# Development and Optimization of Classic Multiplex RT-PCR for the SARS-CoV-2 Detection

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*Abstract— Develop alternative methods for the identification of SARS-CoV-2 is essential for epidemiologic studies and interspecies correlations, allowing the allocation of scant resources to the Real-Time PCR (qRT-PCR) for patient diagnosis. The aim of this study, therefore, was to develop and optimize a multiplex reverse transcription-PCR (RT-PCR) assay to detect SARS-CoV-2 in nasopharynx clinical samples as an alternative to qRT-PCR. Bioinformatics tools to specific primers design analyzed genome sequences of the new coronavirus available on GenBank. The assay was proposed to amplify partial segments of N genes and RdRp region of the orflab gene of SARS-CoV-2, recommended targets by the World Health Organization (WHO). Reaction control used positive commercial control and samples from patients known to be positive and negative. The results of this study demonstrated that it was possible to optimize the RT-PCR for the detection of SARS-CoV-2 through a multiplex assay in agreement with the gold standard, precursors results for validation studies of this alternative method for epidemiological and animal health surveys are until now unclear.*

*Keywords— Coronavirus Disease 2019, Molecular Biology, Diagnosis, Multiplex Polymerase Chain Reaction.*

## I. INTRODUCTION

One of the main responsible for the present severe acute respiratory syndrome cases is the new *Coronavirus* (SARS-CoV-2). The first cases appeared in 2019 in Wuhan, China. The World Health Organization (WHO) classified the virus disease COVID19 as a pandemic in March 2020.

The coronavirus that belongs to the *Coronaviridae* family that is characterized by a positive simple RNA genome and enveloped viruses. Composed of four structural proteins, spike (S), membrane (M), envelope (E), and nucleocapsid (N), being the innermost protein linked to the viral genome [1,2]. RNA-dependent RNA-polymerase (RdRp) region and *N* gene are known

as the most conserved genomic regions, and therefore the best target for the detection of SARS-CoV-2 strains according to the United States and Chinese Centers for Disease Control and Prevention (CDC) and the WHO [3,4].

Biomolecular methods prioritize the detection of viral particles in the acute phase of disease progression. The WHO recommends the use of *real-time* PCR (*q*RT-PCR) for COVID19 diagnosis as it is a highly sensitive and specific method, using as a standard protocol the research of the gene that encodes the *N* protein [4].

Even though *q*RT-PCR is highly sensitive and specific for the acute phase of the infection diagnostic, it is a method whose implementation is arduous, requires high-cost equipment and specifics imported reactants, and

a highly-skilled technical team. It is verified the scarcity of these inputs in the market at the time of vast international demand from the pandemic state. In Brazil, few laboratories have this standard methodology for the SARS-CoV-2 diagnosis, leading to laboratory overload and, consequently, underreporting cases [5].

For isolation measures and medical intervention to be taken, the agent detection must be carried out as soon as possible for viral spread control. Thus, it is essential to develop methods of rapid execution, with lower cost and with high sensitivity and specificity for SARS-CoV-2, to ensure the diagnosis, monitoring, control, and prevention of the disease dispersion in the population. Conventional PCR preceded by viral RNA reverse transcription (RT-PCR), for example, can be performed with national reactants and may have the same detection capability of the agent at the beginning of clinical signs, being a possible methodological alternative for detecting viral particles.

In this context, the present work objective was to develop, standardize, and optimize the classic multiplex RT-PCR for simultaneous detection of SARS-CoV-2 *N* genes and RdRp region of *orflab* gene using positive commercial control and positive and negative clinical, for further validation as a diagnosis method, triage, epidemiological studies, fluctuation, and interspecies correlations.

## II.     METHODS

The *primers* designwas used with eight SARS-CoV-2 genomic sequences held in a public database (*GenBank*), targeting the viral genome *N* genes and RdRp region, listed by WHO for SARS-CoV-2 detection (https://www.who.int/docs/default-source/coronaviruse/whoinhouseassays.pdf?sfvrsn=de3a76aa_2). The reference genomic sequences are those of access in *GenBank* MT081066 (*N* gene) and MT072668 (RdRp of *orflab* gene). For primers designs, *Primer 3* (http://primer3.ut.ee/) and *Primer Blast* (https://www.ncbi.nlm.nih.gov/tools/primer-blast/) programs were used.

