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Evaluation of the effectiveness of SARS-CoV-2 molecular diagnostic tests

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Abstract

Since the emergence of SARS-CoV-2 in late 2019, the virus has spread worldwide and posed a major burden on public health. The rapid and accurate identification of patients infected with the virus by real-time reverse transcription polymerase chain reaction (RT-PCR) is an effective measure to limit the spread of the virus. Many commercial kits have been available, but their efficiencies must be independently evaluated before their use in the laboratory. The objective of this study is to evaluate their effectiveness and stability before proceeding with the diagnosis. Ten SARS-COV-2 molecular diagnostic kits used in the Virology laboratory at the Center for Virology, Infectious and Tropical Diseases (CVMIT) at the Mohammed V Military Instruction Hospital (HMIMV) in Rabat between 2020 and 2024 were evaluated using a quantification standard from viral culture supernatant. The effectiveness of the kits varied from 93% to 168% depending on the manipulators and the target genes.

Keywords: SARS-CoV-2; COVID-19; RT-PCR; Molecular diagnosis.

1. Introduction

Since the appearance of SARS-CoV-2 in China at the end of 2019, the total number of confirmed cases according to statistics from the World Health Organization (WHO) reached more than 776 million in more than 231 countries in November 2024; and the number of deaths reached more than 7 million [1].The first case of COVID-19 in Morocco was reported on March 2, 2020. Morocco has until April 2024 reported more than 1,278,992 confirmed cases and 16,303 deaths [2].

Just after the official announcement in China of cases of pneumonia of unknown etiology in the city of Wuhan on December 30, 2019 [3], a viral genome sequence was released by Zhang Yong-Zhen for immediate health support public

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via the online community resource virological.org [4], followed by four other genomes deposited on January 12, 2020 in the viral sequence database organized by the Global Initiative on Sharing All Influenza Data (GISAID). Real-time polymerase chain reaction technique combined with reverse transcription (RT-PCR) is a rapid method by which we can amplify and detect a specific target sequence in the genome. An internal cellular control such as the human β-globulin gene is often included in the PCR kit to control all stages of the reaction. The most important aspect of using RT-PCR tests is that amplification and detection are performed in a closed system; which reduces the risk of contamination and therefore of obtaining false positive results [5]. The sensitivity and specificity study of this test is based either on the virions of the SARS-CoV-2 coronavirus or on in vitro transcribed RNAs identical to SARS-CoV-2 target sequences. For routine workflow, Corman et al., (2020) recommended the use of PCR targeting the E gene followed by confirmation with RdRp primers combined with a SARS-CoV-2 specific probe [6]. A month later, others were developed by the CDC, the Pasteur Institute in Paris, the companies kogenebiotech, Seegene and Solgent, Primerdesign etc. In September 2021, the FINDDX site lists more than 400 tests intended for the diagnosis of SARS-CoV-2, including 389 marketed and 46 in development (Finddx.org).

At the national level, the MAScIr Research and Development Center succeeded in developing the first Moroccan made kit, tested and validated by the Royal Armed Forces, the Royal Gendarmerie as well as the Pasteur Institute of Paris. This kit has the characteristics of being specific, with a high degree of sensitivity and reliability and at a controlled cost [7].

This work aims to validate the effectiveness of 10 SARS-COV-2 molecular diagnostic kits used at CVMIT between 2020 and 2024, using a quantification standard which is the SARS-COV-2 culture supernatant. Susceptibility testing was performed with dilutions of SARS-CoV-2 viral culture supernatant and PCR efficiency for each kit was determined using standard curves.

2. Material and methods

2.1. Panel of samples tested

The culture supernatant on the cell line isolated from the African green monkey kidney (Vero CCL-81 cells) infected with the SARS-CoV-2 variant presenting the D614G mutation at the Spike or S surface protein was used as a reference for validation. The infectious titer of the virus was determined by the Reed & Muench technique [8] defining the infectious dose which causes a cytopathic effect of 50% on tissue culture (TCID50/ml). The titer of 4.78 log TCID50/ml was used, and eight dilutions of virus in series and in triplicate were carried out by two different manipulators in two different reactions, in order to study the effectiveness of the kits studied.

