table 2).

Table 2. Comparative ROC curve analysis between the RT-qPCR assays for the detection of the SARS-CoV2 virus.

Test result variables	Area	Desv. Error[to]	Asymptotic[significance b]	95% asymptotic confidence interval	
				Lower limit	Upper limit

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BM	93.0%	0.025	<0.001	88%	98%
GF-TM	87.0%	0.033	<0.002	81%	93%
O-S RT-qPCR	79.7%	0.037	<0.003	72%	87%

Abbreviations: GeneFinderTM COVID-19 Plus RealAmp (In Vitro diagnostic): GF-TM, One-Step Real-Time RT-PCR (Vitro Master diagnostica): O-S RT-qPCR, Berlin modified protocol: BM and gold standard Berlin protocol (Berlin Charite Probe One-Step RT-qPCR Kit, New England Biolabs): BR.

When evaluating the optimal Cycle threshold point using the Youden Index, the BM and GF-TM kits had an excellent specificity and good sensitivity while the O-S RT-qPCR Kit had a good sensitivity, but a poor specificity. For the BM Kit a value greater than 7.2 must be considered as positive for COVID 19 having a sensitivity of 89.9% and a specificity of 97.6%. Similarily, the GF-TM Kit values which are higher than 7.7 should be considered positive for COVID with a sensitivity of 79.7% and a specificity of 94%. The O-S RT-qPCR assay is relatively unreliable for the detection of COVID 19 given that at its optimal cut-off point of 8.4 but reaches an important 92.8% sensitivity. Its specificity is only 68.7% which means that it has a false positive rate greater than 30% .

Finally, Table 3 describes the basic advantages and disadvantages of the RT-qPCR assays used for the screening of Severe Acute Respiratory Syndrome-CoV-2 (SARS-CoV-2) regarding the number of genes detected in the kit, processing time, sample volume, and reagent volume.

Table 3. Summary of the basic advantages and disadvantages of the Real-time Polymerase Chain Reaction Assays used for the screening of Severe Acute Respiratory Syndrome-CoV-2 (SARS-CoV-2).

RT-qPCR assays	Advantages	Disadvantages
GF-TM	Identification of 3 target genes gene E, N, RdRp their reagents	Dependency of commercial company
		Kit for 100 tests
	They are easy to handle preparation of the reagents	Higher cost of market availability
O-S RT-qPCR	Shorter amplification time compared to the other kits 1 h 2'	Dependency of commercial company
	Identification of 2 genes target E and N	Kit for 100 tests
		Dependency of commercial company
BM	Identification of two target genes E and N,	Kit for 100 tests
	Easy preparation of reagents for large volumes	Higher cost of market availability
	Reference protocol for molecular detection developed by the virology institute Charitè	Personnel needed to prepare reagents
BR	Recommended by PAHO for the universal monitoring of SARS-CoV-2. Kit for more than 1,000 reactions	Availability of production by manufacture outside the country

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Abbreviations: GeneFinderTM COVID-19 Plus RealAmp (In Vitro diagnostic): **GF-TM**,One-Step Real-Time RT-PCR (Vitro Master diagnostica): **O-S RT-qPCR**, Berlinmodified protocol: **BM** and gold standard Berlin protocol (Berlin Charite Probe One-Step RT-qPCR Kit, New England Biolabs): **BR.**

4. Discussion

Molecular tests based on the identification of specific genes of SARS-CoV-2, which are currently offered on the market, and which are used in both symptomatic and asymptomatic patients, are characterized by high specificity and low sensitivity, sometimes generating false negative results [11]. Although the qRT-PCR technique is highly efficient, some studies have shown that it can generate false negatives [11], which could cause a risk to the patient, their family, the community, and the health system, since an infected person could, by having an erroneous result, spread the infection. On the other hand, in the clinical setting, it should be clear that the accuracy of diagnostic tests can be influenced by the stage of the patient's disease and the quality of the samples [12]. In addition, several authors have shown that false negatives can be determined by multiple factors, including poorly trained personnel, poorly taken samples, possible errors in the batches of primers and other reagents used for analysis, lack of information from manufacturers, and traceability of the reference materials, in the same way, it should be emphasized that the RT-PCR technique is not 100% sensitive and specific for other pathogens of importance in the clinical setting, similar to the data obtained for the diagnosis of SARS-CoV-2 [13][14].

