Since January 2020 Elsevier has created a COVID-19 resource centre with free information in English and Mandarin on the novel coronavirus COVID-19. The COVID-19 resource centre is hosted on Elsevier Connect, the company's public news and information website.

Elsevier hereby grants permission to make all its COVID-19-related research that is available on the COVID-19 resource centre - including this research content - immediately available in PubMed Central and other publicly funded repositories, such as the WHO COVID database with rights for unrestricted research re-use and analyses in any form or by any means with acknowledgement of the original source. These permissions are granted for free by Elsevier for as long as the COVID-19 resource centre remains active.
Keywords: human coronavirus NL63 recombinase polymerase amplification

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 cough 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 rhinovirus 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 and 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 and 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.

* Corresponding author.

E-mail address: Gregory.dame@mhb-fontane.de (G. Dame).

1 These authors contributed equally to this work.

https://doi.org/10.1016/j.jcvp.2022.100115

Received 4 August 2022; Received in revised form 5 October 2022; Accepted 7 October 2022.

2667-0380/© 2022 The Authors. Published by Elsevier Ltd. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/)
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 crup 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 assembles the amplification of RNA as shown for the SARS-CoV-2 RT-RPA assay [26]. RT-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 NPB protocol [35].

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, Ct 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 10⁷ to 10⁹ 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 cm² 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 % CO₂ 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 10⁷ to 10⁹ RNA copies/μl was produced.

Table 1

| Oligonucleotide | Sequence (5’ → 3’) |
|-----------------|--------------------|
| **Forward primer** | RT-RPA |
| Probe           | TCAGAAATGTTGTTAGTGGACAGTTTT |
|                 | CAGCGGGGTTTTTACATTTATCTAC |
| **Reverse primer** | RT-PCR |
| Probe           | TGGAAATGTCAAGACGGTTGCGG |
|                 | [6-FAM-TGCGCAGG-]BMN-Q535 |

The table lists the Oligonucleotide primers and probes sequences 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 nucleotide derivatized with 6-fluorescein in exchange for thymidine, dSpacer as abasic site in exchange for adenine and C3-spacer as alkyl chain with three Carbon atoms.
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 Cₜ 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 (10⁷ - 10⁹ RNA molecules/reaction; n=8) (Figure 1A). A 100 % detection rate was achieved in concentrations down to 10² RNA molecules/reaction for both RPA and PCR. For 10¹ RNA molecules/reaction a 50 % detection rate was observed (4/8 positive) for the RPA and for 10⁰ 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 10² to 10⁷ RNA target copies (Figure 1B) and a threshold time of 8 min for 10² 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 10⁴ synthetic RNA molecules/reaction occurred at a Cₜ 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
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 (10⁷ – 10⁵ 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 × 10⁶ – 5 × 10⁰ HCoV-NL63 RNA molecules/reaction which were analyzed in eight replicate measurements. A 100 % detection rate could be shown for concentrations down to 5 × 10² RNA molecules/reaction, 25 % detection rate for 5 × 10¹ RNA molecules/reaction (2/8 positive) and 12.5 % detection rate for 5 × 10⁰ RNA molecules/reaction (1/8 positive) (Figure 2A). A linear correlation of threshold time and RNA amount was observed in the range from 5 × 10³ to 5 × 10⁷ RNA molecules/reaction (Figure 2B). A threshold time of 7 min was observed for 5 × 10² 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 × 10¹ RNA molecules/reaction isolated from viral particles occurred at a C₅₀-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-PCR 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-PA. 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-genomes, no relevant sequence homology was shown.

In terms of assay run times, the RT-PA 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-PA is considerably faster than the RT-PCR which requires approximately 30 min for detection of the first positive signal for both, 10^3 synthetic RNA molecules/reaction and 5 × 10^3 genomic viral RNA molecules/reaction.

Moreover, the combination of the RT-PA 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 QIAamp 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-PA 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-PA 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-genome sequences found in NCBI GenBank. We detected two point mutations which might affect our HCoV-NL63 RTA, 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 RTA 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 RTA [46, 47].

Altogether, our work demonstrates the successful use of RTA technology for the rapid detection of HCoV-NL63 with high analytical sensitivity and specificity. In the future, we plan to implement our RTA-based HCoV-NL63 assay in microfluidic devices similar to the already established assays for the detection of Clostridoides difficile [48], multidrug resistance Gram negative (MRGN) Klebsiella pneumoniae [49] or avian influenza virus [28, 50]. The combination of RTA 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.

