ACCURATE DETECTION OF SARS-CoV-2 MIGHT BE A CHALLENGE IN THE MOLECULAR BIOLOGY LABORATORY FOR RT-PCR FINAL RESULTS

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Abstract

The challenges we experience professionally always teach us to retreat, to document ourselves, to learn, to become better and to succeed in asserting ourselves in the fields we have trained and perfected throughout several years. This also happened in the pandemic times with Covid-19, when we had to document RT-PCR techniques in order to be able to detect at the molecular level the SARS-CoV-2 virus responsible for disturbing the world worldwide. Thus, in the molecular biology laboratory, analysts must make very important decisions about the final result of the RT-PCR test to determine – given several analytical criteria – whether the result is negative, positive or equivocal. There are situations where the RT-PCR equipment does not detect all the genes responsible for a clearly positive result, or when the cycle threshold (Ct) is higher than specified in the reagent insert kit, and then it is the analyst's duty to decide what is the final result of molecular test. This paper brings to the fore the involvement, duty and the art of researchers and specialists who must assume the final result of the RT-PCR test in the detection of the SARS-CoV-2 virus responsible for the global pandemic of Covid-19.

Introduction

We are in the midst of a COVID-19 pandemic, and in Romania at the moment, we are sad and terrified of the 4th wave of SARS-CoV-2 infection, when the infection rate exceeded 12 positive cases per 1000 inhabitants, and when hospitals have courtyards full of ambulances waiting for a place available in the hospital to transfer critically ill patients, and unfortunately, patients are also getting younger and younger.

We go back a little in time, in 2013, and we remember the onset of the viral disease with the virus SARS-CoV-1 (Severe Acute Respiratory Syndrome Coronavirus), which underwent mutations being named in 2018 MERS-CoV (Middle East Respiratory Syndrome Coronavirus), so that in 2019 we reach the SARS-CoV-2 virus (Severe Acute Respiratory Syndrome Coronavirus) [1, 2, 3].

Testing methods for the detection of SARS-CoV-2 virus are very important, because the accurate molecular result plays a very important role in the correct diagnosis and ideal treatment prescription, enabling the doctor to cure quickly and confident the patients [4, 5]. Thus, the rRT-PCR – the real time reverse transcription polymerase chain reaction is the molecular test mostly used in the detection of the nucleic acid of SARS-CoV-2 virus. This technique detects usually two or three of viral genes responsible for the infection, and one common *Coronaviridae* family. The SARS-CoV-2 virus is a beta coronavirus, single-stranded RNA, positive-sense, part of *Coronaviridae* family, and *Orthocoronavirinae* subfamily. From molecular point of view, the genome encodes some specific glycoproteins (spike – S, membrane – M, envelope – E, and nucleocapsid – N) which can be detected with PCR technique [6-10].

For the first time, the quantitative rRT-PCR technique for detection of SARS-CoV-2 virus was developed in Germany, at the Charité Institute of Virusology, and it was reported also by World Human Organization (WHO) last year (2020) on January 13. Followed this method there were subsequently reported others additional protocols which have as assay target three different specific genes: SARS-CoV-2 RNA-dependent-RNA-polymerase (RdRp), E and N gene [11]. For all RT-PCR techniques, for all protocols, there are necessary approves from Emergency Use Authorization (EUA) for reagents; for extraction, sequences and amplification; analytical sensitivity, specificity, and precision.

The results of RT-PCR are very important for the correct diagnosis, but also some others aspects have to be taken in consideration for the right diagnosis and the best treatment prognosis, such as: collection of nasopharyngeal secretions (swab type, sampling and collection method); transportation and preservation of the samples; blood tests (neutrophils, inflammatory biomarkers, lymphocytes); Ct – cycle threshold value; and if necessary the CT (computer tomographic) examination.

Sometimes the RT-PCR results have to be evaluated taking in consideration not only the final result, but also the detection of both two genes (common family *Coronaviridae* – E gene and also SARS-CoV-2 gene), Ct value for both genes, as well as the graphical shape of all detected genes. But for this it is the task of the analyst to decide if the final result is positive or negative, even the RT-PCR amplification equipment gives the final result "positive", "presumptive positive" or "equivocal". Our paper debates exactly this situation where the final result has to be decided having in view not only the result of the amplification equipment, but also some other aspects regarding the amplification information and the reagent insert kit specifications.

