Using gel-electrophoresis and traditional PCR in RT-PCR assays for the detection of SARS-CoV-2 in resource limited areas

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Abstract

Background. qRT-PCR investigations on patient specimen seem to be the only probable laboratory technology to validate the current individual SARS-CoV-2 status correctly. This kind of nucleic acid amplification technology is available in most countries, but missing in resource limited areas with poor laboratory infrastructure. The most feasible way to build up PCR test capacities for SARS-CoV-2 in these areas is the use of conventional PCR. PCR-thermocycler and gel-electrophoresis systems could be shipped easily by parcel service to the whole world, because they are almost maintenance free and need no engineers for their installation and service.

Methods. In order to reduce the complexity of sensitive reagent kits we have developed a freeze dried test kit for the traditional RT-PCR for the detection of SARS-CoV-2 in single ready to use tubes. Transport and storage of the reagents are now free of any cooling chain.

Results. We have analysed 244 patient samples and compared the results to the SEE-GENE Allplex™ 2019-nCoV assay. The sensitivity and specificity were 95.5% and 98.5%, respectively. Next to these performance parameters we could implement a workflow for processing 96 patient samples in parallel, handled by only 1 laboratory technician in an 8 hours working day.

Conclusion. The use of conventional PCR in combination with gel-electrophoresis for test analysis is a feasible approach to make SARS-CoV-2 diagnostic available in the whole world. This is needed urgently, because SARS-CoV-2 is a question of global health and not only of the well-equipped countries.

Introduction

In December 2019 a considerable number of pneumonia with severe course of the disease was observed in the Chinese region of Wuhan. A single stranded (+) RNA (ribonucleic acid) coronavirus of the genus Betacoronavirus was identified causing this lung diseases [1]. Since 11th February 2020 this respiratory disease was named into Covid-19 (coronavirus disease 2019) by the WHO (World Health Organization). The virus itself was named into SARS-CoV-2 (severe acute respiratory syndrome coronavirus 2) [2]. Meanwhile Covid-19 was observed in all parts of the world [1]. On 12th March 2020 the WHO classified Covid-19 as a pandemic disease [3]. Currently more than 46 million cases of Covid-19 were reported in 219 regions worldwide, 1 04 028 people died due to or with an SARS-CoV-2 infection (<https://www.who.int/emergencies/diseases/novel-coronavirus-2019>; Database request: 2020-11-04, 09:46 a.m.).

To detect an acute infection with SARS-CoV-2 a RT-PCR (Reverse Transcription - Polymerase Chain Reaction) investigation of patient specimens is needed next to the evaluation of further clinical parameters.

Typically naso- or oropharyngeal swabs are used as source to perform a RT-PCR assay. The use of sputum, endotracheal aspirate or bronchoalveolar lavage is also possible, but not recommendable due to the high risk of aerosolization [4].

The worldwide most used NAATs (Nucleic Acid Amplification Technology) are qRT-PCR (quantitative RT-PCR) assays. About 381 commercialized assays are listed by FIND today (https://www.finddx.org/covid-19/pipeline/?avance=all&type=all&test_target=RNA&status=all&section=show-all&action=default; Database request: 2020-11-04, 09:48 a.m.). The qRT-PCR is a well-known and a powerful tool. Several diagnostic and biotechnology companies offer these NAAT tests

in combination with a very high level of laboratory automation for a maximum of “walk-away time” and high sample throughput. Especially the investment into these techniques and machines is very attractive for a large number of diagnostic laboratories around the world, to become more efficient and highly profitable units. The very high initial equipment costs and the need of service engineers for the installation, staff teaching and maintenance limit the availability of qRT-PCR techniques in the whole world and especially in resource restricted areas with poor laboratory infrastructure. Actually the availability of new qRT-PCR capacities is very problematic in these days of SARS-CoV-2 pandemic in resource limited areas, because of strict worldwide quarantine rules and travel restrictions for service engineers. It is not possible and economically questionable to send an engineer from Europe to a laboratory e.g. in sub-Saharan areas to install a qRT-PCR device. This trip which took 3-5 days in the past, now takes 4-5 weeks in the days of SARS-CoV-2 pandemic, because of quarantine and travel restrictions. Next to this logistic problems the costs for the diagnostic equipment did increase drastically. Thus the support for developing countries in fighting against SARS-CoV-2 is very complicated even if financial assistance is provided by the rich countries.

