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Isothermal amplification using sequence-specific fluorescence detection of SARS coronavirus 2 and variants in nasal swabs

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ABSTRACT

Coronavirus disease 2019 is a public health challenge requiring rapid testing for the detection of infections and transmission. Nucleic acid amplification tests targeting SARS coronavirus 2 (CoV2) are used to detect CoV2 in clinical samples. Real-time reverse transcription quantitative PCR is the standard nucleic acid amplification test for CoV2, although reverse transcription loop-mediated isothermal amplification is used in diagnostics. The authors demonstrate a sequence-specific reverse transcription loop-mediated isothermal amplification-based nucleic acid amplification assay that is finished within 30 min using minimally processed clinical nasal swab samples and describe a fluorescence-quenched reverse transcription loop-mediated isothermal amplification assay using labeled primers and a quencher oligonucleotide. This assay can achieve rapid (30 min) and sensitive (1000 plaque-forming units/ml) fluorescence detection of CoV2 (WA1/2020), B.1.1.7 (Alpha) and variants of concern Delta (B.1.617.2) and Omicron (B.1.1.529) in nasal samples.

METHOD SUMMARY

The authors describe a sequence-specific nucleic acid amplification assay (fluorescence-quenched reverse transcription loop-mediated isothermal amplification) based on a modified reverse transcription loop-mediated isothermal amplification assay that utilizes a fluorescence-labeled reporter primer and a short complementary oligonucleotide quencher to detect SARS coronavirus 2 in minimally processed clinical nasal swab samples. The fluorescence-quenched reverse transcription loop-mediated isothermal amplification assay is completed in 30 min without purifying RNA and achieves reproducible, sensitive and specific (1000 plaque-forming units/ml) detection of SARS coronavirus 2 WA1/2020 and three SARS coronavirus 2 variant viruses while not signaling on three closely related human coronaviruses or two other heterologous human respiratory viruses.

KEYWORDS:

diagnostics ● fluorescence ● FQ-LAMP ● POC ● RT-LAMP ● SARS coronavirus 2 ● variant

The coronavirus disease 2019 pandemic has affirmed the importance of rapid, sensitive and accurate diagnostics for aiding patient health and the control of SARS coronavirus 2 (CoV2) outbreaks [1]. The value of *in vitro* diagnostic tests using patient samples (e.g., nasopharyngeal swabs or saliva) is exemplified by the >250 *in vitro* diagnostic tests the US FDA has approved or cleared (www.fda.gov/medical-devices/tests-used-clinical-care/find-all-fda-approved-home-and-lab-tests). Nucleic acid amplification tests (NAATs) are used for screening and pathogen diagnosis in respiratory samples, often involving real-time reverse transcription quantitative PCR (RT-qPCR) assays and versions of isothermal amplification assays [2,3]. Most NAATs for CoV2 detection target the viral nucleocapsid (N) gene or the N gene in combination with the spike or RdRP gene or the envelope gene. Assay sensitivity and specificity vary among the gene targets and are affected by the use of a single gene or multiple gene specificities [4]. CoV2 NAATs targeting the N gene show higher sensitivity in RT-qPCR and isothermal amplification compared with the RdRP gene, likely because the N gene is the most abundant subgenomic CoV2 RNA [5]. Many other factors can impact NAAT sensitivity for a specific target gene, including specific assay employed, sample source, severity of symptoms at time of sampling and potential therapies used.

NAAT results depend on several properties, chief among which is the fact that multiple gene targets provide better sensitivity and specificity than single gene-targeted tests. NAAT results may also vary according to the input sample type (i.e., nasal swabs or saliva), which likely reflects differential virus levels [6]. Lower sensitivity is associated with tests assaying upper respiratory samples alone compared with tests evaluating both upper and lower respiratory samples. Gene mutations associated with the emergence of CoV2 variant viruses can also affect sensitivity and specificity, especially those targeting the CoV2 spike gene [7]. RT-qPCR is the standard NAAT for CoV2 detection, as it provides high sensitivity and specificity attributed to the selection of target-specific amplicons via temperature cycling (melt, anneal/extend) and sequence-specific fluorescence-labeled oligonucleotide (oligo) probe detection [8]. Classical RT-qPCR

assay is amenable to only highly purified target RNA, as the input and assay are susceptible to inhibitors in the sample. DNA amplification and fluorescent signal readout associated with RT-qPCR allow for accurate quantitation of the amount of starting nucleic acid in the assay. Although amenable to automation and high throughput, the process requires specially trained laboratory personnel and expensive laboratory equipment. Alternatively, reverse transcription loop-mediated isothermal amplification (RT-LAMP) is a NAAT based on isothermal amplification of the target nucleic acid. The process employs a strand-displacing DNA polymerase such as Bst 2.0 and four to six target-specific primers in a single reaction mix. Temperature cycling to achieve exponential target amplification is not required since the Bst 2.0 enzyme possesses both polymerases and strand displacement activities and is activated at the same temperature as primer annealing (typically 60–70°C) [9]. However, some specificity may be lost in isothermal DNA amplification since assembly of the active polymerase complex is a stochastically driven process that can sometimes give rise to exponential nonspecific target amplification.

A variety of end point detection means are used with RT-LAMP, including colorimetric analysis, fluorescence and turbidity, making the assay amenable to a range of sample inputs, from highly purified RNA to minimally processed (heat-inactivated) patient saliva, swabs, urine and blood. Importantly, RT-LAMP assay results are ready in 30 min (while eliminating the RNA purification step) compared with 2–3 h for a typical RT-qPCR assay. Simplicity, affordability, high analytical target sensitivity and specificity, fast time to result and sample input flexibility make RT-LAMP an attractive alternative to traditional RT-qPCR for CoV2 detection, allowing for point-of-care (POC) or home diagnostic testing designs.