Experimental

The RT-PCR technique is a molecular method of detection of nucleic acid of SARS-CoV-2 virus in order to establish if the patient is infected or not! The protocol involves the swab-sampling of the nasopharyngeal secretion, transportation and preservation of the samples, RT-PCR extraction and amplification. The analytical work was done in the Laboratory of Molecular biology from Banat's University of Agricultural Sciences and Veterinary Medicine "King Mihai the Ist of Romania" from Timisoara, Romania. The sampling preparation was performed in a biological safety cabinet class II (hood with biosecurity level 2), and the personnel wore protective equipment according to biosafety specifications. The extraction was performed with an automatic RT-PCR extractor 48 from Bioneer, and the amplification was performed with the RT-PCR Exicycler 96 from Bioneer.

We evaluated the RT-PCR results since 1st of January 2021 to 30th of September 2021 from the Laboratory of Molecular Biology from Banat's University of Agricultural Sciences and Veterinary Medicine "King Michael the Ist of Romania" from Timisoara, Romania (BUASVMT). Thus, we performed a number of 2590 total tests, from which the RT-PCR equipment gave us 2440 negative results, 150 positive results, and 57 presumptive positive results – that represents 2.2% from total RT-PCR tests. This means that at least 2.2% (presumptive or inconclusive positive) from total RT-PCR results had to be re-evaluated by the analyst in the laboratory for setting the final RT-PCR result which was reported also to the Ministry of Health from Romania and also to the patients.

Results and discussion

For an accurate RT-PCR result, for each run we include a negative and a positive control, that is part of the insert reagent kit, and is absolutely necessary for an internal quality control. The result for each control run (negative and positive) has to be "valid". Also, each sample amplified

on the RT-PCR equipment contain the Bioneer's AccuPower PCR PreMix reagent and also internal control, and for all samples we have to have a valid result for internal control.
The results of the RT-PCR amplification can be clear-cut, negative or positive for SARS-CoV-2, with valid internal control, with non-detected gens of *Coronaviridae* family and SARS-CoV-2 specific gene – for negative result; and with Ct (cycle threshold) value for both E. and SARS-CoV-2 gene – for positive result.
The Ct value is very important and could be a very important specification, but not singular, in the right diagnosis and best treatment for the patient! The Ct – cycle threshold in RT-PCR is a positive reaction and response, being detected by accumulation of a fluorescent signal. Ct is the number of cycles required for the fluorescent signal to exceed the background level, and is inversely proportional to the amount of target nucleic acid from the sample. Thus, a high Ct value is correlated with a low viral RNA-SARS-CoV-2 load, and of course a lower risk of Covid-19 transmission [12]. The RT-PCR assay undergo up to 40 cycles for amplification, but the maximum value of Ct for positive results is set by the producer of the insert reagent kit. For Bioneer's AccuPower PCR reagent kit, the maximum value of Ct for positive results is lower 33, which means that positive results have Ct values lower than 33.
However, the RT-PCR equipment gives as a final result sometimes a "presumptive SARS-CoV-2 positive" result, that means that the result is equivocal, is not certain positive.
So, what we should do in this case? How we have to evaluate all the data and give a correct final result? In this case we have to evaluate not only the final result, but we have to check the detection of all genes, the values of Ct for E gene and SARS-CoV-2 gene, and also we can evaluate the graphical presentation of the amplification curves. Figure 5 presents a case of "presumptive SARS-CoV-2 positive", but because we can easily see that only the SARS-CoV-2 gene was detected and the Ct was 31.87, and the E gene was non-detected, we can give as a final result as negative for SARS-CoV-2 infection.
Based also on literature data and the specifications of the reagent kit producer, we can consider a result of suspect, inconclusive or weak positive with Ct values higher than 33, as indicating minimal amounts of target nucleic acid, in our case SARS-CoV-2-RNA could represent early or late infection, residual vaccine or even environmental contamination.
Other situations can be identified as a "positive test result from RT-PCR", with valid negative and positive control for presented run.
Even if the final reported result from RT-PCR after amplification is "positive test result", when we check the graphical representation we can observe that E gene is not detected, SARS-CoV-2 gene is detected – but the Ct value exceed the reagent kit recommendation (as maximum 33 value for positive result), and IPC (internal control is valid). So, in this case the final result reported to the patient is "negative for SARS-CoV-2 infection"!
There are many scientific experimental papers published about the Ct value regarding COVID-19, which try to elucidate and make clear the importance and the right evaluation and interpretation of the Ct value. Thus, Polese-Bonatto and her research team evaluated the Ct value for children and adults infected with SARS-CoV-2, using RT-PCR confirmed infection, using a method that detects three gene-targets, and the results were comparable between children and adults. On the other hand, Ct value is correlated with some hematological and blood biochemical parameters, such as: aspartate-aminotransferase (AST), neutrophils, and monocytes [13].
Other scientific reports presented clear conclusive data about strong correlations between Covid-19 and some blood biochemistry, coagulation tests, and inflammatory factors, such as: leucocytes, lymphocytes, neutrophils, platelet, hemoglobin, total protein, globulin, albumin, procalcitonin, C-reactive protein (CRP), erythrocyte sedimentation rate (ESR),