Funding

The authors would like to thank the German Federal Ministry of Education and Research (BMBF; Grant No. FKZ 01ZZ2051B, R&c.net) for funding this work.

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 Dane: Conceptualization, Funding acquisition, Supervision, Writing – review & editing. Frank Hufert: Project administration, Supervision, Writing – review & editing, Resources.

Acknowledgment

We would like to acknowledge the European Virus Archive Global for provision of the coronavirus specificity panel. Furthermore, we would like to thank Labor Staber, Kassel, Germany and Kliniklabor Neuruppin, Germany for the material from human nasopharyngeal swab samples for specificity analysis.

Supplementary materials

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

References

[1] Anthony R. Fehr, Stanley Perlman. Coronaviruses: an overview of their replication and pathogenesis, SpringerLink [Internet]. 2015 [cited 2020 Oct 18]. Available from: https://link.springer.com/protocol/10.1007/978-1-4939-2438-7_1
[2] P Kardos, FA. Malek, Common cold – an umbrella term for acute infections of nose, throat, larynx and bronchi, Pneumol. Stuttg. Ger. 71 (2017) 221–226.
[3] X Sánchez Choos, M Loaiza Martínez, V Vaca Tatamaz, M López Peña, A Manzano Pasquel, J Simón Sotomayor, Medical cost of upper respiratory tract infections in children in ambulatory care, Value Health Reg. Issues 26 (2021) 1–9.
[4] S Weston, MB. Friedman, Respiratory viruses, Environ. Microbiol. (2019) 85–101.
[5] T Heikkinen, A. Järvinen, The common cold, Lancet Lond. Engl. 361 (2003) 51–59.
[6] JW Tang, TT Lam, H Zaraket, WI Lipkin, SJ Drews, TF Hatchette, et al., Global epidemiology of non-influenza RNA respiratory viruses: data gaps and a growing need for surveillance, Lancet Infect. Dis. 17 (2017) e525–e526.
[7] F Hufert, M. Spiegel, Coronaviruses: von der banalen erkältung zum schweren lungenversagen: chronologie einer pandemie, Monatschr. Kinderheilk. (2020) 488–501.
[8] JG Keck, S Makino, LH Soe, JO Fleming, SA Stohlman, MMC. Lai, RNA recombination of coronavirus, in: MMC Lai, SA Stohlman (Eds.), Coronaviruses [Internet], Springer US, Boston, MA, 1987, pp. 99–107, doi:10.1007/978-1-4684-1280-2_11, [cited 2021 Jan 8]Available from:
[9] MM Lai, BS Baric, S Makino, JG Keck, J Egbert, JL Leibowitz, et al., Recombination between nonsegmented RNA genomes of murine coronaviruses, J. Virol. 56 (1985) 449–456.
[10] J Cui, F Li, Z.L. Shi, Origin and evolution of pathogenic coronaviruses, Nat. Rev. Microbiol. 17 (2019) 181–192.
[11] L van der Hoek, K Pyrc, MF Jebbink, W Vermeulen-Oost, RMJ Berkhourt, KC Wolthers, et al., Identification of a new human coronavirus, Nat. Med. 10 (2004) 368–373.
[12] Hoek L van der, Sure K, G Ithorst, A Stang, K Pyrc, MF Jebbink, et al., Group is associated with the novel coronavirus NL63, PLOS Med. Public Lib. Sci. 2 (2005) e240.
[13] SS Chiu, K Hung Chan, K Wing Chu, SW Kwan, Y Guan, LL Man Poon, et al., Human coronavirus NL63 infection and other coronavirus infections in children hospitalized with acute respiratory disease in Hong Kong, China, Clin. Infect. Dis. 40 (2005) 1721–1729.
[14] N Bastien, K Anderson, L Hart, PV Gaseeke, K Brandt, D Milley, et al., Human coronavirus NL63 infection in Canada, J. Infect. Dis. 191 (2005) 503–506.
[15] T Ehshara, R Endo, X Ma, N Ishiguro, H Kikuta, Detection of human coronavirus NL63 in young children with bronchiolitis, J. Med. Virol. 75 (2005) 463–465.
[16] A Valeret, T Mourrez, J Dina, I van der Hoek, S Gouarin, J Petitjean, et al., Human coronavirus NL63, France, Emerg. Infect. Dis. 11 (2005) 1225–1229.
[17] Z-Q Zeng, D-H Chen, W-P Tan, S-Y Qiu, D Xu, H-X Liang, et al., Epidemiology and clinical characteristics of human coronaviruses OC43, 229E, NL63, and HKU1: a study of hospitalized children with acute respiratory tract infection in Guangzhou, China. Eur. J. Clin. Microbiol. Infect. Dis. 37 (2018) 363–369.