Using positive commercial control patterns for *N* gene (2019-NCOV_N Positive Control Kit, IDT, EUA – 200.000 cópias/µL), assays were optimized in uniplex PCR with N1-F (5'-GGTTCACCGCTCTCACTCAA-3') and N2-R (5'-CAAGCAGCAGCAAAGCAAGA-3') primers, beginning on 155 and 673 nucleotides of MT081066 sequence, respectively, with expected products of 519 pb.

Two primers forward (RdRp1F 5'-AATAGAGCTCGCACCGTAGC-3' and RdRp3F 5'-GCCTCACTTGTTCTTGCTCG-3' – start on 76 and 322 nucleotides of MT072668 sequence, respectively) and two *reverse* (RdRp2R: 5'-CCGCCACACATGACCATTTC-3' and RdRp4R: 5'-GCCGTGACAGCTTGACAAAT-3' – start on 434 and 527 nucleotides, respectively) were evaluated for *orflab* gene RdRp region. Each set of primerswas appraised individually. The *primers* synthesis was performed in Brazil (Custom DNA Oligos Synthesis, ThermoFisher) on the scale of 50N.

The standardization and optimization of technic used samples of four patients newly diagnosed with SARS-CoV-2 per *q*RT-PCR (gold standard). The epidemiological surveillance team from the municipality of Araguaína, Tocantins state, Brazil, responsible for monitoring positive patients in the referred locality, collected two positives (samples 1 and 4) and two negatives (samples 2 and 3). The participation of the patients was voluntary. The team of this project made all the material available for these patients collection: a set of three swabs (rayon tip and plastic rod) combined (bicavitary nasopharyngeal and oropharyngeal) stored in the same tube (sterile Falcon type with a capacity of 15 mL) containing 3 mL of sterile saline solution (0,9%). The samples were immediately sent under refrigeration to the Microbiology Laboratoryof Federal University of Tocantins, Araguaína campus.

The DNA extraction was promptly performed in a commercial kit (BioGene Extração de DNA/RNA viral, Bioclin, BR) in a biosafety environment. The reverse transcriptase enzyme (SuperScript™ III, Invitrogen, USA) was used in the reverse transcription according to the manufacturer recommendations with random oligos available in the enzyme kit.

The commercial mix (Platinum™ Hot Start PCR Master Mix, Invitrogen, USA) performed the multiplex PCR reactions to provide higher reproducibility. Each one of the primers was added in 1µL (20 pmol) per sample to each assay. The uni and multiplex reactions final volume was 30 µL/each, with the application of 1 to 4 µL of reverse transcription product.

Amplification conditions were optimized in gradient (T100 Thermal Cycler, Bio-Rad, USA) to *N* gene with temperature/time conditions determined in a cycle of 94ºC/5 min; 40 cycles of 94ºC/45 sec, 52ºC/45 sec and 72ºC/1 min; followed by a final extension cycle at 72ºC/10 min.

Amplification products were submitted to electrophoresis in agarose gel at 2%, stained in ethidium bromide (0,2 mg/mL), and documented under ultraviolet light.The Brazilian National Research Ethics Commission (CONEP) previously approved the research project (Presentation Certificate for Ethical Appreciation n° 33350720.9.0000.5519).

## III. RESULTS AND DISCUSSION

The serial dilution of positive commercial control in TE buffer (Tris-HCl [10mM]: EDTA [1 mM]) sensitized 100 genomic fragments of the primersdetermined for *N* gene amplification.

Uniplex assays tested samples 1 and 4 from patients for each one of the searched genes. The test results for the *N* gene coincided with the diagnosis by the gold standard (*q*RT-PCR), being noticed 519 bp*amplicons* in samples 1 and 4 and negative results in samples 2 and 3, presenting the same result if applied 1μL until 4 μL of the reverse transcription product.

