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Rapid detection of human coronavirus NL63 by isothermal reverse transcription recombinase polymerase amplification

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ABSTRACT

Background: Human coronaviruses are one of the leading causes for respiratory tract infections and for frequent primary care consultation. The human coronavirus NL63 (HCoV-NL63) is one representative of the seasonal coronaviruses and capable of infecting the upper and lower respiratory tract and causative agent for croup in children.

Objectives: For fast detection of HCoV-NL63, we developed an isothermal reverse transcription recombinase polymerase amplification (RT-RPA) assay.

Study design: The analytical sensitivities of the RT-RPA assay were identified for in vitro transcribed ribonucleic acid (RNA) and for genomic viral RNA from cell culture supernatant. Moreover, specificity was tested with nucleic acids from other human coronaviruses and a variety of clinically relevant respiratory viruses. Finally, a clinical nasopharyngeal swab sample with spiked genomic viral HCoV-NL63 RNA was analyzed.

Results: Our HCoV-NL63 RT-RPA assay is highly specific and has an analytical sensitivity of 13 RNA molecules/reaction for in vitro transcribed RNA. For genomic viral RNA from cell culture supernatant spiked into a clinical nasopharyngeal swab sample the assay's analytical sensitivity is 170 RNA molecules/reaction. The assay shows amplification of the lowest detectable target copy number after 8 minutes and 7 minutes, respectively.

Conclusions: We were able to design a sensitive and specific RT-RPA assay for the detection of HCoV-NL63. Additionally, the assay is characterized by short duration, isothermal amplification, and simple instrumentation.

1. Introduction

Respiratory viruses have an immense impact on our healthcare systems as they are a frequent cause for diseases in humans and for medical consultation in primary care [1–4]. Among respiratory viruses such as rhino- or influenza viruses, the different human coronaviruses (HCoV) correspond to a large group [5,6]. The seasonal representatives of the family of *Coronaviridae* and relevant to humans are: HCoV-229E, HCoV-NL63, HCoV-OC43 und HCoV-HKU1 [7]. They mainly cause upper respiratory tract infections with rather mild symptoms but are also capable of seriously infecting the lower respiratory tract, especially in immunocompromised people [1]. The large ribonucleic acid (RNA) genome of coronaviruses is prone to homologous and non-homologous recombination [8,9]. Recombination events together with point mutations enable the viruses to form new variants and allow species crossing. This has led to severe epidemics (SARS-CoV-1 und MERS-CoV) and even an ongoing pandemic (SARS-CoV-2) [7,10]. In 2004, the human coronavirus HCoV-NL63 was first identified in eight individuals suffering from upper or lower respiratory tract illness in the Netherlands [11]. Subsequent studies showed symptoms in children, senior citizen and immunocom-

Abbreviations: HCoV-NL63, human coronavirus NL63; RT-RPA, reverse transcription recombinase polymerase amplification; LAMP, loop-mediated isothermal amplification; N-gene, nucleocapsid gene; exo-IQ, internally quenched exo probe.

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promised people ranging from rhinorrhea, cough, fever to bronchiolitis [12–15]. Extra respiratory tract symptoms included digestive problems, otitis, and conjunctivitis [16]. Data show varying incidences of HCoV-NL63 infections in children and adults with respiratory tract symptoms between 0.5 – 3 % [17–19]. Infections with endemic coronaviruses are not subject to routine testing, resulting in underestimated numbers [20]. Moreover, mild symptoms caused by more pathogenic coronaviruses such as SARS-CoV-2 are similar to HCoV-NL63 symptoms, hence clinical differentiation is difficult. On the other hand, lower tract respiratory infection with HCoV-NL63 in younger children is associated with croup indicating the pathogenic potential of HCoV-NL63 [12]. To differentiate respiratory pathogens and to rapidly distinguish between the human coronaviruses fast and reliable assays are urgently required.