The authors recently described the development of an RT-LAMP assay to detect CoV2 in minimally processed clinical samples [10]. The fluorescent CoV2 RT-LAMP assay used six primers specific for the CoV2 N gene and was based on the fluorescent signal from target DNA amplification over 30 min at 65°C in the presence of a SYBR-like fluorescent dsDNA binding dye. RT-LAMP was shown to be sensitive and specific in comparison with the N gene-specific CDC EUA N1 single-target RT-qPCR assay for detection of CoV2 in minimally processed clinical samples. The authors chose a fluorescence readout for the assay, as it was the least perturbed by the inherent clinical sample heterogeneity. Other isothermal NAAT readouts that rely on pH-sensitive, dye-based colorimetric or turbidity readouts are adversely affected if the nucleic acid target is not purified from the sample [11]. However, a notable limitation to the fluorescence readout is related to the nonspecific binding of SYBR-like dyes to dsDNA. In the case of nonspecific amplification from primer dimers or inappropriate priming of nontarget sequences, the nonspecific DNA amplification product will elicit a fluorescent signal from binding of the SYBR-like dye, giving terminal false-positive results or a rising assay baseline depending on the rate of nonspecific amplification and whether target-specific amplification competes for assay reactants. The analytical sensitivity and specificity of isothermal NAAT assays can be improved by eliminating the detection of any nonspecific amplification product. This strategy has been described in probe/quencher schemes, fluorescence resonance energy transfer and molecular beacons [12,13]; however, these techniques do not eliminate nonspecific amplification, and nonspecific amplification can still compete for reagents in the reaction and possibly limit specific signals in some cases.

In the present study, the authors describe the modification of our former fluorescent CoV2 N gene RT-LAMP assay [10] to eliminate the SYBR-like DNA binding dye to remove detection of nonspecific amplification to enhance the sensitivity and specificity of the assay. The modified assay, termed 'fluorescence-quenched reverse transcription loop-mediated isothermal amplification' (FQ-LAMP), employs a reporter and quencher strategy in the form of a fluorescence-labeled LoopB primer (FLB) and a quencher-labeled short reverse complement oligo (QLB) based on the LoopB primer sequence to provide a target sequence-specific fluorescence end point readout (Figure 1). At the end of the 30-min 65°C incubation time in a real-time thermocycler, the assay is cooled to room temperature and the fluorescent signal is read. If target-specific amplification has occurred, the FLB primer will be incorporated into the DNA product and demonstrate fluorescence upon excitation. Any unincorporated FLB primer is quenched by solution hybridization with the reverse complement QLB oligo and does not contribute to the fluorescent signal. Non-templated isothermal amplification, usually initiated by one of the internal LAMP primers (FIP or BIP), does not result in the incorporation of the FLB primer. Thus, background signals and false-positive signals remain low in this case. Additionally, unlike the former SYBR-like dye-based fluorescent LAMP assay [10], the FQ-LAMP end point fluorescent signal can be determined outside of the real-time thermocycler directly in the assay tube using a simple excitation source, such as a handheld UV lamp, without emission filter, and a standard mobile phone camera to capture an image that can be used for visual discrimination of positive or negative CoV2 assay results. This study highlights the potential for FQ-LAMP application in the POC or home testing space.

Methods

FQ-LAMP reactions

All FQ-LAMP reactions were performed using a commercial 2X RT-LAMP master mix product (New England Biolabs, MA, USA). The reactions were run in an AriaMx Real-Time PCR system (Agilent Technologies, CA, USA) for 30 min at 65°C and then actively cooled to 25°C for 30 s. Finally, end point fluorescence was measured in the FAM channel. FQ-LAMP reactions contained 12.5 µl warm Start LAMP Kit 2X Master Mix (New England Biolabs), 2.5 µl 10X FQ-LAMP oligo mix (Integrated DNA Technologies, IA, USA), 2 µl assay target (live virus or live virus-spiked nasal swab clinical specimen) and 8 µl deionized water for a total of 25 µl.