Once it was expected to develop a multiplex protocol for SARS-CoV-2 detection, the same optimized amplification conditions for the *N* gene performed uniplex assays of clinical samples for the RdRp region. In the individual assays of each set of primers for the *orf1ab* gene was observed that theforwardprimer RdRp1F with both RdRp2R and RdRp4R did not present samples 1 and 4 amplification. It can be related to differences in melting temperature that influences in the annealing temperature. Therefore the primer RdRp1F was discarded from the multiplex test.
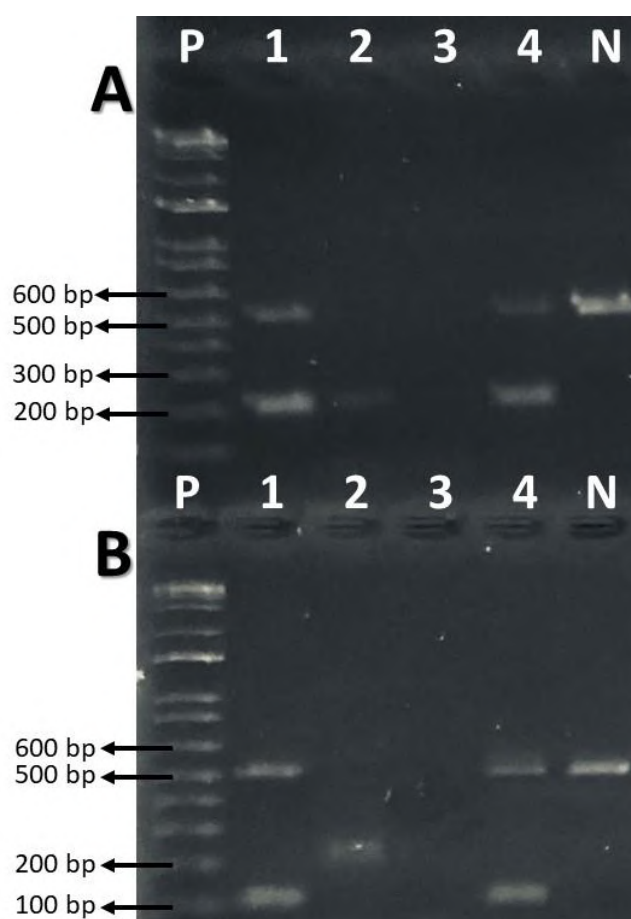


*Fig.1: Multiplex PCR reaction result for SARS-CoV-2, N genes, and orf1ab RdRp. In the A subsection, it is possible to verify the PCR results for the N gene of 519 pb and RdRp of 204 bp search in the samples 1 and 4 positive in the gold standard (qRT-PCR) and negative results in the negative samples 2 and 3. In the B subsection, it is possible to verify the same result for the N gene and 111 pb product of the RdRp gene by changing the primers. P = molecular weight marker and N = positive commercial control for the N gene applied 1.000 multiplex PCR copies.*

However, when uniplex PCR subjected the clinical samples 1 and 4 to the RdRp region utilizing the *forward* primer RdRp3F was observed the products amplification of 111 and 204 bp with theRdRp2R and RdRp4R reverse primers, respectively, in the samples known to be positive (1 and 4) and negative results in the negative samples (2 and 3), enabling them to the amplification protocol established for the *N* gene.

For the optimization of the multiplex assay for the RdRp and *N* gene, the N1-F and N2-R primers were tested with RdRp3F and RdRp2R primers and in another assay with RdRp3F and RdRp4R primers. Figure 1 represents the results. It can be observed that in both assays, the samples known to be positive 1 and 4 simultaneously presented the expected results for the RdRp and *N* gene amplification. Moreover, samples 2 and 3 known to be negative, did not show size amplification of the expected genetic fragment.

The researchers involved in this study elaborated on a research protocol to validate the referred method based on local epidemiological data and have a favorable ethical decision of CONEP for testing other 342 patients suspects of COVID19 cared by the Brazilian public health system. The prospection of this study is to validate this alternative methodology for triage in the case of high sensitivity and low specificity, create a molecular tool for epidemiological studies of incidence/prevalence of SARS-CoV-2 in populations, in addition to epidemiological correlations evaluation with domestic or production animals species.

## IV. CONCLUSION

Considering positive commercial control for the *N* gene and this pilot study clinical samples, it was possible to determine primers and amplification conditions for the detection of SARS-CoV-2 in positive patient samples for COVID19 in classic multiplex RT-PCR assay for *N* genes and RdRp region. However, it is still necessary to carry out a validation study with a bigger number of samples to obtain more robust and significant results to establish this method as an alternative to *q*RT-PCR. This will provide methodological resources for the execution of infection rates fluctuation and interspecies epidemiological correlations studies that are until now unclear.

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