antistreptolysis O (ASO), D-dimer, fibrinogen, lactate dehydrogenase (LDH), interleukin-6 (IL-6), interleukin-2 receptor (IL-2R) [14-18].
There is another situation where the RT-PCR result is "invalid". In this case, first we have to evaluate if the IPC (internal control) is valid or not for that sample, if the negative and positive control for that run is valid, and also to check if there is any of the specific gene detected.
An example of this situation presents the final RT-PCR test result as "invalid", the IPC is also "invalid", and none of the E gene and SARS-CoV-2 gene is detected. In this case the final RT-PCR result is "Equivocal" that means that the test has to be performed again, and we have to repeat extraction and amplification to be sure for the final result.
More and more researchers try to make correlations between different blood tests results, computed tomography (CT) evaluation, radiographic evaluation (RX), and magnetic resonance imaging (MRI) evaluation, encephalography technique, just to find new and new findings which can in recent future to prevent and treat with success this pandemic disease of Covid-19!
Bairwa and his team found strong association with Covid-19 mortality of some hematological and biochemical markers. Thus, they evaluated 249 hospitalized patients infected with SARS-CoV-2 which presented elevated procalcitonin, C-reactive protein, AST, serum potassium, neutrophils count, RBC (red blood cells) count, and prothrombin time; while the lymphocyte count, oxygen saturation, partial oxygen pressure, alanine-aminotransferase (ALT), and lactate dehydrogenase decreased – most of the situations having strong association with mortality [19].
World Human Organization (WHO) Working Group on the Clinical Characterization and Management of Covid-19 infection publishes regular reports about the new findings regarding this new and pandemic disease published in Lancet – Infectious disease a clinical report where they underlined some endpoints used in clinical studies planned and done during Covid-19 outbreak and also clinical progression scale, presented a score of severity of disease from "uninfected" to "dead" of Covid-19 [20].
Having all this very complex data in our view, trying to bring new experimental proves and findings into the world attention, we are part of this scientific work, and, also with our experimental experience we come and add information that can help in this fight against the SARS-CoV-2 virus and help as much as we can for all people to return to their previous lives, to get through the disease easily or to get vaccinated and thus to develop antibodies that protect us in the near future.

Conclusion

RT-PCR technique is used with success for qualitative and quantitative detection of SARS-CoV-2 RNA viral infection and Covid-19 disease. The samples represents swab nasopharyngeal secretions and the molecular test involves the extraction of the RNA and the amplification on RT-PCR.
Most of the time the results from RT-PCR technique is very clear, is "positive SARS-CoV-2" or "negative", but sometimes the result could be "invalid" or "presumptive SARS-CoV-2 positive" – and in these situations the analyst from the laboratory, usually geneticist specialist (medical doctor, biologist, biochemist, or chemist) has to evaluate all data and information from the equipment and to give the final medical report.
The geneticist plays a critical role in the final decision of the final medical report and result of the RT-PCR test. But there are more characteristics that should be taken in view and should be evaluated. Because all these, the cooperation between the medical doctor that follow the medical state of the patient, the laboratory specialists, and the family doctor for the historical medical data for the patient in critical and very important.

Prevention, correct diagnosis, medical prognosis, and right treatment could be the difference between a medical success and the medical failure!
Not the last, very important is the pre-analytical phase (sampling the biological specimen – in our case the nasopharyngeal secretions), analytical and post-analytical phase plays all very important role in the great achievements and professional recognition!

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