Currently, nucleic acid amplification techniques for the detection of HCoV-NL63 are either based on polymerase chain reaction (PCR) [20,21] or on loop-mediated isothermal amplification (LAMP) [22]. Both assays need specialized instrumentation and the time-to-diagnosis takes more than one hour [22,23]. Recombinase polymerase amplification (RPA) is a nucleic acid amplification technique that combines simple reaction conditions and short amplification times. In contrast to LAMP and similar to PCR, RPA requires only two primers and one probe for amplification of target RNA. Different studies show that RPA technology has a high analytical sensitivity and specificity which is in many cases comparable to PCR as benchmark method [24]. Due to isothermal amplification at 42 °C and enzymatic processes for all steps of nucleic acid amplification the RPA assays are completed within 10 – 20 min. By labeling the target regions with a fluorescence probe, amplification is made visible in real-time [24,25]. A reverse transcription (RT) step allows the amplification of RNA as shown for the SARS-CoV-2 RT-RPA assay [26]. RPA assays were successfully employed for the point-of-care-testing of Ebola virus [27], avian influenza A (H7N9) virus [28] and Dengue virus [29] as well as for the coronaviruses MERS-CoV, SARS-CoV-2 and bovine coronavirus [26,30,31].

Here we present a newly developed RT-RPA assay based on the rapid detection of the nucleocapsid gene (N-gene) of human coronavirus HCoV-NL63 RNA suitable for point-of-care-testing. Sensitive amplification is demonstrated for *in vitro* transcribed RNA as well as for the genomic viral RNA from cell culture supernatant spiked into nasopharyngeal swab samples. Furthermore, our assay is highly specific: It neither detected other human coronavirus RNA including SARS-CoV-2 nor DNA or RNA from 27 different respiratory viruses.

2. Material and methods

2.1. Primer and probes, RPA settings and *in silico* analyses

RPA primers and internally quenched exo probes (exo-IQ) [26,32] for the detection of HCoV-NL63 N-gene (GenBank RefSeq: NC_005831.2) were designed with PrimedRPA software [33] in accordance with the recommendations given in the TwistDx assay design manual [34]. All oligonucleotides were evaluated *in silico* regarding secondary structure formation (Mfold program from UNAFold Web Server) and dimer formation (Multiple Primer Analyzer, Thermo Fisher). Moreover, each sequence was aligned with NCBI blast tool to check specificity towards the selected target sequence. Additionally, the RPA amplicon within the reference sequence (RefSeq NC_005831.2) was aligned to 136 gene sequences (NCBI GenBank search and alignment) of the nucleocapsid protein of circulating HCoV-NL63 strains (Supplemental figure 1) to check for sequence variations.

PCR primers and probes were designed (Primer3 software) and synthesized accordingly. All HCoV-NL63 N-gene specific primers and probes (listed in Table 1) were provided by biomers.net (biomers.net GmbH). Low volume single tube RT-RPA, was performed in the ESE-Quant isothermal fluorescence reader (Qiagen) using the TwistAmp exo kit (TwistDx) at 42 °C as described elsewhere [26,32]. RT-PCR was performed with the LightCycler 480 II instrument (Roche Diagnostics) using one-Step RT-PCR Mix (New England Biolabs) according to NEB protocol [35].

Table 1
Oligonucleotide primers and probes sequences and modifications for HCoV-NL63 RT-RPA and RT- PCR assays. Post-synthetic modification of the RT-RPA exo-IQ probes and for RT-PCR according to [26] for real-time detection. BMN-Q535 as quencher, dT-FAM deoxythymidine nucleoside derivatized with 6-flourescein in exchange for thymidine, dSpacer as abasic site in exchange for adenine and C3-spacer as alkyl chain with three Carbon atoms.

Oligonucleotide	Sequence (5' → 3')
	RT-RPA
Forward primer	TCAGAATGGTGTTGATGCCAAAGGTTTT
Probe	CAGGCTGCGTTATTCTTTGATAGTGAGGT[-dT-FAM-][-dSpacer-]G[-BMN-Q535-]CACTGATGAAGTGGGTGA[-C3-spacer]
Reverse primer	ACAAGCATTTTGTAGGTGTAGGTAATCT
	RT-PCR
Forward primer	TGGAATGTTCAAGAGCGTTGGCGT
Probe	[6-FAM-]TGCGCAGG[-BMN-Q535-]GGGCAACGTGTTGATTTGC[-BMN-Q535]
Reverse primer	GCAACCCAAACAACACCATCAGAACG