2.2. RNA extraction

Viral RNA is extracted from 140 µl of each dilution of the virus. The RNA extraction kit (QIAGEN, viral RNA mini kit; Hilden, Germany) was used according to the manufacturer's instructions. The viral supernatants lysis step was carried out under a microbiological safety station at the level 3 or BSL-3 safety laboratory. Ultrapure water was used as an elution buffer.

2.3. Amplification by qRT-PCR

The kits used in this work are all CE-IVD certified. They all required transport and storage at -20°C (Table 1). The amplification of the RNA extracts was carried out by the two manipulators according to the kits and the recommendations provided. Technician 1 is a laboratory professional, while technician 2 is a trainee in Master's training. Viral RNA was added to the prepared mix, according to the instructions of each kit and RT-PCR was carried out using the Q-Tower 3 thermal cycler (Germany).

Table 1 The kits selected for this work and their target genes

Kits	Manufacturers	Countries	Regulatory status	Target genes
A	Eurobio scientific	France	CE-IVD	RdRp, N
B	LabGenomics	Republic of South Korea	USFDAEUA-Australia TGA –South Africa SAHPRA - CE-IVD	RdRp,E
C	Osang Healthcare	Republic of South Korea	Health Canada- Brazil ANVISA – Singapore HSA-CE-IVD	RdRp,N, E
D	A* Agency for Science, Technology and Research	Singapour	Singapore HSA	RdRp
E	TAIGEN Bioscience Corp	Taiwan	-	N,E
F	GeneProofa.s.	Czech Republic	CE-IVD	RdRp,E, N
G	PCL	Republic of South Korea	Korea MFDS EUA – Health Canada - Singapore HSA –South Africa SAHPRA-Brazil ANVISA-CE-IVD	N
H	MAScIR-Moldiag	Maroc	CE-IVD	E,N, S
I	Foregene Co., Ltd	Chine	-	ORF1ab,N,E
J	Sansure Biotech	Chine	USFDAEUA–China, NMPA EUA - SingaporeHSA - Australie TGA –Sud d'Afrique SAHPRA -CE-IVD	ORF-1ab, N

2.4. Interpretation of results

Amplification curves were automatically generated by the thermal cycler after the end of each PCR reaction, with the threshold cycles (Ct) of all tested dilutions of the viral supernatant. These curves must be regularly shifted to the right with a ΔCt, which corresponds to the difference between the Ct of a given dilution and that of the previous dilution. These results were interpreted according to the kit used and its target gene, the median Ct of the triplicate produced for each dilution, the infectious titer of each dilution, and the handler. These elements are therefore used to draw the standard curves of the kits, after the linearity of the Ct obtained is verified, and a trend curve of the Ct as a function of the infectious titers is automatically generated according to the formula Y=aX+b, with a = the slope of the curve, Y = the Ct, and X = the viral titer. These standard curves allowed us to calculate the efficiency E of each kit for its corresponding target gene, according to the following formula: $E = (10^{-1/a} -1) \times 100\%$.

2.5. Limit of Detection (LOD)

The limit of detection of RT-PCR (95% PCR or LOD95) is defined as the lowest number of DNA copies per unit volume that can be reliably detected. Typically, the LOD refers to the limit associated with a 95% probability of obtaining a correct result [9].

3. Results and discussion

All the Real-Time PCR Coronavirus (SARS-CoV-2) kits used in this study are CE-IVD certified. They are in vitro diagnostic tests based on real-time PCR technology, developed for the specific detection of SARS-CoV-2 viral RNA extracted from nasopharyngeal swabs, oropharyngeal swabs and sputum of suspected Covid-19 patients. We were not interested in the specificity control due to the absence of any cross-reactivity with other coronaviruses reported by all the manufacturers of the kits evaluated. However, we were interested in their sensitivity, and therefore in the chemical stability of the reaction mixtures of the different kits by studying their efficiency based on a reference which was the virus on cell culture. The performance of real-time PCR was evaluated by the following parameters: dynamic range, precision, R^2 coefficient and sensitivity which mainly determine the efficiency.

