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Short Communication

Pitfalls found in SARS CoV-2 specific test performance during the comparison between WHO recommended method and a commercial test

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Abstract

Aim: One of the important methods to detect the SARS CoV-2 is polymerase chain reaction (PCR) during the pandemic outbreak. There are a number of real time PCR tests, which are recommended from World Health Organization (WHO). Usually, the WHO recommended test should give correct and reproducible results.
Material and Methods: The real time PCR tests are conducted to perform the comparisons between WHO test for N gene and ready to use PCR kit FR475 of Genekam Biotechnology AG.
Results: It was found that WHO test gave false positive in the negative samples and correct positive results in positive samples. As this method is used from many laboratories as in-house method and many commercial companies around the world have developed their kits based on WHO tests. The results of this research work are showing that there should be around 30% false positive results as well as there are problems of reproducibility of the results.
Conclusion: There is a need that the laboratories should run the tests to show that these in-house or commercial assays are free from these above-mentioned pitfalls.

Keywords: SARS CoV2, real time PCR, virology, false positive test, World Health Organization

INTRODUCTION

Since January 2020, there is pandemic outbreak of coronaviruses from a Chinese city called Wuhan. (1-3). Real time PCR is an important method to be used in pandemic outbreak as done in H1N1 outbreak in 2009 (4,5).

World Health Organization (WHO) has published a number of test protocols to be used to detect SARS CoV-2 in clinical settings. At the same time, a number of companies have developed various tests, which are approved through different authorities around the world (World Health Organization 2020). In USA, FDA has given a number of real time PCR kits approval to be used as diagnostic tools in different laboratories in USA. WHO has its own approval system, where a number of real time PCR kits are approved and proposed to be used as diagnostic tools around the world (6).

Almost all countries in the world are following these instructions, but regretfully the pandemic outbreak is not under control in spite of the fact more than 80% of the populations of many countries are vaccinated with emergency approved vaccines. Today end of December of 2021, the number of infected cases is increasing, and they are three to four times more in many countries like Norway, Denmark, Finland, Germany, USA and other countries, they are more than number of cases in December 2020 as nobody with emergency approved was vaccinated (7). The main reason behind the failure of these vaccination is mutations arising from different countries like India, South Africa, Brazil etc. (8, 9). There are several real time PCR tests being used in different laboratories as the primers and probes are available in internet. CDC tests have been removed from the list of FDA emergency authorization use (10).

Regretfully there is suboptimal quality controls during use of such primers and probes as in-house assays in different diagnostics laboratories against the commercial tests, which are to be produced under strong quality control conditions. Despite the fact many of these laboratories carries ISO certifications for conducting diagnostic, but they have rarely any experience in the production and quality control of real time PCR kits, such

practices should be leading to the tests being conducted with substandard quality and many of results may be questionable. This publication may be going to give a proof that many of laboratories running real time PCR testing are perhaps likely to have substandard testing system as they are using questionable test systems. During approval and validation of a kit in quality management system of Genekam Biotechnology AG as examples, each kit must be tested for cross reactions with related and non-related pathogens along with a number of tests, where only negative controls are being tested to find whether the cross-contamination problem or false positive tests are being taking place. There is strong quality control during the early phase of development of tests i.e., in silico testing. If the kit passes these important steps, it will be purposed to proceed further steps for approval process. It seems that none of the public as well as private laboratories are following these along with that, they may have no experience in the production of kits in the diagnostic laboratories, which are running as in-house assays.

One cannot produce kits in the same area, where a laboratory is running the diagnostics as there are many risks in PCR technology, which can lead to wrong results (11,12).

During the detecting of unknown coronavirus in a post vaccinated sample, it was found that WHO primer and probes are not giving stable and optimal results. Therefore, we decided to conduct a thorough comparison between FR475 and the primers and probe for N gene from WHO for their performance in a research work to find the problem of stability as well as false positive results (13).

MATERIALS AND METHODS

The positive reference samples from NHS (UK) are used to perform the comparison studies between kit FR475 and the primer and probes from WHO to detect SARS CoV-2 in samples. Test with WHO primers and probe for N gene are conducted as one step-in real-time machine ABI7500 (Thermofischer) as shown in the WHO protocol. The mastermix was used from Genekam Biotechnology AG. The tests were conducted as follows:

One plate with 9 wells, where only one positive sample was added to one well and one negative control was added. This test was conducted minimum 4 times. This was conducted with WHO primer and probes. In another test, 8 wells in A row, 8 wells in B and 4 wells in C row were used, where in A row, the positive controls were added in 6th well, in B row in well 4 and in C row in well 4 also to see how these positive controls impact the results. Negative controls were added. Outlay of this testing has been shown as Figure 1.