2.2. Statistical methods

For fluorescence data analysis from RT-RPA, the first value detected after mixing is subtracted from itself and from all other values after mixing for background correction. Additionally, a fluorescence threshold needed to be set for defining a sample as positively or negatively detected. To do so, fluorescence values of eight no template control (NTC) runs were averaged, and the standard deviation was calculated. For each data point, the triple of the standard deviation was added to the average value and the highest value was set as threshold [26]. The threshold times were defined as the point of intersection between the sigmoidal regression curve from amplification, generated using five-parameter logistic regression of qpcR package with R language [36], and the calculated fluorescence threshold. For RT-PCR, C_t values were calculated with LightCycler 480 software (Roche Diagnostics). Probit analysis was used to estimate lower limit of detection (analytical sensitivity) for both amplification methods and was performed with Analyse-it software (Analyse-it Software, Ltd.) [37].

2.3. *In vitro* transcribed RNA

RNA was synthesized from plasmid DNA (Invitrogen) by *in vitro* transcription (HiScribe T7 High Yield RNA Synthesis Kit, NEB), followed by digestion of the DNA template (DNAse I, NEB), purification (RNA Clean-Up Kit, Norgen Biotek) and quantification of the RNA (RiboGreen assay, Invitrogen). Finally, a dilution series from 10^7 to 10^0 RNA copies/µl was produced.

2.4. Virus culture

Subconfluent LLC-MK2 cells (CCL-7™, ATCC American Type Culture Collection) were infected with HCoV-NL63, Amsterdam I isolate, (purchased from National Collection of Pathogenic Viruses operated by Public Health England, catalog number: 2008102v) with a multiplicity of infection of 0.01. Prior to infection, cells were grown in 175 cm^2 tissue culture flasks using Dulbecco's Modified Eagle's Medium (DMEM), supplemented with 4 mM Glutamine, 1 mM sodium pyruvate (everything purchased from Biowest) and 10 % fetal calf serum (c.c.pro) at 37 °C in a 5 % CO_2 atmosphere. Virus supernatant was harvested seven days post infection. Viral RNA from cell culture supernatant was purified (QIAamp Viral RNA Mini Kit, Qiagen), eluted in 50 µl PCR-grade water (Roth) and quantified by RT-PCR. A dilution series from 10^7 to 10^0 RNA copies/µl was produced.