To reduce the tension in the availability of SARS-CoV-2 diagnostics in resource limited areas we developed a trial to use conventional PCR assays analysed by the means of gel-electrophoresis. Based on the primer sequences from Corman *et al.* [5] from University Hospital Charité in Berlin and Chu *et al.* [6] from Hongkong University we developed a freeze dried, 2-step, RT-PCR for the detection of SARS-CoV-2. The advantage of freeze dried, ready to use reagents are, that no cooling chain for transport and storage is needed. In case of PCR reagents “cooling chain” does mean permanent cooling (e.g. during shipment) at 4 to -20 °C for master mix reagents and molecular probes and -80 °C for the enzymes and molecular

probes. With our new ready to use tests, each individual number of patient samples could be run. A collection of a minimum of samples to use the individual kit size of third party supplier or the space of a 96 well plate is not necessary. Also there are no dead volumes of expensive reagents associated with the preparation of master mixes.

After setup of our test regimen, there are some other suggestions for SARS-CoV-2 RT-PCR diagnostics in resource limited areas like nested PCR [7], colorimetric LAMP (Loop-mediated isothermal amplification) assay [8] or special miniature PCR machines in combination with well-plate readers [9].

In this study we want to investigate the following things:

1. What is the total financial investment to build up a new SARS-CoV-2 diagnosis unit?
2. How many patient samples can be analysed by 1 lab technician in a normal 8 hours working day?
3. What is the diagnostically sensitivity and specificity of the test?
4. What are the total costs per single test including all consumables (RNA extraction, RT-PCR, gloves, plastics, etc.)?

Method

RNA extraction

The specimen collection was performed by naso- and/ or oropharyngeal swabs. Each swab was washed out in 1 ml 0.9% NaCl for about 5 to 10 seconds. Afterwards 350 µl of NaCl-specimen solution was transferred to the Nimbus (SEEGENE, Seoul, South Korea) RNA

extractor. RNA extraction was performed with STARMag 96 (SEEGENE) magnetic silica beads. The purified RNA was used in the routine-diagnostic for SARS-CoV-2 and subsequently stored at -80 °C.

qRT-PCR for routinely SARS-CoV-2 status determination

The purified RNA was set into the Allplex™ 2019-nCoV (SEEGENE) assay. qRT-PCR preparation was performed on the micro lab Nimbus (SEEGENE) PCR setup liquid handling system and the qRT-PCR was run on the CFX96™ (BioRad, Hercules, USA) qRT-PCR device with 45 repetitions.

Cytects SARS-CoV-2 RT-PCR

The Cytects SARS-CoV-2 RT-PCR is a lyophilized 2-step RT-PCR. Master mixes for cDNA synthesis and PCR are already prepared and freeze dried in 0.2 ml reaction tubes. The PCRs were performed as two independent assays, E N and ORF1b-nsp14. After cDNA synthesis and amplification the PCR products has to be analysed utilizing conventional DNA agarose gel-electrophoresis. As the most SARS-CoV-2 tests, the Cytects SARS-CoV-2 RT-PCR is a dual target test, performed as single plex batches, two PCR products could be seen on an agarose gel for a positive test result (figure 1).

Cytects SARS-CoV-2 RT-PCR cDNA synthesis

For cDNA synthesis random and oligo dT primer were used. We used our own reverse transcriptase supplied by Invigate (Jena, Germany). The cDNA tubes of the Cytects SARS-CoV-2 RT-PCR (Cytects, Münster, Germany) were rehydrated with 15 µl of PCR grade water. RNA samples of positive and negative patient samples from routinely SARS-CoV-2 testing were

thawed and 5 µl of purified RNA were set in into the cDNA synthesis. The cDNA synthesis was performed on Flex Cycler (Analytik Jena, Jena, Germany) PCR device.

E N and ORF1b-nsp14 assay

The primer sequences for this assay are in accordance to the publications of Corman *et al.* [5] and Chu *et al.* [6]. DNA Taq-Polymerase was provided from segenetic (Borken, Germany). Tubes for E N and ORF1b-nsp14 assays were rehydrated with 23 µl PCR grade water. Afterwards 2 µl of cDNA was added as template. The amplification was carried out on a T-Personal 48 (Analytik Jena) PCR-thermocycler with 45 repetitions.

Gel-electrophoresis

The DNA agarose gel-electrophoresis was performed on the E-CUBE (Cytecs) all in one gel-electrophoresis and documentation system. We used 3% metaphor agarose gels in TBE buffer system. Gels and reagents were used from E-CUBE Gel-Electrophoresis Kit (Cytecs). PCR products were separated for 30 minutes and analysed by comparing PCR product bands to a DNA size marker.

