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Comparative study of two SARS-COV 2 qRT-PCR kits with two types of internal controls

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ABSTRACT

Coronavirus is a virus that was unknown before the outbreak in Wuhan (China) in December 2019 but today it has become a major public health problem. Indeed, Covid-19 is an infectious and acute disease but it can also be very deadly especially with certain comorbidities such as hypertension, diabetes, coronary heart disease, cerebral infarction or even chronic bronchitis. The qRT-PCR is the reference method for the diagnosis of Covid-19. Nevertheless, it is a delicate technique whose result depends on several parameters such as the quality of the sample, the RNA extraction steps, the cDNA synthesis, the choice of the amplification kit, the choice of controls, the analysis and the validation of the results. Any anomalies in these steps may hinder the obtaining of relevant and reliable biological results. The objective of this paper is to verify the importance and analytical efficiency of the endogenous and exogenous controls used in two diagnostic tests for Sars-cov2 by qRT-PCR commonly used in the laboratory of medical analysis and biology of reproduction, Labomac Casablanca.

1. Introduction

In early December 2019, several local health facilities reported for the first-time cases of pneumonia of unknown origin in Wuhan, China (Rasmussen *et al*., 2020). This novel coronavirus infectious disease (Covid-19), caused by severe acute respiratory syndrome coronavirus 2 (Sars-cov2), was first reported on 1 December 2019 and identified as a beta-coronavirus (Costagliola *et al.,* 2021). Since then, studies have increasingly demonstrated that Sars-cov2 can be efficiently transmitted between humans via aerosols or passive vectors (Poggio *et al*., 2020). With transmission capabilities even before the onset of symptoms, this pandemic is evolving and spreading rapidly (Del Rio and Malani (2020).

The pathogenicity, uncontrolled transmission of the virus and its immeasurable damage has led to a critical need for accurate and rapid diagnostic tests to trigger effective clinical interventions (Schwartz, Y. (2015). Thus, there was a rush to develop real-time qRT-PCR nucleic acid detection kits for coronavirus (Sars-cov2).

Since qRT-PCR has become the gold standard for RNA quantification, several molecular tests have been developed to detect covid-19 cases (Bivins *et al*., 2020). However, many clinical and public health research laboratories are unclear which test to adapt or whether the data are comparable. Although robust and reliable, the technique can generate variable results which may be due to several factors such as sampling conditions and quality, RNA quality, choice of kit and analysis or validation of results. Therefore, independent evaluations of the component sets of the main Sars-cov2 qRT-PCR assays are needed to compare results from different studies and to select appropriate assays for in-house testing (Sil *et al*., 2020; Smyrlaki *et al*., 2020).

The objective of this paper is to compare the analytical efficiency and success of the endogenous and exogenous control sets used in two Sars-cov2 qRT-PCR assays commonly used in the medical analysis and reproductive biology laboratory, Labomac Casablanca.

Patients and Methods

Patients and specimens

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The samples were taken at the laboratory of medical analysis and reproductive biology, Labomac Casablanca, Morocco. We established a study group of 26 nasopharyngeal samples from volunteers who came to the laboratory for covid-19 testing.

Informed consent was obtained from all patients included in this study before their samples were used.

Sample treatment and inactivation

Nasopharyngeal swabs are considered potentially infectious samples (Kojima *et al*., 2020). As such, they must undergo inactivation well before RNA extraction (Kojima *et al*., 2020). This is achieved by placing the samples in 5% diluted bleach before placing them in an incubator at 65°C for 10 min to inactivate the virus.

Extraction

Viral RNA extraction was performed using the Nextractor® NX-48S automated system using the magnetic bead extraction principle. The process is very simple and allows the purification of up to 48 samples in 15 minutes. Methods using magnetic beads or particles functionalised with silica surfaces allow selective RNA binding in the presence of high salt concentrations.

QRT-PCR

For our qRT-PCR, we used two amplification kits, namely:

- The Sars-cov2 Specific and Analytical PCR Kit, 100% GeneProof with the E (envelope protein gene), N (nucleocapsid protein gene) and RdRp (RNA-dependent RNA polymerase) gene as target sequences. Its detection requires the FAM, HEX, Cy5 detection channels (Table 2).

- The Sars-cov2 Specific and Analytical PCR Kit, 100% Speedy PCL with the N and E gene as target sequence. Its detection requires the FAM, HEX, Cy5 detection channels (table 2).