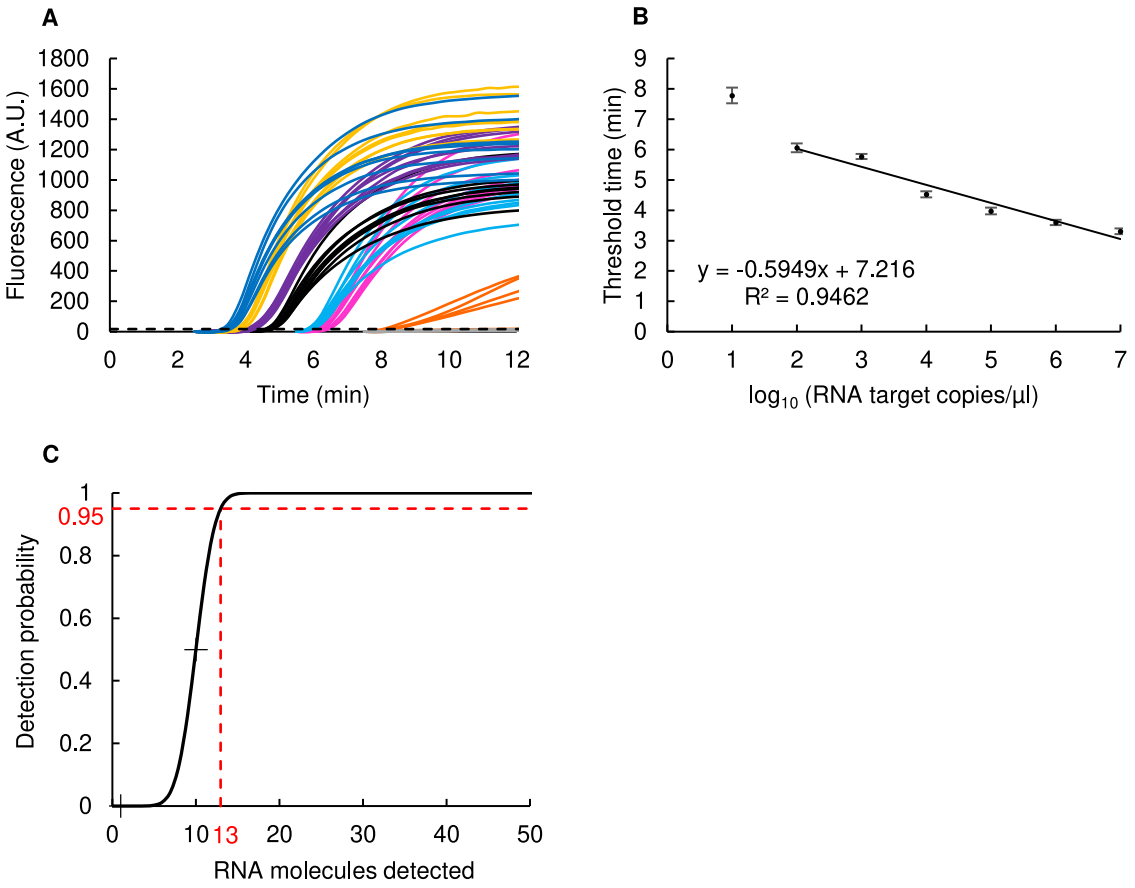

Fig. 1. Real-time RT-RPA sensitivity assay with synthetic HCoV-NL63 RNA. A) Fluorescence over time was measured in an RT-RPA running at 42 °C for 20 min. Each standard concentration containing synthetic HCoV-NL63 RNA (10^7 RNA molecules/reaction represented by dark blue line; 10^6, yellow; 10^5, purple; 10^4, black; 10^3, light blue; 10^2, magenta; 10^1, orange) as well as PCR-grade water as no template-control (NTC; grey line) was assessed in 8 replicates (n=8). Normalized data are shown, and threshold is indicated as dotted black line. B) Calibration line for the detection of HCoV-NL63 RNA in a real-time RT-RPA assay. Shown is the linear correlation of threshold times over the decadic logarithm of the RNA molecules/reaction. C) Probit regression for synthetic HCOV-NL63 RNA in PCR-grade water revealing a 95 % detection probability (analytical sensitivity) of 13 RNA copies/reaction (dotted red line).

2.5. Specificity testing

For specificity testing, viral RNA of the human coronaviruses HCoV-OC43, HCoV-229E, SARS-CoV, MERS-CoV and SARS-CoV-2 (European Virus Archive global) were analyzed in HCoV-NL63 RT-RPA. Furthermore, 27 nucleic acid samples from a variety of respiratory viruses (Friedrich Loeffler institute, Germany and Quality Control for Molecular Diagnostics (QCMD), UK) were also analyzed (list of used viral nucleic acids for specificity analysis see Supplemental table 1).

2.6. Clinical samples

50 nasopharyngeal swab samples, collected for the purpose of routine screening for SARS-CoV-2, were analyzed with HCoV-NL63 RT-RPA assay (Labor Staber, Kassel, Germany and Kliniklabor Neuruppin, Germany). Viral RNA was extracted with QIAamp Viral RNA Mini Kit (Qiagen) or QuickExtract DNA Extraction Solution (Lucigen). Quality of samples was assessed by detecting the human RNAse P with RT-PCR according to CDC protocol yielding C_t values between 25 to 34 [38,39].

3. Results

Analytical assay sensitivity (lower limit of detection) of the newly established HCoV-NL63 RT-RPA were assessed and compared to HCoV-NL63 RT-PCR using eight concentrations of the synthetic HCoV-NL63 RNA (10^7 – 10^0 RNA molecules/reaction; n=8) (Figure 1A). A 100 % detection rate was achieved in concentrations down to 10^2 RNA molecules/reaction for both RPA and PCR. For 10^1 RNA molecules/reaction a 50 % detection rate was observed (4/8 positive) for the RPA and for 10^0 RNA molecules/reaction no amplification could be detected (0/8 positive) (Figure 1A). A linear correlation of threshold times and amount of RNA was observed for 10^2 to 10^7 RNA target copies (Figure 1B) and a threshold time of 8 min for 10^2 RNA molecules. Using Probit regression, the analytical sensitivity was determined to be 13 RNA molecules/reaction (Figure 1C) for the RPA assay. This was in the same order of magnitude when compared to HCoV-NL63 RT-PCR which was 5 RNA molecules/reaction (Supplemental figure 2A and B). First detection of 10^1 synthetic RNA molecules/reaction occurred at a C_t-value of 33 which corresponds to 33 min according PCR run times (NEB protocol [35]; Supplemental figure 2A).