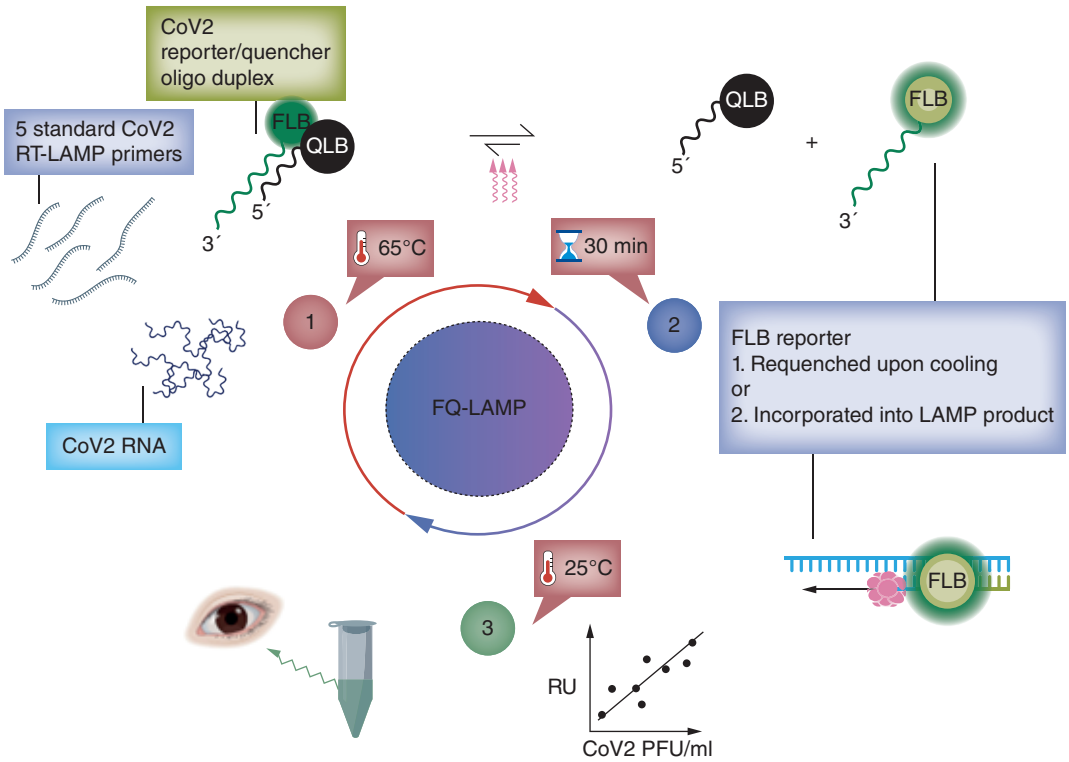

Figure 1. Fluorescence-quenched reverse transcription loop-mediated isothermal amplification for detection of SARS coronavirus 2. In addition to the conventional RT-LAMP reagents and five standard RT-LAMP primers (FIP, BIP, F3, B3 and LoopF), FQ-LAMP employs a 5′[6-FAM] fluorescence-labeled LoopB primer reporter (FLB) and a short complementary quencher oligo (QLB) modified at the 3′ end with IBHQ to achieve target sequence-specific detection of CoV2. Upon heating to 65°C, the reporter (FLB):quencher (QLB) oligo duplex is melted, allowing FLB to base pair with available complementary CoV2 target sequence, thereby initiating Bst 2.0-mediated isothermal amplification and incorporation of the FLB reporter into the exponentially expanding FQ-LAMP dsDNA product. After 30-min incubation at 65°C, the reaction is cooled to 25°C. FLB reporter pairs with QLB to regenerate the quenched oligo duplex, not contributing to the signal in CoV2-negative samples or in the presence of CoV2 target sequence. FLB reporter is incorporated into the RT-LAMP dsDNA product, with fluorescent signal magnitude directly proportional to the amount of CoV2 starting material. RFUs are recorded in the FAM channel using a real-time thermocycler or visualized with the unaided eye.
CoV2: SARS coronavirus 2; FQ-LAMP: Fluorescence-quenched reverse transcription loop-mediated isothermal amplification; oligo: Oligonucleotide; RFUs: Relative fluorescence units; RT-LAMP: Reverse transcription loop-mediated isothermal amplification.
Image created with BioRender.com.

FQ-LAMP assay targets & sample preparation solution protocol

The FQ-LAMP assay targets were either live CoV2 alone or CoV2-negative nasal swab clinical samples spiked with live virus. Samples were prepared by mixing a 20-μl aliquot of the liquid sample 1:1 (v:v) with sample prep solution (SPS), 500 mM guanidine HCl, 0.1% Triton X-100, 1 mM EDTA (Sigma-Aldrich, MO, USA) adjusted to a pH of 7.8 with 2 M Tris HCl (Sigma-Aldrich) and 80 U/ml Proteinase K (New England Biolabs) followed by incubation at 37°C for 15 min and incubation at 95°C for 10 min to inactivate the Proteinase K. The sample lysate (2 μl) was used as target directly in the FQ-LAMP reactions without further processing.

FQ-LAMP primers & signal oligos

FQ-LAMP primers and signal oligos (Supplementary Table 2) were used at the following final concentrations: FIP 0.1 μM, BIP 1.0 μM, F3/B3 at 0.2 μM each and LF 0.4 μM. The FLB (0.4 μM) primer was covalently labeled at the 5′ end with a 6-FAM moiety to provide a fluorescent signal when incorporated into the target-specific FQ-LAMP DNA amplification product. The quencher oligo, QLB (0.6 μM), is an 11-mer reverse complement sequence of the FLB primer designed with a melting temperature of 40°C and covalently labeled at the 3′ end with IBHQ. All primer/oligo solutions were prepared in deionized water. The primer sequences were synthesized by Integrated DNA Technologies and created using free PrimerExplorer software. The sequence for USA-WA1/2020 CoV2 (WA1; GenBank: MN985325.1) was obtained from the National Center for Biotechnology Information database. Nucleotides 251–468 of the N gene of WA1 were used to generate an FQ-LAMP primer set specific for the N gene and selected based on the amplification results obtained at 65°C for 30 min.

Visual detection & imaging of FQ-LAMP

FQ-LAMP reactions were prepared in eight-tube PCR strips with caps (Agilent Technologies), and at the end of FQ-LAMP incubation in the thermocycler, the tube strips were removed and imaged. The tube strips remained capped to mitigate possible contamination. A

