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Dr. Margaret Harris
The World Health Organization's coronavirus response team
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Dr Eduardo Guerrero
WHO Regional Office for the Americas
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Dr. Anthony S Fauci
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Extremely sensitive, no false-positive tests needed for SARS-CoV-2

Dear Drs. Harris, Guerrero and Fauci:

It has been widely reported in the social media that the RT-qPCR test kits used to detect SARS-CoV-2 RNA in human specimens are generating many false positive results and are not sensitive enough to detect some real positive cases, especially during convalescence.

RT-qPCR is known to generate false positive results when used to detect influenza A virus [1] and MERS-CoV, [2] another Coronavirus.

Without a nested (two-round) PCR, a single round RT-PCR may miss real infections caused by SARS-CoV [3] and by SARS-CoV-2 [4].

The major technical flaw of RT-qPCR for molecular diagnosis is the limitation of the length of its DNA probe which is about 25 bases long or shorter. And hybridization is not an accurate method to determine nucleotide sequences, the foundation of all nucleic acid-based diagnostics.

This letter recommends that the WHO coronavirus response team adopt or develop a nested RT-qPCR protocol to generate a cDNA PCR amplicon to be used as the template for bi-directional sequencing. As demonstrated in this letter, nested RT-PCR is an extremely sensitive detection method and DNA sequencing will guarantee no-false positive results if all positive reports are accompanied by two-directional sequencing electropherograms, like an EKG for the diagnosis of Left Bundle Branch Block in a cardiologist's consultation.

Based on information retrieved from the GenBank databases and available in the public domain, there is a unique 398-base segment in the SARS-CoV-2 nucleocapsid (N) gene which not only has a 100% match with that in the Wuhan seafood market pneumonia virus, but also contains four single-nucleotide mutations found in the viruses isolated from patients in the states of

California, Texas and Massachusetts of the U.S.A. This segment of the gene can be targeted for accurate molecular diagnosis.

The nucleotide sequence of this 398-base gene segment is copied from the GenBank and re-printed here with the 4 mutated bases typed in red color. Identification of these virus isolates each with a single-base mutation in this segment may be useful in tracing the immediate source of the pathogen among patients and carriers tested positive for SARS-CoV-2.

Severe acute respiratory syndrome coronavirus 2 SARS-CoV-2 RNA Isolated from throat swab of patient in cruise ship, Japan, 02-10-2020

Sequence ID: LC528233.1

Score	Expect	Identities	Gaps	Strand
736 bits(398)	0.0	398/398(100%)	0/398(0%)	Plus/Plus

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Query  1      CAATCCTGCTAACAATGCTGCAATCGTGCTACAACTTCCTCAAGGAACAACATTGCCAAA  60
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  28728  CAATCCTGCTAACAATGCTGCAATCGTGCTACAACTTCCTCAAGGAACAACATTGCCAAA  28787

Query  61     AGGCTTCTACGCAGAAGGGAGCAGAGGCGGCAGTCAAGCCTCTTCTCGTTCCTCATCACG  120
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  28788  AGGCTTCTACGCAGAAGGGAGCAGAGGCGGCAGTCAAGCCTCTTCTCGTTCCTCATCACG  28847

Query  121    TAGTCGCAACAGTTCAAGAAATTCAACTCCAGGCAGCAGTAGGGGAACTTCTCCTGCTAG  180
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  28848  TAGTCGCAACAGTTCAAGAAATTCAACTCCAGGCAGCAGTAGGGGAACTTCTCCTGCTAG  28907

Query  181    AATGGCTGGCAATGGCGGTGATGCTGCTCTTGCTTTGCTGCTGCTTGACAGATTGAACCA  240
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  28908  AATGGCTGGCAATGGCGGTGATGCTGCTCTTGCTTTGCTGCTGCTTGACAGATTGAACCA  28967

Query  241    GCTTGAGAGCAAAATGTCTGGTAAAGGCCAACAACAACAAGGCCAAACTGTCACTAAGAA  300
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  28968  GCTTGAGAGCAAAATGTCTGGTAAAGGCCAACAACAACAAGGCCAAACTGTCACTAAGAA  29027

Query  301    ATCTGCTGCTGAGGCTTCTAAGAAGCCTCGGCAAAAACGTACTGCCACTAAAGCATACAA  360
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  29028  ATCTGCTGCTGAGGCTTCTAAGAAGCCTCGGCAAAAACGTACTGCCACTAAAGCATACAA  29087