[18] I Heimdal, N Moe, S Krostad, A Christensen, LH Skanke, SA Nordhe, et al., Human coronavirus in hospitalized children with respiratory tract infections: a 9-year population-based study from Norway. J. Infect. Dis. Oxford Acad. 219 (2019) 1198–1206.

[19] K Akagi, T Kudo, R Miyashita, A Kondo, N Ehara, T Takazono, et al., Clinical evaluation of conventional human coronavirus infection in adults, Jpn. J. Infect. Dis. 75 (2022) 121–126.

[20] LJR van Elden, Anton M. AM, F van Alphen, KAW Hendriksen, AIM Hoepelman, M2L van Kraaij, et al., Frequent detection of human coronaviruses in clinical specimens from patients with respiratory tract infection by use of a novel real-time reverse-transcriptase polymerase chain reaction, J. Infect. Dis. 189 (2004) 652–657.

[21] S Abdul-Rasool, BC, Fielding, Understanding human coronavirus HCov-NL63, Open Virol. J. 4 (2010) 76–84.

[22] K Pyrc, A Milewska, J Potempa, Development of loop-mediated isothermal amplification assay for detection of human coronavirus-NL63, J. Virol. Methods 175 (2011) 133–136.

[23] DX Liu, QJ Liang, TS, Fung, Human Coronavirus-229E, -OC43, -NL63, and -HKU1 (Coronaviridae), Eucycl. Virol. (2021) 428–440.

[24] J Li, J Macdonald, F. von Stetten, Review: a comprehensive summary of a decade development of the recombinase polymerase amplification, Anal. R. Soc. Chem. 145 (2020) 1590–1960.

[25] O Piiponen, CH Williams, DL Stemple, NA, Armes, DNA detection using recombinase proteins, PLoS Biol. Public Lib. Sci. 4 (2006) e204.

[26] O Behrmann, I Bachmann, M Spiegel, M Schramm, A Abd El Wahed, G Dobler, et al., Rapid detection of SARS-CoV-2 by low volume real-time single tube reverse transcription recombinase polymerase amplification using an Eko probe with an internally linked quencher (Eko-IQ), Clin. Chem. 66 (2020) 1047–1054.

[27] M Yang, Y Ke, X Wang, H Ren, W Liu, H Lu, et al., Development and evaluation of a rapid and sensitive EBOV-RPA test for rapid diagnosis of Ebola virus disease. Sci Rep. Nat. Publ. Group 6 (2016) 26943.

[28] A Abd El Wahed, M Weidmann, FT Hufert, Diagnostics-in-a-suitecase: development of a portable and rapid assay for the detection of the emerging avian influenza A (H7N9) virus, J. Clin. Virol. 69 (2015) 16–21.

[29] AAE Wahed, P Patel, O Faye, S Thaolengsok, D Heidenreich, P Matangkasombut, et al., Recombinase polymerase amplification assay for rapid diagnostics of dengue infection, PLoS ONE. Public Lib. Sci. 10 (2015) e0129682.

[30] A Abd El Wahed, P Patel, D Heidenreich, FT Hufert, M Weidmann, Reverse transcription recombinase polymerase amplification assay for the detection of middle east respiratory syndrome coronavirus, PLoS Curr. [Internet] (2013) 5 [cited 2020 Oct 18]Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3871419/.

[31] HM Amer, A Abd El Wahed, MA Shalaby, FN Almajhdi, FT Hufert, M Weidmann, A new approach for diagnosis of bovine coronavirus using a reverse transcription recombinase polymerase amplification assay, J. Virol. Methods 193 (2013) 337–340.

[32] L Lillis, J Siverson, A Lee, J Canteria, M Parker, O Piiponen, et al., Factors influencing recombinase polymerase amplification (RPA) assay outcomes at point of care, Mol. Cell. Probes 30 (2016) 74–78.

[33] M Higgin, M Ravenhall, D Ward, J Phelan, A Ibrahim, MS Forrest, J Hancock, et al., PrimeredRPA: primer design for recombinase polymerase amplification assays, Bioinformatics 35 (2019) 682–684.

[34] TwistDx LimitedTwist Amp® DNA Amplification Kits - Assay Design Manual [Internet], 2019 Available from: www.twistdx.co.uk.