The SARS-CoV-2 culture supernatant of the same viral strain, called stock solution stored at -80°C, was used after extraction of viral RNA by the Qiagen kit that we have validated on viral culture. The cytopathic effect (CPE) of SARS-

CoV-2 viral infection on cells is characterized by morphological alterations of cells infected by the virus. We thus observe rounded cells, fused in the form of giant cells which, after their lysis, detach from the cell layer (Figure 1.B).

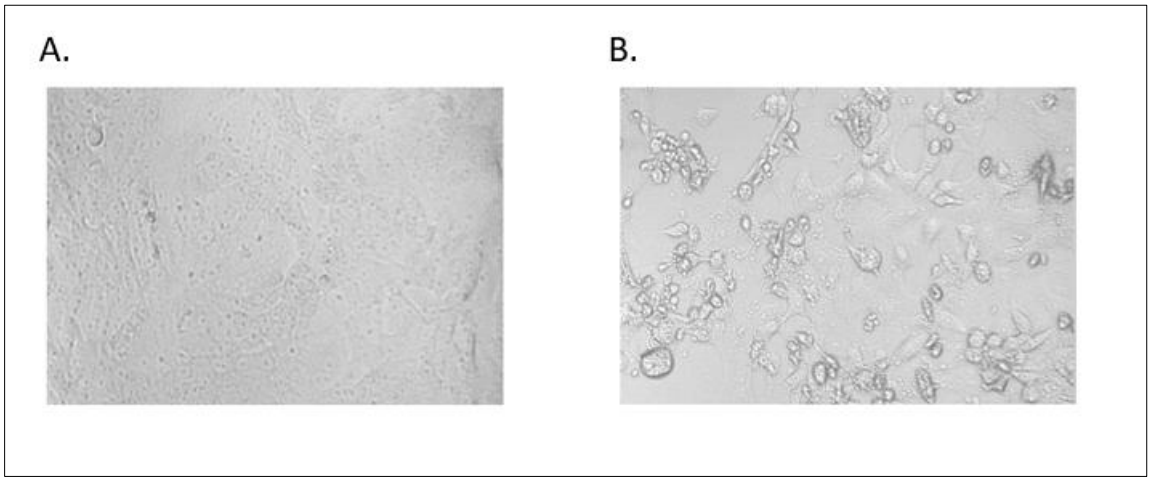

Figure 1 A: Uninfected Vero cells. **B**: Cytopathic effect of the SARS-CoV-2 virus on the Vero cell layer**.**

For each kit, the dynamic range, which is the interval of the efficiency calculated from the linearity of the Ct data, is determined by plotting a standard curve. Dilutions of supernatant that titrate approximately 5 log DICT50/mL are tested 3 times by two different handlers (an experienced handler and a Master's trainee). All dilutions are titrated and their concentrations in number of infectious viral particles are determined (titer in DICT50/mL). They are quantified by PCR and the data are recorded as Ct values. A ΔCt that separates two successive amplification curves with a dilution step of 10 (i.e. 1 log DICT50/mL) must be constant and ideally of a value of 3.3 [9].

In our study, the ΔCt obtained for kits A, B, D, E, F, G and H weren't constant and vary from 0.59 to 4.69, probably reflecting poor pipetting precision and extract handling. On the other hand, the amplification curves of the dilutions obtained by manipulator 1 using kit C respect a constant ΔCt of 3 (Figure 2) for all target genes: RdRp (3.17 to 3.48), E (3.41 to 3.86), and N (3.16-3.82).