Results

The analysis of 244 RNA samples showed a correlation of 97.9 % between Cytecs SARS-CoV-2 RT-PCR and previously characterised RNA extracts from patient specimen with Allplex™ 2019-nCoV. From these 244 RNA samples, 46 were branded as SARS-CoV-2 positive and 198 as SARS-CoV-2 negative by routinely SARS-CoV-2 testing (Tab. 1). This is in accordance to the FIND evaluation protocol for molecular tests [10]. The Cytecs SARS-CoV-2 RT-PCR failed in 2 of 46 SARS-CoV-2 positive cases and in 3 of 198 SARS-CoV-2 negative cases. The calculated

sensitivity compared to Allplex™ 2019-nCoV is 95.5 % and the specificity is 98.5 %. Positive and negative predicted values are 0.93 and 0.99, respectively.

Table 1: Results of 244 comparative measurements (n=244) of SEEGENE Allplex™ 2019-nCoV and Cytects SARS-CoV-2 RT-PCR.

	SEEGENE positive	SEEGENE negative	TOTAL
Cytects positive	44	3	47
Cytects negative	2	195	197
TOTAL	46	198	244

The evaluation of an agarose gel, which is loaded with SARS-CoV-2 PCR products is easy to perform, because SARS-CoV-2 positive samples show clear bands for the E N and ORF1b-nsp14 assay (figure 1).

As described above the Cytects SARS-CoV-2 RT-PCR is a dual target test, performed in two single batches. For each patient sample two lanes on an agarose gel are needed. Positive samples like sample 5, 6, 8, 9 and 10 (figure 1) show the E N amplicon with a size of 110 base pairs in the left lane and the ORF1b-nsp14 amplicon with a size of 132 base pairs in the right lane.

Negative sample like samples 1-4, 7 and 11-18 (figure 1) show no fluorescence signal for this specific fragment size. Sometimes weak bands of primer dimers could be seen for a fragment size of 50 base pairs.

Figure 1: 18 patient samples could be loaded on a 40 slot 3% TBE-agarose gel. Patient samples 1-4, 7 and 11-18 are negative for SARS-CoV-2, because no bands could be seen in the lanes. Sample 5, 6, 8, 9 and 10 are positive for SARS-CoV-2. The bands for E N in the left lane and ORF1b-nsp14 in the right lane are clearly detectable.

All of these samples were handled without any automation and were performed in a process of 96 RNA extracts in 7.25 hours by only 1 technician.

Discussion

We demonstrate a simple and feasible technique to perform SARS-CoV-2 RT-PCR in a system using conventional RT-PCR and subsequent gel-electrophoresis.

There is a discrepancy between the results of the Cytects SARS-CoV-2 RT-PCR and the in advanced characterised RNA extracts of 5 from 244 patient samples. It was noticeable that the three false positive results were recognised in three consecutively samples. This indicates that there could have been a worse sample handling e.g. in PCR preparation, cross contamination while splitting or simple confusion, because of the high stress level in the daily routine.

Two samples were presumed to be false negative. The RNA extracts were collected and stored in the freezer due to a temporary reduced number of positive patient samples, which made a parallel sample measurement impossible. Both unclear samples were stored for longer time and had c_t values >29 and >34 during their characterization in routinely qRT-PCR. A degradation of RNA in these samples can be assumed. In summary, we are satisfied with these results, because the data was collected in the daily routine laboratory process during a pandemic with a maximum daily throughput of swabs and testing. The samples derived from patients and from obviously healthy persons, who were suspected to have had contact to SARS-CoV-2 positive people. Several positive samples had low c_t values, which indicates a low viral load, bad specimen collection or bad sample handling. However, people who are in the beginning or at the end of a SARS-CoV-2 infection have also a high level of false negative test results [11,12].

Next to the performance study of the Cytects SARS-CoV-2 RT-PCR in daily routine this investigation deals with the question, how many samples one lab technician can handle in parallel in an 8 hours working day? In contrast to the workflow, shown in figure 2, we started the procedure with cDNA synthesis and completed the handling of 96 samples in 7.25 hours, including the runtime for the PCR thermocyclers. Of course, the RNA extraction has to be considered in this workflow. The RNA extraction of 96 samples is calculated with 2 h of manual work. We suggest to use a silica membrane based system on a 96-well plate format. For buffer handling a 12-channel pipette should be used. The whole extraction can be done with about 40 steps of pipetting for 96 samples. Buffers will be removed by a vacuum pump.