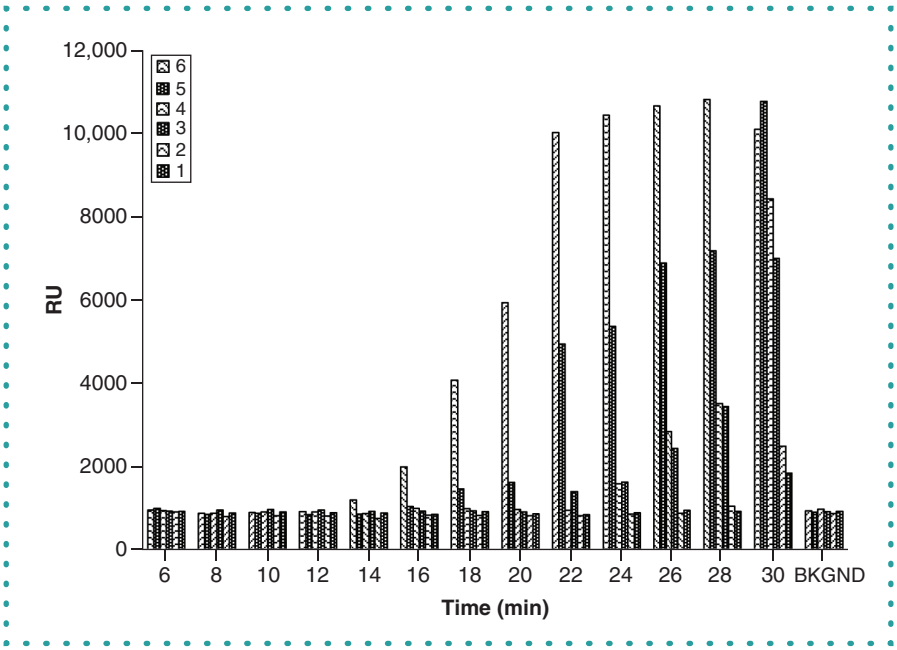

Figure 2. Fluorescence-quenched reverse transcription loop-mediated isothermal amplification SARS coronavirus 2 detection kinetics time to positive. FQ-LAMP assay targeted WA1 from infected Vero cells. Cell culture supernatants were log serially diluted from 10^6 to 10 PFUs/ml in SPS buffer. FQ-LAMP was then performed and end point fluorescence measured in the FAM channel. RFUs were plotted against the elapsed reaction time (min) to generate the amplification plot for each target dilution. Background fluorescence values were determined for each target dilution using identical FQ-LAMP reactions that were first heat-inactivated at 80°C for 20 min.
FQ-LAMP: Fluorescence-quenched reverse transcription loop-mediated isothermal amplification; PFU: Plaque-forming unit; RFU: Relative fluorescence unit; SPS: Sample prep solution.

standard laboratory handheld UV lamp (302 nm) (Thermo Fisher Scientific, MA, USA) was held 5 cm above the tube strip to excite the 5′[6FAM] moiety of the unquenched reporter (FLB). Reactions with sufficient unquenched reporter emit a green-colored fluorescence that is apparent to the unaided eye and easily imaged using a mobile phone camera without filters. Negative reactions do not appear fluorescent to the naked eye under these conditions.

Clinical nasal swab samples

Nasal swab samples from consenting adult volunteers were collected using a sterile swab applicator that was placed in 1 ml of saline. The University of Georgia Veterinary Diagnostic Laboratories (GA, USA) determined the CoV2 status of the nasal swab samples using an Applied Biosystems TaqPath coronavirus disease 2019 EUA assay (Thermo Fisher Scientific) to detect CoV2 RNA in a multiplex RT-qPCR format. Pooled CoV2-negative nasal swab samples were spiked with wild-type CoV2 (WA1; GenBank: MN985325.1), Alpha variant (B.1.1.7; GenBank: MW422255.1), Delta variant (B.1.617.2; GenBank: MZ208926.1) or Omicron variant (B.1.1.529; GenBank: MT380725.1) – all of which were purchased from BEI Resources (VA, USA), which acquires, authenticates and produces reagents scientists need to carry out basic research and develop improved diagnostic tests, vaccines and therapies – in Vero cell culture supernatant (MOI = 0.1) to determine the analytical sensitivity and specificity of the FQ-LAMP assay in the nasal swab sample background. To confirm FQ-LAMP specificity, CoV2-negative nasal swab samples were spiked with culture supernatant from human respiratory syncytial virus (RSV) strain A2 (GenBank: KT992094.1), influenza A/Guangdong-Maonan/SWL1536/2019 (H1N1) (GISAID: EPI_ISL_419003) and human coronavirus strains OC43 (GenBank: AY585228.1), 229E (GenBank: AF304460.1) and NL63 (GenBank: AY567487.2).

Results & discussion

Kinetics & sensitivity of CoV2 FQ-LAMP

The authors determined the kinetics of the FQ-LAMP assay using WA1-infected (MOI = 1) Vero cell-free culture supernatants. A log dilution series ranging from 10^6 to 10 plaque-forming units (PFUs)/ml of cell culture supernatant was prepared in SPS buffer and processed as described earlier. Separate FQ-LAMP reactions were incubated at 65°C between 6 and 30 min. End point fluorescence, as measured in the FAM channel, was recorded after cooling the reactions to 25°C for 30 s. The magnitude of the fluorescent signals for each virus dilution was dependent on the amount of starting material (Figure 2). As expected, the lowest dilution containing the most virus was detected first (within 15 min) at 65°C. Successive target dilutions required a longer incubation time to signal, and at 30 min the 1000-PFU/ml sample had considerable fluorescent signal.

Table 1. Fluorescence-quenched reverse transcription loop-mediated isothermal amplification assay targeting WA1 from infected Vero cells.

WA1 (PFUs/ml)	FQ-LAMP, mean ± SD (%) [95% CI] (n = 32 replicates)
10^6	14,046 ± 279 (±2.0) [13,766–14,325]
10^5	14,089 ± 357 (±2.5) [13,731–14,446]
10^4	13,811 ± 376 (±2.7) [13,434–14,187]
10^3	8069 ± 550 (±6.8) [7518–8619]
10^2	2716 ± 409 (±15.1) [2306–3125]
10	1219 ± 239 (±19.6) [979–1458]
NTC	1235 ± 281 (±22.8) [953–1516]

Culture supernatants were log serially diluted from 10^6 to 10 PFUs/ml in SPS buffer. FQ-LAMP was then performed and end point fluorescence measured in the FAM channel. Identically processed samples (but lacking WA1, with deionized water added to the SPS buffer) served as NTC. The 95% CIs were calculated using n = 32 replicates for each target virus dilution.
FQ-LAMP: Fluorescence-quenched reverse transcription loop-mediated isothermal amplification; NTC: No template control; PFU: Plaque-forming unit; SD: Standard deviation; SPS: Sample prep solution.