9 wells, where one positive sample was added to one well and one negative control was added. This test was conducted with FR475. It was repeated minimum 4 times (13).

As there were positive results in the wells, where no positive samples were added in WHO test, therefore the test was conducted with FR475 to see find whether the primers and probes are being contaminated during the production process in the manufacturer. 2 ul of primer and probes are added to each well each and run at 42 0C for 60 minutes and 70 0C for 10 minutes for cDNA synthesis and 95 0C for 15 seconds and 60 0C for 1 minute for 45 cycles in real time machine ABI 7500.

RESULTS

Primers and Probe for N gene are originally developed in the publication from Corman et al. and they are recommended from WHO as diagnostic test for detection of SARS CoV-2 (14). It was found that adding of positive RNA samples in plate with WHO test showed positive results in negative controls and in many wells, where no positive samples were added (Figure 2 and 4).

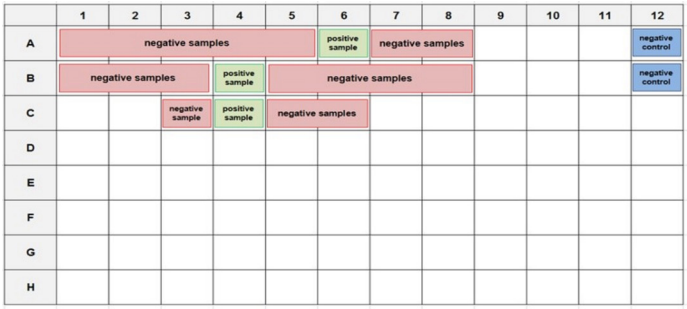

Figure 1. Outlay of plate to conduct the studies as an example

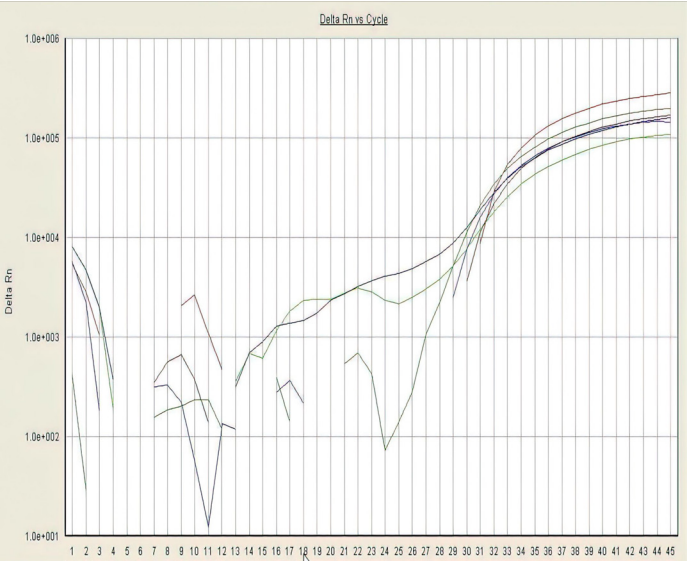

Figure 2. Positive signals in wells, where no positive samples were added. WHO test for N gene

The positive samples also gave the positive results, but the same wells tested with FR475 have shown no positive results, where no positive samples were added. They showed only positive results in the wells, where the positive samples were added and there were negative results in negative controls (Figure 3 and 5). The WHO tests were observed with linear and log modus of the analysis software of real time machine. It was found that

there was hardly any fluorescence in false positive samples oft, but there was fluorescence in the positive samples with both methods. It means that WHO test shows correct results, if the sample is really positive, but the positive sample is creating positive signals in wells, which are negative in reality i.e., false positive results. It was found that 3 to 4 samples were false positive out of 8 wells. It means that WHO test is giving around 30% false positive results in the lane, where one real positive sample is present. Data of tests conducted as shown in outlay in Figure 1 are analyzed (Table 1).

The tests conducted on the primers and probes from WHO with FR475 have shown no positive signals. The negative control gave no positive signal. This shows that there is no contamination during the production process of these primers and probes.