The sequences of the primers we used are:

Table 2: Information about amplification kits

Manufacturer	Country	Storage conditions	Regulatory status	Target genes
PCL Chip	South Korea	-20°C	CE-IVD	N, E
GeneProof	Croatie	-20°C	CE-IVD	Rdrp, E, N

The programmes used for detection were: 42°C for 15 min; 7 cycles of 42°C for 15 min; 95°C for 5 sec, 55°C for 40 sec, 72°C for 20 sec and 35 cycles of 95°C for 5 sec, 60°C for 40 sec and 72°C for 20 sec for the Gene Proof Sars-Cov2 kit and 50°C for 5 min; 95°C for 20 sec and 40 cycles of 95°C for 5 sec and 55°C for 30 sec for the PCL Speedy kit.

Statistical analysis

The data obtained in our experiment were subjected to statistical study. The results were done by Student's t-test.

Results

In the present work we diagnosed Covid-19 in 26 patients by qRT-PCR using two kits GeneProof and PCL SPD. The results are shown in Table 3.

From this table we can see that the results of our amplifications gave very different threshold cycles (TC). The TCs of the GeneProof internal control are much lower than those of PCL-SPD. GeneProof gave TCs between 18 and 25. PCL SPD, on the other hand, had TCs between 28 and 33. As for the detection of positivity of Sars-cov2 cases, the two kits give comparable results, for example P08 which is positive.

For P19, the results were not consistent in the series run by the Geneproof kit due to the non-detection of the internal control of the reaction despite its negativity, but the sample was considered valid for the PCL SPD kit.

Finally, in patients P25 and P26, the reverse occurred when the sample came back invalid with the PCL SPD kit, whereas for the Geneproof kit no non-compliant elements are reported.

Table 1: les amorces et les sondes qui sont utilisées pour la RT-PCR du SRAS-CoV-2

Name	Amplicon length (bp)	Description	Sequence
N	72	Forwad Reverse Probe	GACCCCAAAATCAGCGAAAT TCTGGTTACTGCCAGTTGAATCTG FAM-ACCCCGCATTACGTTTGGTGGACC-BHQ1
E	113	Forwad Reverse Probe	GGAAGAGACAGGTACGTTAATA AGCAGTACGCACACAATCGAA FAM-ACACTAGCCATCCTTACTGCGCTTCG -BHQ1
RdRP	81	Forwad Reverse Probe	GTCATGTGTGGCGGTTCACT CAACACTATTAGCATAAGCAGTTGT FAM-CAGGTGGAACCTCATCAGGAGATGC -BHQ1
Rnase P	65	Forwad Reverse Probe	AGATTTGGACCTGCGAGCG GAGCGGCTGTCTCCACCAAGT FAM-TTCTGACCTGAAGGCTCTGCGCG -BHQ1

Table3 : The results of our amplifications of our two kits

	GENEPROOF				PCL Speedy			
Patient	**RdRP/FAM**	**IC/HEX**	**N/CY5**	**Interpretation**	**E/CY5**	**IC/HEX**	**N/FAM**	**Interpretation**
01	-	24.96	-	Negative	-	33.22	-	Negative
02	-	24.77	-	Negative	-	33.42	-	Negative
03	-	23.62	-	Negative	-	30.53	-	Negative
04	-	24.67	-	Negative	-	31.45	-	Negative
05	-	24.61	-	Negative	-	31.72	-	Negative
06	-	25.5	-	Negative	-	31.9	-	Negative
07	-	24.47	-	Negative	-	31.63	-	Negative
08	18.56	22.94	21.46	Negative	29.66	31.25	29.88	Negative
09	-	19.58	-	Negative	-	29.7	-	Negative
10	-	24.48	-	Negative	-	27.75	-	Negative
11	-	19.58	-	Negative	-	27.18	-	Negative
12	-	20.09	-	Negative	-	26.06	-	Negative
13	-	19.14	-	Negative	-	26.15	-	Negative
14	-	18.46	-	Negative	-	29.04	-	Negative
15	-	19.16	-	Negative	-	27.08	-	Negative
16	-	20.88	-	Negative	-	27.69	-	Negative
17	-	26.23	-	Negative	-	30.92	-	Negative
18	-	26.32	-	Negative	-	31.44	-	Negative
19	-	-	-	Invalid	-	31.74	-	Negative
20	-	24.43	-	Negative	-	30.2	-	Negative
21	-	23.23	-	Negative	-	28.95	-	Negative
22	-	26.61	-	Negative	-	32.58	-	Negative
23	-	24.72	-	Negative	-	29.78	-	Negative
24	-	24	-	Negative	-	28.45	-	Negative
25	-	23.25	-	Negative	-	-	-	Invalid
26	-	20.08	-	Negative	-	-	-	Invalid

Discussion

The objective of this study was to compare the internal controls of the GeneProof and PCL SPD amplification kits, to raise the importance of the choice of the type of internal control and also to deduce the strong points in the detection of the SARS-COV2 virus.