Next, the authors determined the sensitivity of the FQ-LAMP assay using WA1-infected Vero cell culture supernatants. A log dilution series ranging from 10^6 to 10 PFUs/ml of culture supernatant was prepared in SPS buffer and processed as described earlier. Identically processed samples with SPS buffer alone served as the no template control (NTC). Prepared FQ-LAMP assays were incubated at 65°C for 30 min and cooled to 25°C for 30 s, and end point fluorescence was recorded in the FAM channel (Figure 3A). To determine the assay positive/negative cut-off fluorescence value, the authors considered the results from n = 32 replicate assays of the NTC sample containing SPS buffer only (Table 1). First, the authors calculated the standard deviation from the mean for the NTC population (standard deviation = 812). The authors multiplied the standard deviation by 3 (812 × 3 = 2436) and added the product to the value previously calculated as the upper bound of the 95% CI (1516) for the fluorescent signals from the same NTC dataset (1516 + 2436 = 3952). This calculation was used to assess the relative fluorescence units (RFUs) $\geq$4000 represent a positive result, whereas RFUs $<$4000 represent a negative result for the FQ-LAMP assay performed in the authors' real-time thermocycler in SPS buffer.

The authors continued analysis of LOD at the 10^3-PFU/ml virus dilution since this was the last dilution to yield a positive result in the log dilution series. A twofold dilution series of WA1-infected Vero cell culture supernatant in SPS buffer ranging from 63 to 1000 PFUs/ml was processed with the SPS protocol and analyzed by FQ-LAMP (Figure 3B). As expected, the 1000-PFU/ml sample resulted in a significant positive signal with tight confidence. The first twofold dilution sample (500 PFUs/ml) resulted in borderline signal from replicate samples just above the authors' positive/negative cut-off value (RFUs = 4000). Successive samples in the twofold dilution series produced only negative results. The 95% CIs were calculated using n = 32 replicates for each target virus dilution in SPS buffer measured in a thermocycler (Tables 1 & 2). The results from these data suggested the LOD for WA1 in the FQ-LAMP assay to be 1000 PFUs/ml in SPS buffer. The mobile phone camera images of the FQ-LAMP assay tubes supported this finding (Supplementary Figure 1A–C).

To validate the LOD, the authors examined pooled CoV2-negative nasal swab samples spiked with WA1-infected Vero cell culture supernatants. The authors examined n = 20 replicates of each virus dilution at 2X, 1X and 0.5X multiples of the LOD to determine at which dilution FQ-LAMP was positive in at least 19 of 20 replicates (Figure 4A). Replicate control samples (n = 20) consisted of the SPS-processed nasal swab sample material alone without spiked virus. The data showed that at the 0.5X dilution, the target virus concentration (500 PFUs/ml) was insufficient to produce a consistent fluorescent signal. By contrast, virus target at 1X and 2X LOD levels (1000 and 2000 PFUs/ml, respectively) was positive (RFUs $\geq$4000) in at least 19 of 20 replicates when measured in a real-time thermocycler (Supplementary Table 1). Images of the FQ-LAMP reaction tubes from a mobile phone camera recapitulated these results (Supplementary Figure 2A–D).

FQ-LAMP reproducibility & specificity

To determine FQ-LAMP reproducibility and specificity, the authors repeatedly (n = 20 replicates) tested three CoV2 variant viruses – namely, Delta (B.1.617.2), Alpha (B.1.1.7) and Omicron (B.1.1.529) – at 2X LOD (2000 PFUs/ml) as well as three common human coronaviruses (OC43, 229E and NL63) at 10^5 PFUs/ml and two heterologous human respiratory viruses (RSV and influenza A virus) at 10^5 PFUs/ml spiked into pooled nasal swab samples and processed with SPS buffer (Figure 4B & C). Replicate control samples (n = 20)