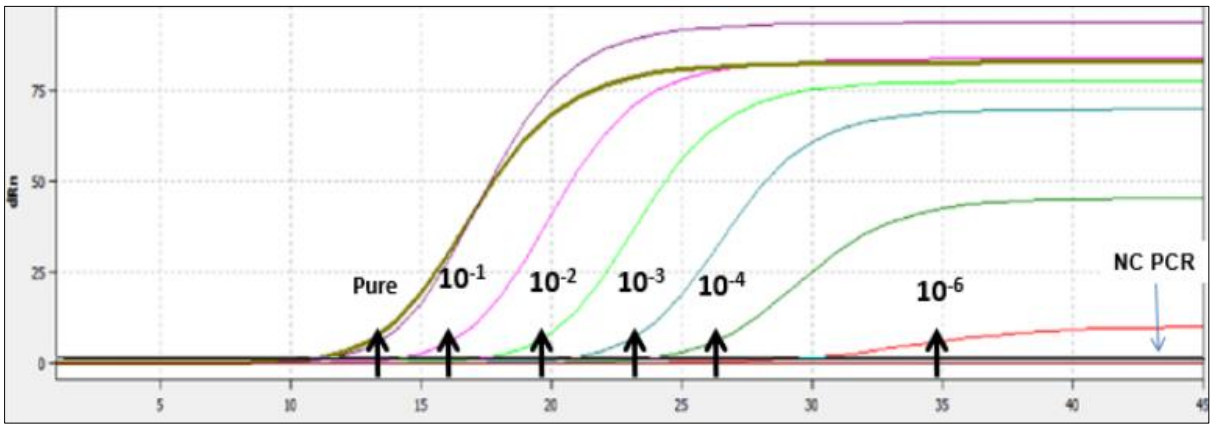

Figure 2 Example of RdRp gene amplification graph for kit C for the majority of dilutions (10-1 to 10-8), by manipulator 1. The cascade viral culture dilutions display parallel curves offset by a constant ΔCt of approximately 3 corresponding to 1 logDICT50/ml in terms of viral titer.

Furthermore, we determined calibration curves (standard curves) for all kits that show a linear trend, and therefore can be fitted using the least squares linear regression model. The correlation coefficients R^2 presented in Table 2 demonstrate the acceptability of the linearity of the data; all R^2 coefficients approach 1 with a better linearity for handler 1. Indeed, the minimum R^2 value obtained for handler 1 is 0.96, while that of handler 2 is 0.94. Kits A, C, E, F, G, H, I, and J target the N gene, and their standard curves are therefore presented together in Figures 3 and 4, except for kit F (Geneproof) whose results for the N gene are invalid. Its standard curve is plotted from the results for the RdRp gene. The remaining two kits (kits B and D) are also represented here by their RdRp gene results (Figure 5 and 6).

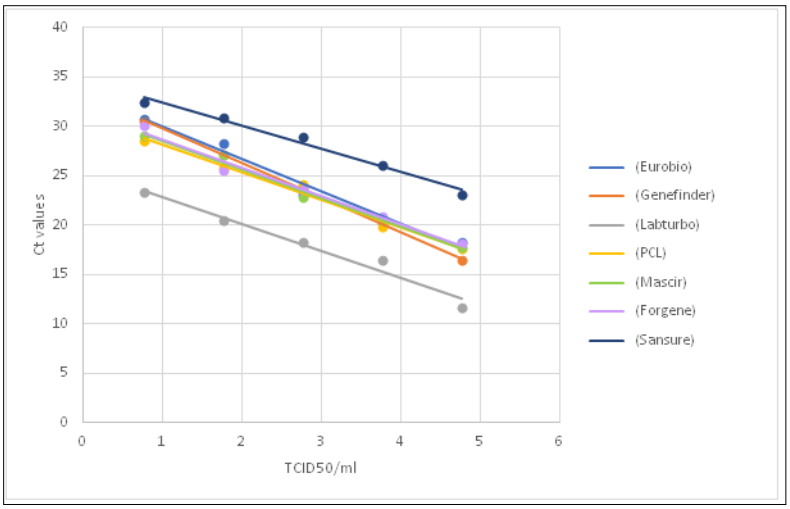

Figure 3 Standard curves of the kits from the results of manipulator 1 (Gene N).