Query  361    TGTAACACAAGCTTTCGGCAGACGTGGTCCAGAACAAA  398
              |||||||||||||||||||||||||||||||||||||
Sbjct  29088  TGTAACACAAGCTTTCGGCAGACGTGGTCCAGAACAAA  29125
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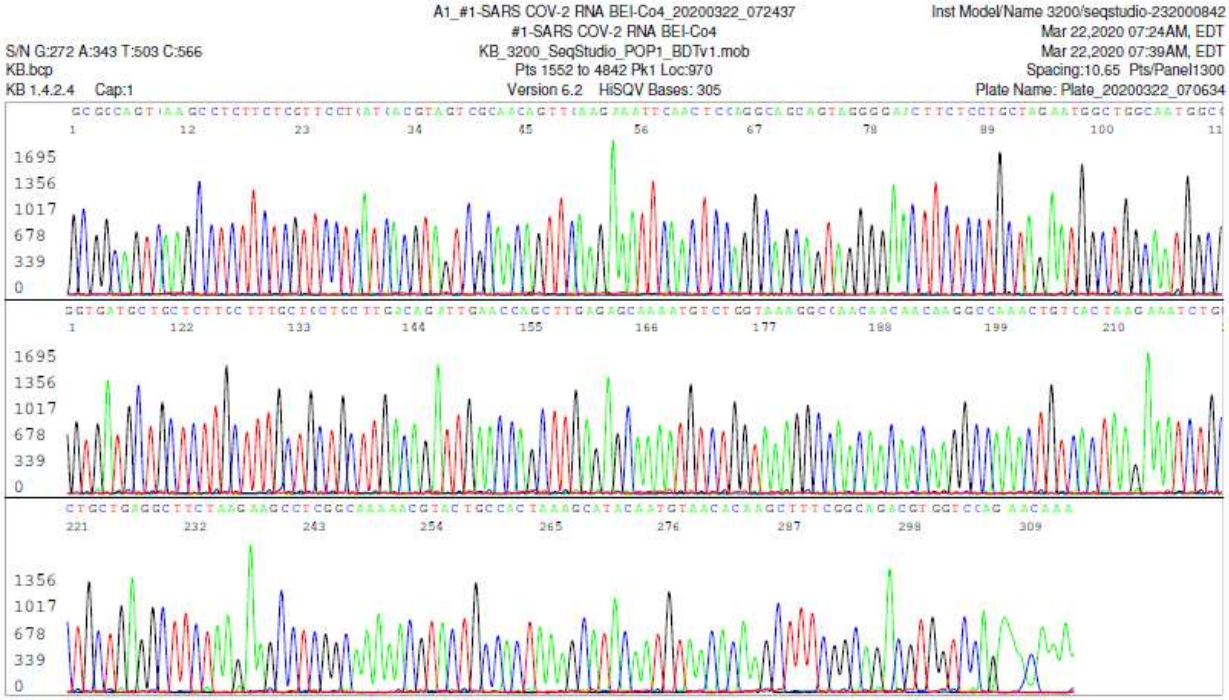
NOTE: This 398-base sequence is identical to that of the Wuhan seafood market pneumonia virus, isolated in December 2019, GenBank Sequence ID: NC_045512.2