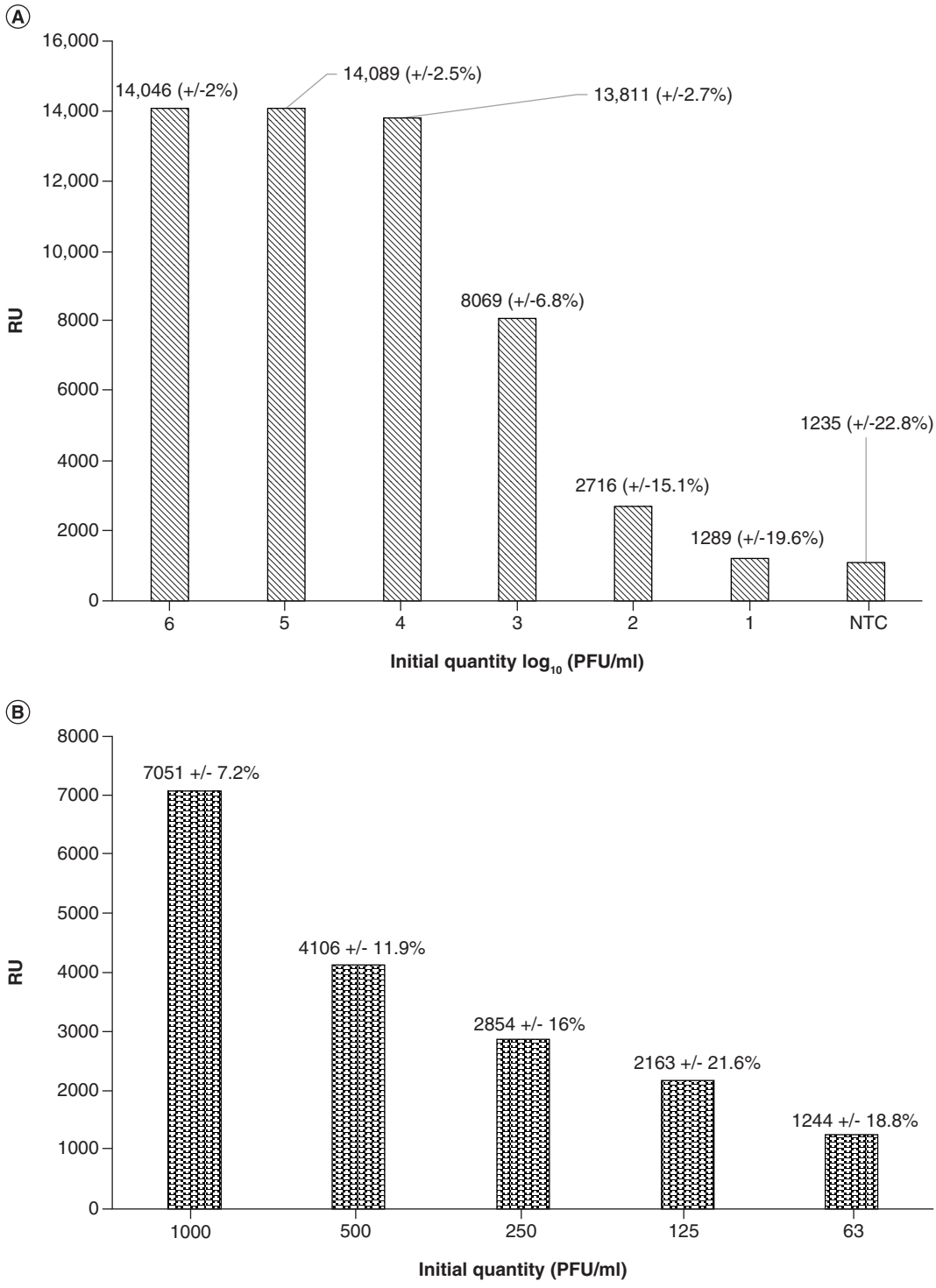

Figure 3. Fluorescence-quenched reverse transcription loop-mediated isothermal amplification LOD for WA1. (A) WA1 target log dilution in SPS. FQ-LAMP assay targeted WA1 from infected Vero cells. Culture supernatants were log serially diluted from 10^6 to 10 PFUs/ml in SPS buffer. FQ-LAMP was then performed and end point fluorescence measured in the FAM channel. Identically processed samples (but lacking WA1, with deionized water added to the SPS buffer) served as NTC. The 95% CIs were calculated using n = 32 replicates for each target virus dilution and values indicated in the plot above each entry. **(B)** WA1 target twofold dilution in SPS. Twofold dilution series of WA1-infected Vero cell culture supernatant in SPS buffer from 1000 to 63 PFUs/ml was processed with the SPS protocol and analyzed by FQ-LAMP. The 95% CIs were calculated using n = 32 replicates for each target virus dilution and values indicated in the plot above each entry.
FQ-LAMP: Fluorescence-quenched reverse transcription loop-mediated isothermal amplification; NTC: No template control; PFU: Plaque-forming unit; SPS: Sample prep solution.

Table 2. Fluorescence-quenched reverse transcription loop-mediated isothermal amplification assay targeting WA1 from infected Vero cells.

WA1 (PFUs/ml)	FQ-LAMP, mean ± SD (%) [95% CI] (n = 32 replicates)
1000	7051 ± 507 (±7.2) [6543–7558]
500	4106 ± 488 (±11.9) [3617–4594]
250	2854 ± 456 (±16.0) [2397–3310]
125	2163 ± 466 (±21.6) [1696–2629]
63	1244 ± 233 (±18.8) [1010–1477]

A twofold dilution series of WA1-infected Vero cell culture supernatant in SPS buffer from 1000 to 63 PFUs/ml was processed with the SPS protocol and analyzed by FQ-LAMP. The 95% CIs were calculated using n = 32 replicates for each target virus dilution.
FQ-LAMP: Fluorescence-quenched reverse transcription loop-mediated isothermal amplification; PFU: Plaque-forming unit; SD: Standard deviation; SPS: Sample prep solution.

consisted of the SPS-processed nasal swab sample material alone without virus spike. The assay repeatedly detected all three CoV2 variants at equal target concentrations (2X LOD) compared with WA1 (Figure 3A), as measured in a real-time thermocycler (Supplementary Table 1). Importantly, FQ-LAMP did not detect irrelevant viruses, even at high concentrations (10^5 PFUs/ml), for human coronaviruses (OC43, 229E and NL63) or heterologous respiratory viruses (RSV and influenza A virus) when spiked in nasal swab material. Mobile phone camera images of the FQ-LAMP reaction tubes showed that all three CoV2 variants could be visually detected (Supplementary Figure 3A–C), whereas the three human coronaviruses and two heterologous respiratory viruses were not detected by the unaided eye (images not shown), reflecting the fluorescence data collected in the real-time thermocycler.