Figure 2: Simplified timeline and workflow for the handling of 96 patient samples in parallel, processed by one lab technician in 8.25 hours.

During cDNA synthesis the lab technician prepares the PCR tubes and during PCR run the lab technician will have a coffee break, cleans up and prepares the documentation.

Most laboratories in the world are equipped with simple PCR machines and gel-electrophoresis systems. If not, the whole equipment is available for about 35.000 USD. We summarized the current list prices for the equipment in table 2. The high affordability makes it possible to install a lot of these laboratory units in the periphery – “point of care diagnostic”. The lower equipment costs for the devices is not the only key argument. Currently no company can send their engineers around the world, because of quarantine rules or even travel restrictions. The listed devices can be send by parcel service, because they are maintenance free and need no service engineer for installation - plug and play.

Table2: Overview about needed equipment to perform Cytects SARS-CoV-2 RT-PCR

	Units	Single price	Total
Vacuum pump	1	1,500.00 USD	1,500.00 USD
Vacuum regulator	1	150.00 USD	150.00 USD
Vacuum manifold (for plate)	1	550.00 USD	550.00 USD
Centrifuge	1	4,000.00 USD	4,000.00 USD
Heating block	1	2,500.00 USD	2,500.00 USD
12-chanel pipette	3	980.00 USD	2,940.00 USD
Pipette set	2	710.00 USD	1,420.00 USD
PCR-Thermocycler	3	3,500.00 USD	10,500.00 USD
E-CUBE gel-electrophoresis	2	5,600.00 USD	11,200.00 USD
TOTAL			<u>34,760.00 USD</u>

Next to available and affordable equipment, the test itself, including all needed reagents and consumables have to be affordable, too. We expect a price of around 17.00 USD (supplier fee not included) for one SARS-CoV-2 test, including all necessary consumables and reagents. Summarizing these results, it is possible to equip a new laboratory unit for routinely SARS-CoV-2 RT-PCR testing for around 35,000.00 USD and costs per test of around 17.00 USD. In single shift use one unit processes 96 samples. But what is about increasing these capacities? Of course, it is possible to increase the number of equipment and the laboratory staff. But this needs a larger investment into more machines and personnel without any possibility to reduce the costs for each test itself. A probable approach could be the sample pooling as suggested by Lohse *et al.* in April 2020 [13]. Pools of up to 30 samples did not show any significant decrease of the clinical sensitivity. In a further study Schmidt *et al.* showed that it is possible to pool up to 50 samples with their novel swab method [14]. The approach of Lohse *et al.* was validated by the German Bone Marrow Donation Agency (DKMS). They could implement a strategy of sample pooling for the daily routine [15]. In this study the smallest investigated pool size were sample pools of 8 specimen. If this strategy of sample pooling will be applied it is possible to increase the test capacity of such a small lab unit 8-fold. This will decrease the cost per test to 2.00 USD and wide testing for SARS-CoV-2 becomes realistic in poor areas.

Summary:

1. The total price for the necessary equipment is in the range of 35,000.00 USD. This includes three thermocyclers and two all-in-one gel electrophoresis and documentation systems as well as small devices like multichannel pipettes etc.

This equipment can be shipped by parcel service. The installation can be done by the

local lab technicians, traveling of service engineers is not needed anymore.

2. With this equipment one lab technician can run 96 patient samples in a normal 8 hours working day by analysing each patient sample individually.

We strongly recommend pooling of samples, which will increase the number of samples one lab technician can analyse up to 800 samples within 8 hours.

3. The sensitivity and specificity of the Cytects SARS-CoV-2 RT-PCR are 95.45 % and 98.46 % based on the investigation of 244 patient samples.
4. Based on the single testing, the test costs are around 17.00 USD. If always 8 samples would be pooled the costs per test could be reduced to around 2.00 USD per patient.

Conclusion

SARS-CoV-2 is a question of global health. This new scourge of humanity could only be defeated, if all people in the world have access to a high standard of diagnostic – RT-PCR.

Currently it is not possible to offer qRT-PCR capacities in levels, which are needed.

Therefore, it makes sense to enter an unorthodox way. Currently we cannot see any efforts of competitors to offer SARS-CoV-2 PCR diagnostic for remote areas at affordable prices.

Competing interests

The authors declare that they have no competing interests.

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