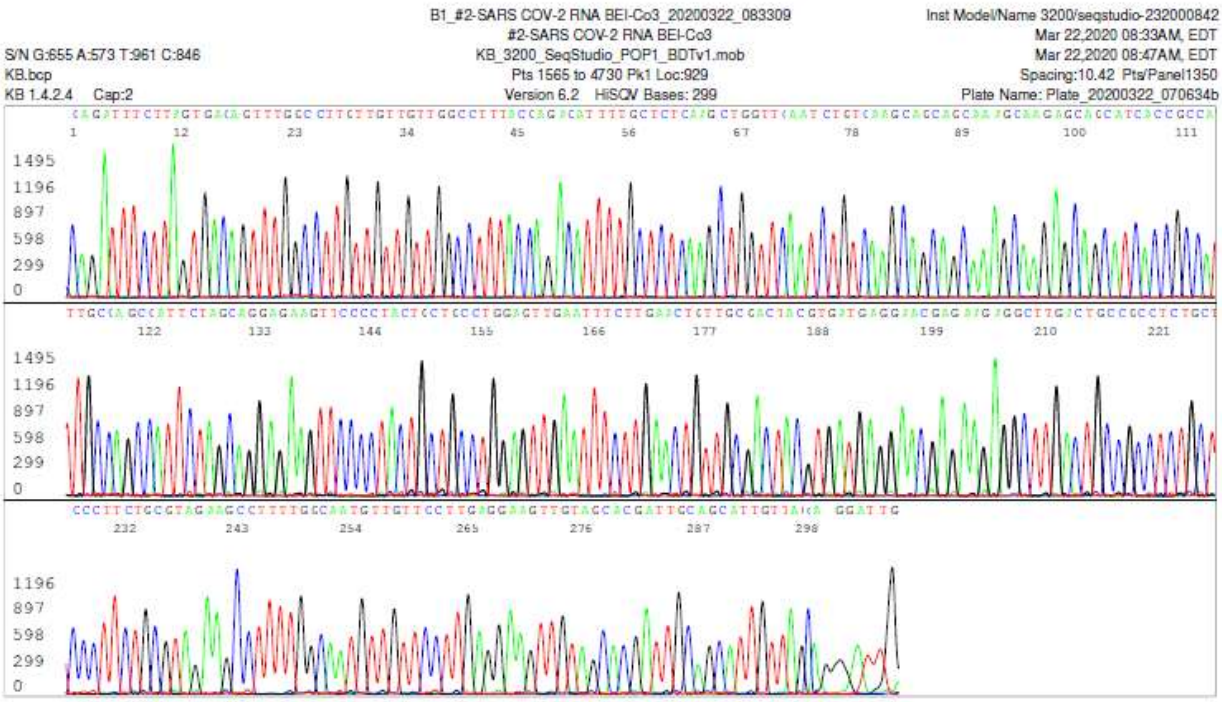
SARS CoV-2 isolates in the USA may have following single-base mutations in this segment at the positions typed in red (Sequences were retrieved from NCBI Databases).

29103 C>T Sputum of patient, TX, USA, 02-11-2020 Sequence ID: MT106054
28886 G>A Nasopharyngeal swab, CA, USA, 02-06-2020 Sequence ID: MT106052
28862 C>T Oropharyngeal swab, MA, USA, 01-29-2020 Sequence ID: MT039888
28792 A>T Nasopharyngeal swab, CA, USA, 01-23-2020 Sequence ID: MN994467

Left is an image of gel electrophoresis of the products of primary RT-PCR (upper half) and nested PCR (lower half) showing that nested PCR increases the sensitivity of RT-PCR at least 1,000 times in detecting SARS-CoV-2 RNA. The copy number of synthetic viral RNA added to each 25 µL primary RT-PCR mixture was calculated based on the analysis data supplied by BEI Resources, NIAID, NIH: Quantitative Synthetic RNA from SARS-Related Coronavirus 2, NR-52358. As demonstrated, this protocol can detect a single copy of viral RNA.

The 398-bp nested PCR amplicon shown in Lane 6 was used as the template for Sanger sequencing. The bi-directional sequences are pasted below.





Please inform your affiliated laboratories that we are now in position to assist them to resolve their questionable RT-qPCR test results with high Ct values (between 37 and 40) if they are able to send us 10 µL of the residual RNA extract kept at -80°C in dry ice package. We will perform a nested RT-PCR on each of received residual samples, and perform a bi-directional Sanger sequencing on all positive cases and report the results back to the sender.

Contact person is: Sin Hang Lee, MD email shlee01@snet.net

Sincerely,

Sin Hang Lee, MD, F.R.C.P.(C)

References

1. Martí NB, Del pozo ES, Casals AA, Garrote JI, Masferrer NM. False-positive results obtained by following a commonly used reverse transcription-PCR protocol for detection of influenza A virus. *J Clin Microbiol*. 2006;44(10):3845.
2. Pas SD, Patel P, Reusken C, et al. First international external quality assessment of molecular diagnostics for Mers-CoV. *J Clin Virol*. 2015;69:81–85.
3. Jiang SS, Chen TC, Yang JY, et al. Sensitive and quantitative detection of severe acute respiratory syndrome coronavirus infection by real-time nested polymerase chain reaction. Clin Infect Dis. 2004;38(2):293–296.
4. Nao, N., et al. Detection of second case of 2019-nCoV infection in Japan. 2020. https://www.who.int/docs/default-source/coronaviruse/method-niid-20200123-2.pdf?sfvrsn=fbf75320_7