The coronavirus disease 2019 pandemic has reaffirmed that rapid and accurate testing for detection of infections and control of transmission is a major public health challenge. RT-qPCR is the gold standard NAAT for CoV2 detection, but implementation of widespread testing is costly, and return of results may not be timely with regard to adequately affecting transmission control measures. CoV2 control methods require accurate and widely accessible tests that are robust enough to accept heterologous, minimally processed clinical samples without perturbation of results. Ideally, tests are performed in the POC or in-home setting so that determination of results is immediate. As a step toward this end, the authors developed the FQ-LAMP assay with high analytical sensitivity and specificity. Reaction kinetics (Figure 2) showed that the highest target concentrations signaled within 10–15 min post-incubation. By 30 min, the assay LOD of 1000 PFUs/ml CoV2 was positive, with a stringent 95% CI (Table 1) using repeat samples (n = 32) of the serial dilution of WA1 in SPS buffer (Figure 3A). The presence of nasal swab material did not adversely affect assay sensitivity. The LOD for nasal swab samples spiked with CoV2 was also revealed to be 1000 PFUs/ml, and 19 of 20 repeat assays had a positive signal in the presence of nasal swab sample material (Figure 4A & Supplementary Table 1). At the assay LOD (1000 PFUs/ml), FQ-LAMP is robust and repeatable, demonstrating the ability to cope with complex sample material in minimally processed nasal swab samples while maintaining analytical sensitivity and specificity. FQ-LAMP assay repeatability and specificity were shown by replicate testing of three CoV2 variants (Alpha, Delta and Omicron) at 2X LOD (2000 PFUs/ml) spiked in nasal swab sample material in which all viruses were readily detected by the authors' thermocycler-based measurements. By contrast, three common human coronaviruses (i.e., OC43, 229E and NL63) [14] and two heterologous human respiratory viruses (RSV and influenza A virus) assayed at high concentrations (10^5 PFUs/ml) were undetected in spiked nasal swab sample material (Figure 4B & Supplementary Table 1).

Notably, FQ-LAMP results can be visually detected, as shown by imaging of the post-incubation FQ-LAMP assay tubes, using a mobile phone camera while under excitation by UV light (302 nm). The authors showed that an image from a WA1 log dilution series (Supplementary Figure 2A) and an image from a WA1 twofold dilution series (Supplementary Figure 2B & C) recapitulated the results from identical samples, as measured in our real-time thermocycler (Figure 3A & B). The assay LOD (1000 PFUs/ml) was brightly positive, but the signal from the first twofold dilution (500 PFUs/ml) was not apparent to the unaided eye. Visual detection of positive FQ-LAMP reactions was shown to be consistent and reproducible over repeat testing of WA1 in nasal swab sample material at the LOD and 2X LOD (Supplementary Figure 3A & B) and was in agreement with real-time thermocycler measurements (Figure 4A) for identical spiked nasal swab samples. Repeat testing of low target concentration samples revealed inconsistent detection by the unaided eye (Supplementary Figure 2C). Negative control nasal swab samples (without virus spike) did not visually fluoresce over repeat testing (Supplementary Figure 2D), whereas CoV2 variants (Delta, Alpha and Omicron) spiked in nasal swab sample material at 2X LOD (Supplementary Figure 3A–C) showed repeatable and specific detection by the unaided eye.