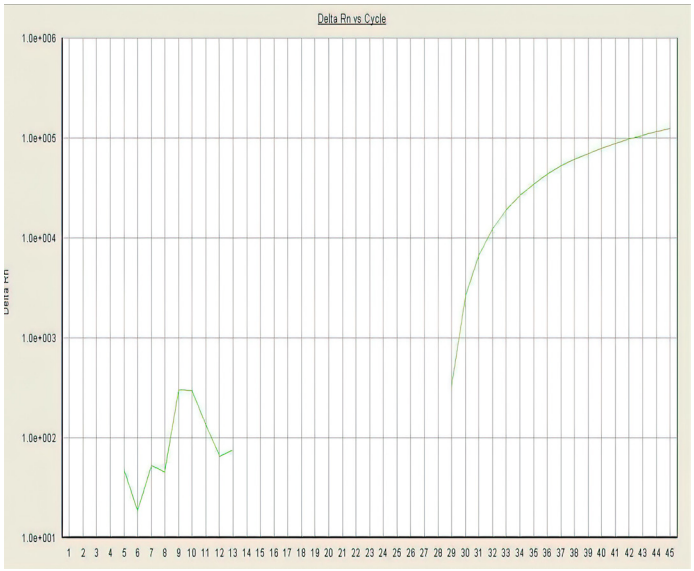

Figure 3. Positive signal in reference positive sample (NHS, UL) with Genekam kit FR475

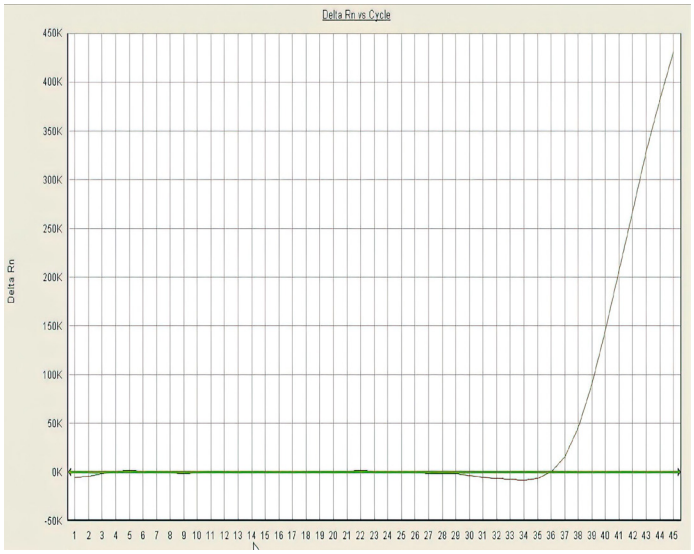

Figure 4. Positive signal in negative control with WHO test for N gene

Figure 5. No signal in wells without positive samples with real time PCR kit FR475

Table 1. Total number of samples and false positive results with WHO test

Total samples	21
Positive samples	3
Negative samples	18
Total false positive samples (percentage)	7 (33.33 %)

DISCUSSION

Corman et al. tested 120 times without any positive control and found that there is no positive signal as it was being discussed under the chemical stability for all assays (14). This quality control is not with agreement with the work provided in this publication. The primers and probes from Corman et al were recommended from WHO to provide the whole world specific tests for detecting the SARS CoV-2 (15). Moreover, it is mentioned in the publication that E protein is thought to be used as specific test. WHO protocol does not mention anywhere that user has to use E protein test.

Further E gene test is found to detect 6 pathogens found in different bats; hence it is not written anywhere in WHO protocol that this may lead to false positive results too, in case the person may carry any of these viruses, which may or may not be pathogenic to human beings. In another research work, it is shown that mutations in E gene have negative impact the detection capabilities of many commercial tests (16). WHO primers and probes were being distributed throughout the world through one of biggest biopharma company from Switzerland along with a number of companies, which synthesize the oligos. There are companies around the world, which developed primers and probes based on knowledge of Corman et al. publication, which is being endorsed through WHO. WHO carries a number

of kits from different manufacturers around the world. The question is whether these kits need to be reviewed again for these pitfalls shown in this research work? In a new document found in internet from WHO is showing the relevance of E gene in bat coronaviruses and the test for N gene is removed from the list from Corman et al. research work. It is summary of all tests, which are recommended from WHO (17). It is very difficult to confirm the exact date of upload of this new file. It seems that it should be late 2021, but it may not be true. CDC has withdrawn its emergency authorization for its tests, but these tests are on this list, therefore it may be uploaded before this withdrawal.