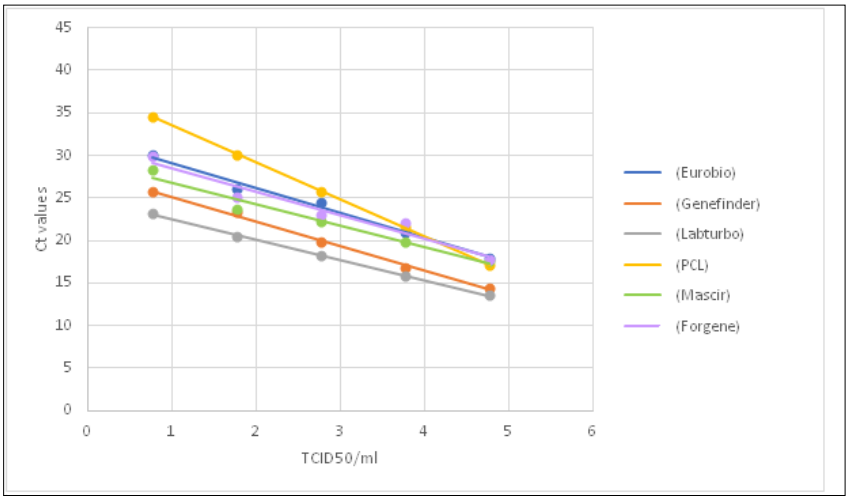

Figure 4 Standard curves of the kits from the results of manipulator 2 (Gene N).

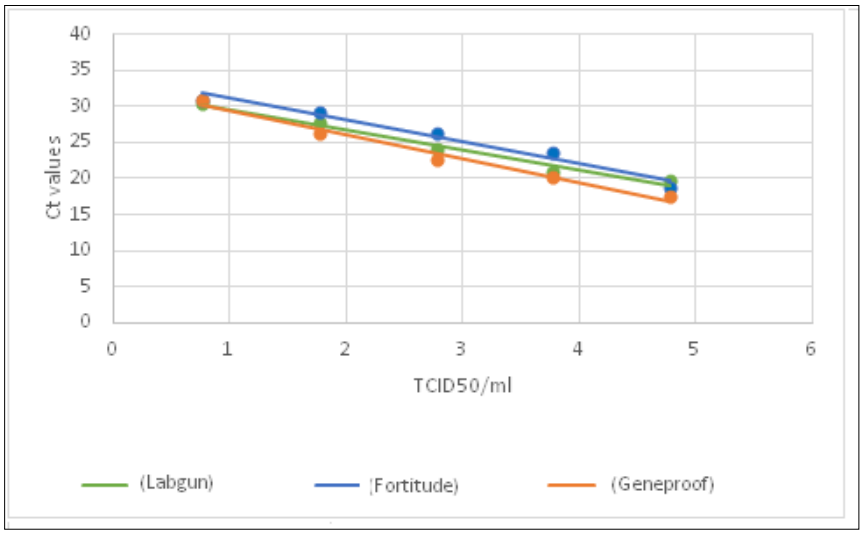

Figure 5 Standard curves of the kits from the results of manipulator 1 (RdRp gene).

The results from each handler are displayed separately, in order to elucidate the impact of the handler on the Ct values and reaction efficiency. The results from kits B and J by handler 2 are invalid and therefore are not included with the other results.

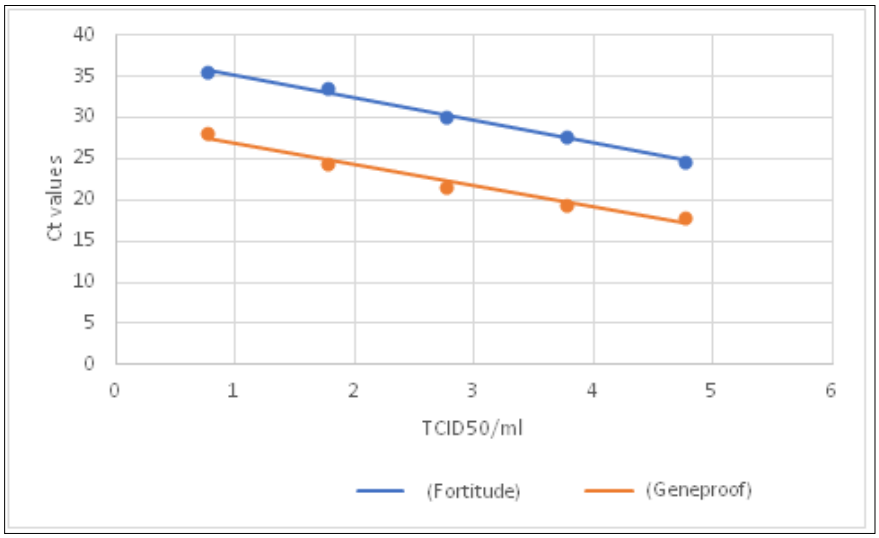

Figure 6 Standard curves of the kits from the results of manipulator 2 (RdRp gene).