In clinical samples, a positive sample is considered when the Ct value of any specific gene for SARS-CoV-2 is less than or equal to 43, on the contrary, if the amplification is greater than 43, it is considered negative. As an internal control, RNAse P must be present in each sample and its Ct must be less than 35 to validate the test. If this gene does not amplify, the test must be invalidated and the extraction repeated (https://www.aidian.eu/uploads/NO-Dokumenter-og-materiell/ES-Products/ELITech/GeneFinder-COVID-19-RealAmp-Plus-Kit_Full-manual_V1_IVD.PDF). Based on the results obtained in this study, discordant samples can be observed in 37 of the 152 samples analyzed. This discrepancy could be associated with the design of the primers by the manufacturers, and because the target genes to be identified vary between the kits of diagnosis, which might cause changes of the Ct values due to the amount of RNA assessed, likewise, the viral load of the patient can affect the results obtained. In addition to this, to date, there is no standard methodology such as calibrators, or reference material, among others, that allows standardizing values between the kits offered on the market.

The evaluation of the diagnostic accuracy of four commercially available RT-qPCR methods for the detection of SARS-CoV2 from respiratory samples referred to the Laboratorio Carvajal IPS, SAS of Tunja, Boyacá – Colombia was performed. Our results ensure that the tests offered for the screening of SARS-CoV-2 in Colombian patients who are suspected of having COVID-19 meet the criteria for their optimal performance.

The current study provides a comprehensive and independent comparison of the analytical performance of primer–probe sets for SARS-CoV-2 testing in several parts of the world. Our findings show a high similarity in the analytical sensitivities for SARS-CoV-2 detection which indicates that the outcomes of different assays are comparable. The primary exception to this is the One-Step Real-Time RT-PCR (Vitro Master diagnostics, Spain) (O-S RT-qPCR), which had the lowest sensitivity and is consistent with a previous study [15].

This study demonstrates that RT-qPCR significantly improves accuracy and reduces the false negative rate in the diagnosis of SARS-CoV-2 in pharyngeal swab specimens which is more convenient and simpler to sample. Furthermore, qPCR is more sensitive and suitable for low virus load specimens from the patients under isolation and observation who may not be exhibiting clinical symptoms. Finally, RT-qPCR could be used in the quantitative monitoring of patients to evaluate dis ease progression [16].

We conclude that the GF-TM and BM protocols had optimal sensitivity, specificity, and a very similar results to the gold standard BR protocol which could be due to the design of the primes. We recommend evaluating the diagnostic accuracy of the OS-RT-qPCR protocol in future studies with a larger number of samples. We recommend that laboratories evaluate the diagnostic accuracy of RT-qPCR assays used for the detection of the SARS-CoV2 virus to ensure reliable results for patients who are suspected of being COVID-19 positive.

Supplementary Materials: The following are available online at www.mdpi.com/xxx/s1, Table S1: Primary data obtained from Four Real-time Polymerase Chain Reaction Assays for the Detection of SARS-CoV-2 on respiratory samples from Tunja, Boyacá – Colombia, Table S2. Description of the concordant and discordant results found in 152 samples analyzed. A. Concordant results of positive samples between the kits used and their amplification Ct, B. Concordant results of negative samples between the different kits used, C. Discordant results between the kits used.

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Institutional Review Board Statement: The study was conducted according to the guidelines of the Declaration of Helsinki, the protocol was approved by the Ethics Review Committee of the Universidad Pedagógica y Tecnológica de Colombia on November 18, 2021.

Informed Consent Statement: No informed consent was given for the research due to the fact that previously characterized RNAs from the biobank of the molecular biology area of Carvajal, IPS laboratory were used.

Data Availability Statement: In this section, please provide details regarding where data supporting reported results can be found, including links to publicly archived datasets analyzed or generated during the study. Please refer to suggested Data Availability Statements in section "MDPI Research Data Policies" at https://www.mdpi.com/ethics. You might choose to exclude this statement if the study did not report any data. The Supplementary Material for this article can be found online at:

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