It is found in this research work that there are false positive results with these WHO recommended test for N gene. If one analyzes the results very carefully, one comes to conclusion that the tests give false positive results with the samples, which were really negative and this test gives correct positive results with the samples, which were from the positive patients. As there are many reports that the test is positive and the test person is symptomless (18). The research work shown in this publication may perhaps indicate the reason for such results, why there are so many symptomless positive cases? In the other words, these WHO tests are leading to higher number of SARS CoV-2 positive cases, which is likely to create fear among the people and feeling that there are so many people are positive. Many innocent tested persons may perhaps be subjected to isolation and quarantine.

Many Governments around the world have put movement restrictions. The whole countries are put under conditions, where human basic rights are restricted as everybody is believing that the results shown through the laboratories around the world are correct. These WHO tests are being used throughout the world as there are tenders floated for these tests as multiplex, but in the publication, these tests are not developed as multiplex, hence they are for single use. Many Governments like India, Syria and many have modified according to their requirements in spite of fact that many times multiplex PCR does not oft work properly. These multiplex tests may be another contributing factor for questionable results. This needs to be investigated. Tax prayer money is being used for questionable results and the pandemic outbreak is not under control.

Many laboratories around the world for diagnostic testing carry certifications, which define how to control the quality of conducting and performing the tests. This example gives some indication that there is no real quality control in these laboratories, hence it is important that these laboratories must have some quality control process as manufacturer of in vitro diagnostic tests, if they want to use in-house tests. This publication points out a big pitfall in quality control system of many diagnostic laboratories around the world, which needs to be tackled immediately.

If the results of this publication are correct, there may be around more than 30% samples, which should be considered wrong, particularly those, who never have symptoms or those, who have symptoms, which have any other respiratory pathogen involved. In this research work, there were 4 or more samples per 9 samples, were positive for N gene test. There are already reports of false positive cases (19). We compared the performance of WHO test and Genekam FR 475 kit, which led to finding that there was not even a single false positive test with FR 475 and positive samples were always recognized correctly. The negative control was not giving any positive signal; hence this kit should be used in other laboratories to make a comparison. This kit can be used to reconfirm the results of symptomless people, who were positive with WHO test in order to have any idea, what is the percentage of false positive results. Such comparison can be done in WHO or national reference laboratories. Genekam put a petition to German parliament (Bundestag) to do so in June, 2021, but they never cared as it was suspected that the results of many kits on the market may be wrong. There are some reports of comparison of performance of different kits of SARS CoV-2 in the literature.

In these publications, there was hardly a test conducted, where one made efforts to see whether these kits are giving false positive results as shown in this research work (20-23). It is highly recommended to compare the results of E gene test from WHO and other commercial supplies with the results of FR475 as this will make WHO test system better. There is need of a plan for laboratories how to produce kits based on primers and probes as in-house tests, if WHO wants to recommend such tests? This plan must define the documents needed for production and quality control of such in-house assays as done through commercial companies, so that these laboratories can learn that production process of a kit needs a lot of preparations. documentations, precautions and testing. In-house assays must be subjected to regular check through the authorities in order to avoid such false results. During pandemic outbreak, some of laboratories may be conducting thousands of tests as mistakes during production of in-house assays can have a big impact on the results.

There are a number of companies around the world, which developed their own primers and probes from N gene, their kits must be tested whether they give false positive results as shown in this research work. These commercial kits should have approvals in USA, Europe and many other countries. All commercial tests or in-house test being used for detection SARS CoV-2 must run through a test, where at least 20 samples are without positive control and 20 samples with 3 positive controls in different rows along with negative controls in order to see whether the test is working properly and not giving false signals. This should be part of quality control process with the manufacturers as well as quality control of diagnostic laboratories. This should be performed once a month in each laboratory and results must be reported. In this process, Genekam FR475 kit may be used as standard for comparison. These steps can help to contain the pandemic outbreak faster. As the number of infections are rising in spite of fact that up to 90 % populations are vaccinated. The question will be, which role are being played through these tests

mentioned in this research to give false positive results, this must be examined urgently to get the clear Figure of pandemic outbreak. The reduction in false positive test is going to help the Governments around the world to save money and reduce the restrictions and fear.

Conflict of interests

The authors declare that there is no conflict of interest in the study.

Financial Disclosure

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Ethical approval

Ethics committee approval is not required.

Contributions

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