[35] Luna® Universal Probe One-Step RT-qPCR Kit Protocol (E3006) | NEB [Internet], 2022 [citedMay 10]Available from: https://www.neb.com/protocols/2016/11/11/luna-universal-probe-one-step-rt-qpcr-kit-protocol-e3006.

[36] C Ritz, A.-N. Spiess, gpcR: an R package for sigmoidal model selection in quantitative real-time polymerase chain reaction analysis, Bioinf. Ox. Engl. 24 (2008) 1549–1551.

[37] COVID-19: calculating the detection limit for a SARS-CoV-2 RT-PCR assay using analyse-it | blog | analyse-it® [Internet], [cited 2022 Apr 28], Available from: https://analyse-it.com/blog/2020/5/covid-19-calculating-the-detection-limit-for-a-sars-cov-2-rt-PCR-assay-using-analyse-it.

[38] AG Rowan, P May, A Badhan, C Herrera, P Watber, R Penn, et al., Optimized protocol for a quantitative SARS-CoV-2 duplex RT-qPCR assay with internal human sample sufficiency control, J. Virol. Methods 294 (2021) 114174.

[39] CDC. Labs [Internet]. Cent. Dis. Control Prev. 2020 [cited 2022 May 29], Available from: https://www.cdc.gov/coronavirus/2019-ncov/lab/rt-pcr-panel-primer-probes.html

[40] K Pyrc, AC Sims, R Dijkman, M Jubbink, C Long, D Deming, et al., Culturing the unculturable: human coronavirus HKU1 infects, replicates, and produces progeny virions in human ciliated airway epithelial cell cultures, J. Virol. Am. Soc. Microbiol. 84 (2010) 11255–11263.

[41] HE Groves, P-P Piché-Renaud, A Peci, DS Farrar, S Buckrell, C Bancej, et al., The impact of the COVID-19 pandemic on influenza, respiratory syncytial virus, and other seasonal respiratory virus circulation in Canada: A population-based study, Lancet Reg. Health - Am. 1 (2021) 100015.

[42] SJ Olsen, AK Wiin, AP Budd, MM Prill, J Steel, CM Midgley, et al., Changes in influenza and other respiratory virus activity during the COVID-19 pandemic — United States, 2020–2021, Morb. Mortal. Wkly. Rep. 70 (2021) 1013–1019.

[43] A Abd El Wahed, A El-Drehy, M El Tholoth, H Abd El Kader, A Ahmed, S Hassan, et al., A portable reverse transcription recombinase polymerase amplification assay for rapid detection of foot-and-mouth disease virus, PLoS ONE 8 (2013) e71642.

[44] RK Daher, G Stewart, M Boissinet, DK Boudreau, MG, Bergeron, Influence of sequence mismatches on the specificity of recombinase polymerase amplification technology, Mol. Cell. Probes 29 (2015) 116–121.

[45] X Liu, Q Yan, J Huang, J Chen, Z Guo, Z Liu, et al., Influence of design probe and sequence mismatches on the efficiency of fluorescent RPA, World J. Microbiol. Biotechnol. 35 (2019) 95.

[46] EK Rames, J. Macdonald, Rapid assessment of viral water quality using a novel recombinase polymerase amplification test for human adenovirus, Appl. Microbiol. Biotechnol. (2019) 11.

[47] J Coertse, J Weyer, LH Nel, W Markotter, Reverse transcription recombinase polymerase amplification assay for rapid detection of canine associated rabies virus in Africa, PLOS ONE. Public Lib. Sci. 14 (2019) e0219292.

[48] M-N Tsalougou, J. Watson, R. M, Rushworth C, Y Zhao, X Niu, M S Sutton, J, et al., Real-time microfluidic recombinase polymerase amplification for the toxin B gene of Clostridium difficile on a SlipChip platform, Anal. R. Soc. Chem. 140 (2015) 258–264.

[49] O Behrmann, M Hügle, F Eckardt, I Bachmann, C Heller, M Schramm, et al., 3D printed monolithic microreactors for real-time detection of klebsiella pneumoniae and the resistance gene blaNDM-1 by recombinase polymerase amplification, Micro-machines 11 (2020) 395.

[50] N Yehia, A-S Araf, A Abd El Wahed, AA El-Sonoui, M Weidmann, MA Shalaby, Development of reverse transcription recombinase polymerase amplification assay for avian influenza H5N1 HA gene detection, J. Virol. Methods 223 (2015) 45–49.