In a specificity experiment, it was assessed if this newly designed real-time RT-RPA detects HCoV-NL63 RNA only. Three respiratory virus DNAs and 29 different respiratory virus RNAs (Supplemental table 1) including RNA from SARS-CoV-1, SARS-CoV-2, MERS-CoV, HCoV-OC43 and HCoV-229E were used as template for the RT-RPA reaction. The RT-RPA assay neither detected any of the other human coronavirus RNAs nor the other respiratory virus DNAs or RNAs indicating that the assay is highly specific for HCoV-NL63 RNA (data not shown).

The newly designed HCoV-NL63 assay was then used to analyze 50 nasopharyngeal swab samples from patients originally tested for SARS-CoV-2 infection. RT-PCR revealed that all 50 samples were negative for

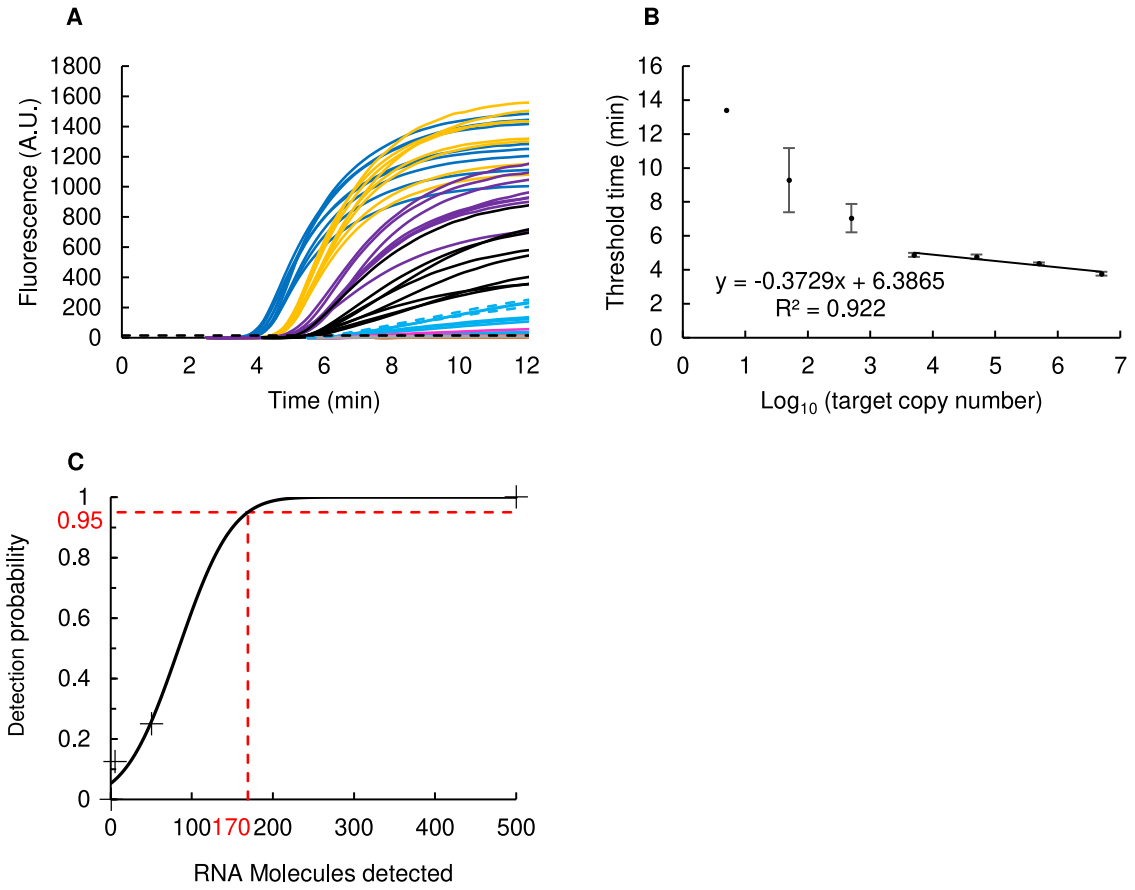

Fig. 2. Real-time RT-RPA sensitivity assay with genomic viral HCoV-NL63 RNA in human nasopharyngeal background. A) Fluorescence over time was measured in an RT-RPA running at 42 °C for 20 min. Each standard concentration containing viral HCoV-NL63 RNA from cell culture and human RNA from swab sample material (5×10^6 RNA molecules/reaction represented by dark blue line; 5×10^5, yellow; 5×10^4, purple; 5×10^3, black; 5×10^2, light blue; 5×10^1, magenta; 5×10^0, orange) as well as PCR-grade water as no template-control (NTC; grey line) was assessed in 8 replicates (n=8) and 2 replicates (n=2) containing 5×10^2 synthetic RNA molecules/reaction as positive control (dotted light blue line). Normalized data are shown, and threshold is indicated as dotted black line. B) Calibration line for the detection of viral HCoV-NL63 RNA in a real-time RT-RPA assay. Shown is the linear correlation of threshold times over the decadic logarithm of the RNA target concentration. C) Probit regression for viral HCoV-NL63 RNA from cell culture and human RNA from swab sample material revealing a 95 % detection probability (analytical sensitivity) of 170 RNA molecules/reaction.

HCoV-NL63. Similarly, HCoV-NL63 RT-RPA analysis of 48 swab samples showed no amplification at all, and in 2 samples fluorescence signals appeared marginally above calculated threshold but did not show exponential amplification and were thus interpreted as negative (data not shown).