Indeed, the GeneProof kit includes an Internal Control (IC) that serves as a control for the entire diagnostic process, RNA extraction efficiency, reverse transcription step efficiency (transcription of RNA into cDNA) and PCR amplification efficiency. The amplification of the IC internal control is detected in the fluorescence channel for the HEX fluorophore. The Speedy kit does not include an exogenous IC Internal Control but relies on the detection of the RNase P gene present in human cells which serves as an endogenous control that not only informs us about the diagnostic process (RNA extraction efficiency, reverse transcription efficiency, and PCR inhibition), but also about the pre-analytical step demonstrating sample quality first.

Analysis of the characteristics of our standard curves such as correlation coefficient (R^2), slope and efficiency showed that our amplifications worked perfectly well and resulted in satisfactory amplifications. Knowing that a PCR efficiency of 100% corresponds to a slope of -3.32 and is determined by the following equation: Efficiency = $10^{(-1/\text{slope})-1}$.

The GeneProof 100% Sars-cov2 Specific and Analytical PCR Kit has the E, N and RdRp gene as its target sequence and the PCL Speedy kit has the N, E gene as its target sequence. The detection system for both kits requires the same FAM, HEX, Cy5 detection channels (Table 1).

The temperature profiles of our two kits are different, PCL Speedy has the shorter temperature profile than Geneproof. The GeneProof detection system is based on the detection of 3 Sars-cov2 specific genes in 3 independent channels namely: RdRp gene, E gene, N gene to avoid false negative results.

The low CT values of the GeneProof internal control compared to PCL Speedy is explained by the fact that the GeneProof internal control mimics the natural viral particle hence named -Exogen, this controls the whole diagnostic process from RNA extraction, reverse transcription and PCR amplification, thus increasing the detection sensitivity hence the difference in CT between the two kits.

The choice of an exogenous internal control in the Geneproof kit also allows rapid detection of the virus and therefore immediate isolation of positive cases after confirmation.

We also noted the presence of a typical S Shape amplification curve with the Geneproof kit. As for the PCL SPD kit, the pattern is sometimes atypical given the number of cycles and the reduced reaction time.

The absence of detection of the GeneProof internal control in the P19 patient well when it was detected with the PCL Speedy well suggests that a pipetting problem occurred when the internal control was added during extraction.

The absence of the PCL Speedy internal control CT signal in the wells of patients P25 and P26 when it was detected with GeneProof is explained by the fact that the sampling procedure was not well performed, and therefore no cells and RNAse P gene were present.

For patient P08, the difference in sensitivity between the two kits expressed by the CT values did not affect the result and its interpretation.

According to the results obtained, the Geneproof kit represents the most reliable kit, with better specificity and sensitivity of detection than PCL SPD. It also provides more confirmed results based on typical amplification curves.

Although the Geneproof kit has a longer temperature profile, and does not allow verification of the sampling step, its advantages are more interesting from a biological and clinical monitoring point of view to face the pandemic.

Conclusion

The real-time PCR coronavirus assay is an in vitro diagnostic test based on qRT-PCR for the qualitative detection of Sars-cov2 viral RNA.

In the two assays we used, three Sars-cov2 genes, E, N and RdRp, are targeted and the primers and Taqman probes are designed in the conserved region of the Sars-cov2 virus-specific genome to allow sensitive and specific amplification and detection of the virus.

To ensure the performance and reliability of the assay it is essential to introduce controls, in our study we investigated the analytical efficiency and success of endogenous and exogenous control sets used in two qRT-PCR assays GeneProof and PCL-SPD. According to the results obtained, the Geneproof kit is the more reliable kit, with better specificity and sensitivity of detection than PCL SPD. It also provides more confirmed results based on typical amplification curves.

According to our study qRT-PCR is a test that can be very accurate, detecting the virus in 95% of cases. But this high specificity is also a weakness. Thus, a new variant of the virus may be less well detected, or even escape amplification completely. It is therefore recommended to use all negative and positive controls, review the characteristics of the tests used, compare them with the available genetic data and adapt them to the new variants to ensure the reliability and performance of the result.

Contributions of the authors: This work is carried out in collaboration between the different authors who are equally involved in the review, drafting and correction. They also wrote, read and approved the manuscript.

Limitations of our study: In future studies, it is recommended to increase the number of samples.

Ethical statement: All subjects gave their informed consent for inclusion before they participated in the study. The study was conducted in accordance with the Declaration of Helsinki, and the protocol was approved by the Ethics Committee of Hassan II University with a code of public health: 2008 – 321.

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Conflicts of Interest: The authors declare no conflict of interest.

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