Table 2 presents the different efficiencies calculated from the slopes of the standard curves for the two manipulators. The standard curves of manipulator 1 have slopes (a) closer to -3.3 for the N gene compared to those obtained for manipulator 2. This aspect is particularly proven by the efficiencies obtained by manipulator 1 for kit C (93% against 120% for manipulator 2) .It can be seen that manipulator 2 obtains a false positive result at the 10-7 dilution of the standard using Kit C. The study of the efficiency therefore allows us to determine the false positives that can occur due to contamination during manipulations.

Table 2 Results of RT-PCR efficiencies for all kits calculated by the slopes of the standard curves**.**

Kit	**Gene**	**Manipulator 1**			**Manipulator 2**		
		Pente	Efficacité	R^2	Pente	Efficacité	R^2
A (Eurobio)	N	-3.287	101.479	0.9761	-2.933	119.254	0.9909
B (Labgun)	RdRp	-2.833	125.415	0.9817			
C (Genefinder)	N	-3.489	93.4706	0.9991	-2.909	120.679	0.9967
D (Fortitude)	RdRp	-2.995	115.72	0.967	-2.93	119.43	0.9954
E (Labturbo)	N	-2.738	131.862	0.9705	-2.397	161.329	0.9992
F (Geneproof)	RdRp	-3.296	101.094	0.9797	-2.581	144.032	0.9745
G (PCL)	N	-2.778	129.072	0.9836	-4.334	70.1115	0.9999
H (Mascir)	N	-2.901	121.161	0.9883	-2.524	148.998	0.962
I (Forgene)	N	-2.861	123.629	0.9839	-2.724	132.867	0.9454
J (Sansure)	N	-2.333	168.307	0.9822			

Based on all the results obtained by the laboratory professional (manipulator 1) and the parameters to be evaluated to judge the performance of the kits studied, kits A (Eurobio), C (Genefinder), D (Fortitude), and F (Geneproof) have the best efficiencies for the target genes, ranging from 93% to 115% (Table 2), with kit C (Genefinder) being the most efficient due to its 93% efficiency, its ΔCt remaining constant for all target genes, and its very high R^2 coefficient of 0.9991.This allows us to extrapolate the Ct value from the viral titer on the standard curve of kit C (gene N) with minimal

artifact, and vice versa.However, this is not entirely valid at all points of the standard curve, since in viral culture, beyond a Ct of 28, the virus suspension is no longer infectious and may contain many defective viral particles (unpublished data), and therefore the Y axis of the Ct will no longer be proportional to the actual infectious titer of the virus.

The high and aberrant values of the efficiency of the other kits B, E, G, H, I and J may reflect their low sensitivity or the instability of their reagents due to poor preservation of the kits during transport. For example, during the preparation of the Master Mix of kit B (Labgun), the reagents did not thaw quickly, which may affect handling. As for the LOD95, a comparison could not be made with our results and the original LOD95s listed in each kit studied, due to the difference in the standard used to determine the detection limits; the marketed kits use reverse transcripts (complementary DNA) while we used a viral culture on cells.

In conclusion, standard curves are very useful for evaluating the performance of the kits used for the diagnosis of COVID-19, extrapolating the Ct value from a viral concentration, or finding the infectious titer from the PCR Ct result. Moreover, with the increasing number of commercial COVID-19 kits, there is a need for researchers to share results of their kit evaluation to decide on the sensitivities of various RT-PCR diagnostic kits used in their countries.

Compliance with ethical standards

Acknowledgments

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Disclosure of conflict of interest

No conflict of interest to be disclosed.

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