In a next step, extracted RNA from one of the collected nasopharyngeal swab samples was spiked with genomic viral HCoV-NL63 RNA extracted from cell culture supernatant. Therefore, eight standard concentrations of viral HCoV-NL63 RNA (10^7 – 10^0 HCoV-NL63 RNA molecules/reaction) were mixed in the same ratio with extracted human RNA from the swab sample. Thus, concentrations used for RT-RPA measurement ranged from 5×10^6 – 5×10^0 HCoV-NL63 RNA molecules/reaction which were analyzed in eight replicate measurements. A 100 % detection rate could be shown for concentrations down to 5×10^2 RNA molecules/reaction, 25 % detection rate for 5×10^1 RNA molecules/reaction (2/8 positive) and 12.5 % detection rate for 5×10^0 RNA molecules/reaction (1/8 positive) (Figure 2A). A linear correlation of threshold time and RNA amount was observed in the range from 5×10^3 to 5×10^6 RNA molecules/reaction (Figure 2B). A threshold time of 7 min was observed for 5×10^2 RNA molecules (Figure 2B) and the analytical sensitivity for RT-RPA is at 170 RNA molecules/reaction (Figure 2C) determined by Probit regression. For the RT-PCR assay the analytical sensitivity is at 25 RNA molecules/reaction and first detection of 5×10^1 RNA molecules/reaction isolated from viral particles occurred at a C_t-value of 32 (corresponding to 32 min; Supplemental figure 2C and D).

4. Discussion

HCoV-NL63 is a human coronavirus which usually causes mild infections in humans. However, it is also able to cause more severe disease, e.g. in younger children where it is associated with croup [12]. Moreover, symptoms caused by HCoV-NL63 are often not very specific and similar to symptoms caused by other respiratory pathogens. HCoV-NL63 in clinical samples is usually detected by real-time RT-PCR, which requires sophisticated instrumentation in a laboratory environment. In this work a reliable and simple isothermal amplification method for the detection of human coronavirus NL63 was developed. The analytical sensitivity was determined to be 13 RNA molecules/reaction using *in vitro* transcribed HCoV-NL63 N-gene derived synthetic RNA. This is comparable to values obtained with MERS-CoV and SARS-CoV-2 RT-RPA assays, being at 21 RNA molecules/reaction and 8 RNA copies/reaction, respectively [30,26]. The analytical sensitivity of our HCoV-NL63 RT-RPA assay for RNA isolated from nasopharyngeal swab samples and spiked with genomic viral RNA was determined to be 170 RNA molecules/reaction and was therefore somewhat less sensitive in

comparison to RT-PCR (25 RNA molecules/reaction). Nevertheless, this newly designed HCoV-NL63 RT-RPA assay should be sensitive enough for the detection of HCoV-NL63 in clinical samples since Van der Hoek *et al.* showed that samples from children with HCoV-NL63 infection had a median viral load of 2.1×10^6 copies/ml [12].

100 % specificity was shown by analyzing 32 nucleic acid samples from a variety of respiratory viruses including the other coronaviruses in RT-RPA. HCoV-HKU1 was not included in the specificity panel due to its problematic cultivation performance in cell culture [40]. When performing a Basic Local Alignment Search Tool (BLAST) alignment between HCoV-NL63 (RefSeq: NC_005831.2) and HCoV-HKU1 (RefSeq: NC_006577.2) N-genes, no relevant sequence homology was shown.

In terms of assay run times, the RT-RPA shows fast amplification with the first positive signal at 8 min for 10^2 synthetic RNA molecules/reaction and at 7 min for 5×10^2 genomic viral RNA molecules/reaction. Therefore, the RT-RPA is considerably faster than the RT-PCR which requires approximately 30 min for detection of the first positive signal for both, 10^1 synthetic RNA molecules/reaction and 5×10^1 genomic viral RNA molecules/reaction.

Moreover, the combination of the RT-RPA with a simple and quick RNA extraction protocol is essential and was shown in this study to function with both, QIAamp (Qiagen) or QuickExtract (Lucigen) extraction methods. While the QIAmp protocol still requires a laboratory setting, the QuickExtract protocol is a suitable method for quick nucleic acid extraction at the point-of-care. In combination with such a simple nucleic acid extraction method, the RT-RPA could represent a rapid diagnostics alternative compared to other methods such as PCR or LAMP [22,24].

A limitation of this study is, however, that no clinical samples positive for HCoV-NL63 were analyzed. This is due to a reduced transmission of respiratory viruses, including the seasonal coronaviruses, within the last two years during Covid-19 pandemic [41,42]. This, together with a lack of routine testing for HCoV-NL63, made acquisition of positive clinical samples very difficult. Future studies should contain patient recruiting and parallel sample testing with this RT-RPA assay.

Due to the length of their RNA-genome, coronaviruses are prone to the emergence of mutations. Therefore, we aligned the reference sequence which was used for primer design to 136 HCoV-NL63 N-gene sequences found in NCBI GenBank. We detected two point mutations which might affect our HCoV-NL63 RPA, one located in the probe (45 of 136 sequences), and the other in the reverse primer (47 of 136 sequences, for details refer to supplemental figure 1). Interestingly, these two mutations are mutually exclusive, i.e. they cannot be found together in one HCoV-NL63 isolate. Since different studies have shown that RPA tolerates a small number of mismatches within the primers and probes [43–45] we do not expect a major impact on amplification performance if one of these two mismatches is present. For future studies, we plan to test if mixtures of degenerated reverse primers and probes can improve the detection of different HCoV-NL63 strains. Similar approaches were already used successfully for virus detection by RPA [46,47].

Altogether, our work demonstrates the successful use of RPA technology for the rapid detection of HCoV-NL63 with high analytical sensitivity and specificity. In the future, we plan to implement our RPA-based HCoV-NL63 assay in microfluidic devices similar to the already established assays for the detection of *Clostridioides difficile* [48], multidrug resistance Gram negative (MRGN) *Klebsiella pneumoniae* [49] or avian influenza virus [28,50]. The combination of RPA and microfluidics simplifies pathogen detection to a level where centralized laboratories and trained personnel are no longer required and thus allows the development of rapid and simple but yet sensitive and specific methods for the point-of-care-testing of pathogens.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

CRediT authorship contribution statement

Aline Dorendorf: Formal analysis, Investigation, Visualization, Writing – original draft. **Iris Bachmann:** Conceptualization, Validation, Supervision, Writing – review & editing, Resources. **Martin Spiegel:** Conceptualization, Software, Validation, Writing – review & editing. **Ahmed Abd El Wahed:** Validation, Resources. **Gregory Dame:** Conceptualization, Funding acquisition, Supervision, Writing – review & editing. **Frank Hufert:** Project administration, Supervision, Writing – review & editing, Resources.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.jcvp.2022.100115.

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