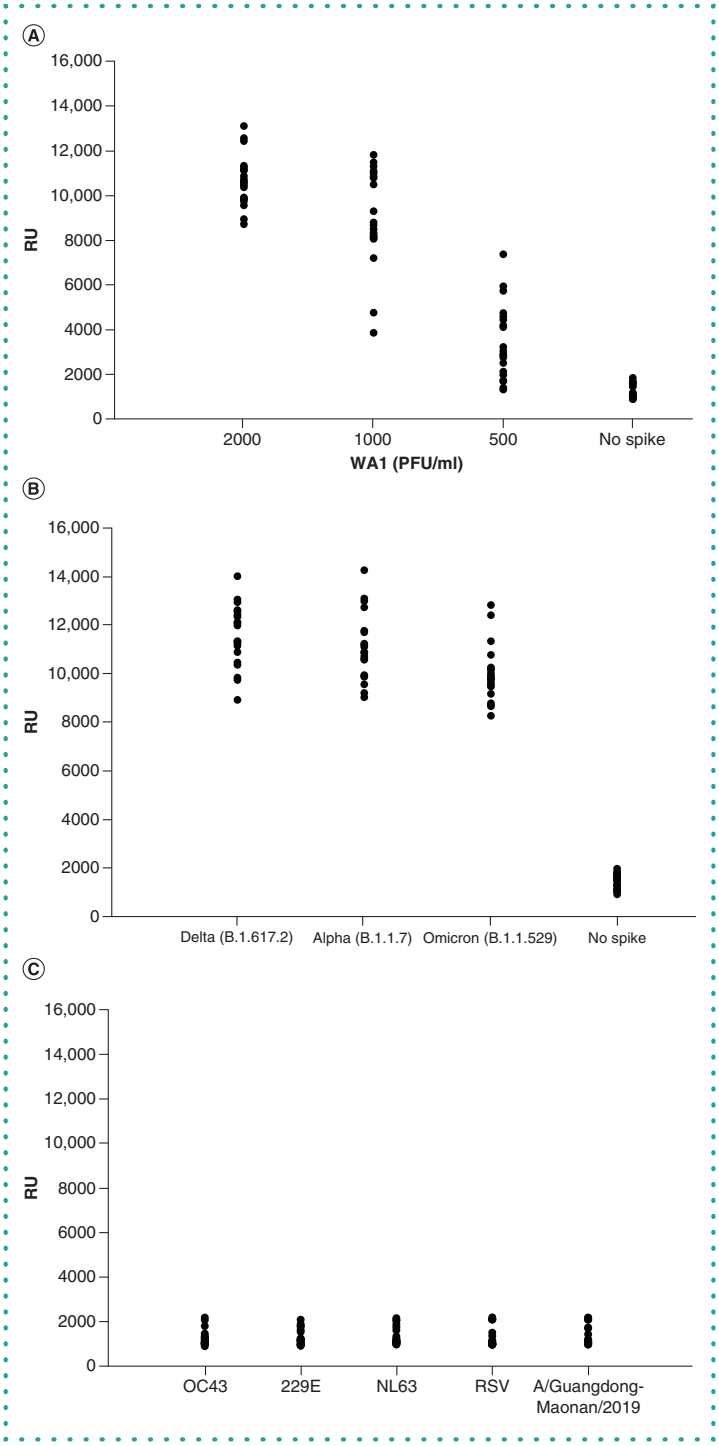

Figure 4. Fluorescence-quenched reverse transcription loop-mediated isothermal amplification LOD for WA1 in nasal swab samples. (A) WA1 target multiples to confirm LOD. FQ-LAMP assay targeted WA1 from infected Vero cells. Culture supernatants were spiked into pooled CoV2-negative nasal swab samples at 2000, 1000 and 500 PFUs/ml representing 2X, 1X and 0.5X LOD target concentrations, respectively, and processed with SPS buffer as described earlier using n = 20 replicates for each target dilution. Negative control assays (no spike) (n = 20 replicates) used identically processed nasal swab samples, but without WA1. End point fluorescence values for each of the n = 20 replicates were plotted for the three WA1 target concentrations to observe the range of responses. The 1000-PFU/ml WA1 target concentration was the highest dilution, displaying at least 19 of 20 replicates with positive signal, and was determined to be the assay LOD. **(B)** CoV2 variant viruses in nasal swab samples. FQ-LAMP assay targeted CoV2 variant viruses Delta (B.1.617.2), Alpha (B.1.1.7) and Omicron (B.1.1.529) from infected Vero cell culture. Culture supernatants were spiked into pooled CoV2-negative nasal swab samples at 2000 PFUs/ml (2X LOD for WA1) and processed with SPS buffer as described earlier using n = 20 replicates for each target virus. Negative control assays (no spike) (n = 20 replicates) consisted of identically processed nasal swab samples, but without CoV2 variant virus. **(C)** Human coronaviruses and heterologous human viruses in nasal swab samples. FQ-LAMP assay targeted three human coronavirus strains – OC43 (GenBank: AY585228.1), 229E (GenBank: AF304460.1) and NL63 (GenBank: AY567487.2) – from infected Vero cells as well as human RSV strain A2 (GenBank: KT992094.1) from infected Vero cells and influenza A/Guangdong-Maonan/SWL1536/2019 (H1N1) (GISAID: EPI_ISL_419003) from infected embryonated hen eggs. Culture supernatants, or egg lysate in the case of IAV, were spiked into pooled CoV2-negative nasal swab samples at 10^5 PFUs/ml and processed with SPS buffer as described earlier using n = 20 replicates for each target virus. All of the virus targets, even at very high concentrations, failed to produce fluorescent signals over the background in the FQ-LAMP assay.
CoV2: SARS coronavirus 2; FQ-LAMP: Fluorescence-quenched reverse transcription loop-mediated isothermal amplification; IAV: Influenza A virus; PFUs: Plaque-forming units; RSV: Respiratory syncytial virus; SPS: Sample prep solution.

Conclusion

In summary, using either real-time thermocycler determination or visual detection with the unaided eye, the FQ-LAMP assay readily detects all three CoV2 variants at equal target concentrations (2X LOD) compared to WA1 (Figure 4A). Furthermore, FQ-LAMP has very good specificity, as it does not erroneously detect three related human coronaviruses or two heterologous respiratory viruses – a finding the authors expected, as contrary to the three CoV2 variants tested, these viruses do not share sufficient sequence homology with the WA1 N gene in the region targeted by assay primers. Owing to a sequence-specific detection strategy that uses a reporter and quencher scheme, FQ-LAMP circumvents false-positive results that can plague other isothermal amplification-based NAATs. The fluorescence readout of positive results is easily detectable by the unaided eye, and negative results are clear and unambiguous in the

presence of nasal swab sample material in the assay. In addition to CoV2, FQ-LAMP demonstrates potential application for the detection of other respiratory viruses, such as influenza and RSV, in the POC or in-home setting.

Future perspective

The standard method for CoV2 detection is the RT-qPCR method, which is laborious, requires special equipment and trained personnel and suffers from delayed (2–3 h) results. Consequently, RT-LAMP and its derivatives, including FQ-LAMP, have emerged as an alternative to the RT-qPCR method. Not only are these methods quicker at amplifying viral transcripts, but they also have fundamental advantages, such as amplification at a constant temperature, exclusion of a thermocycler and potentially larger diagnostic capacity. Recent improvements, including new primer features, have resulted in similar sensitivity and specificity [15], making these methods more suitable than RT-qPCR for diagnostic monitoring of CoV2. FQ-LAMP is faster and, unlike RT-qPCR, amenable to a variety of unpurified, minimally processed sample inputs. Compared with RT-qPCR, this expands the potential for POC and in-home testing applications for FQ-LAMP and other RT-LAMP-based NAATs in relation to CoV2 diagnosis.

However, compared with RT-qPCR, RT-LAMP is an emerging technology with several issues. For example, it is currently a challenge to design robust RT-LAMP assays for pathogen detection, as a typical RT-LAMP assay requires six primers specific for a relatively small region (typically 200–250 bp) of the target sequence. Compromised primer design frequently leads to unacceptable false-positive results stemming from nonspecific, primer-driven amplification events. Such events can be associated with the RT-LAMP DNA polymerase (e.g., Bst 2.0), which demonstrates strand-displacing isothermal polymerase activity essential to RT-LAMP but lacks 3′ to 5′ proofreading capability and also possesses terminal transferase activity [13]. Sequence-specific detection strategies such as FQ-LAMP can aid in overcoming nonspecific amplification events associated with isothermal amplification. Standard primer selection software based on average (ΔG) free energy calculations may not account for these kinds of events. Currently, this uncertainty necessitates the empirical selection of RT-LAMP primer sets for reliable and robust detection of a given target.

Given the need for improved diagnostics, we believe that the field will rapidly evolve to create publicly available tools for RT-LAMP primer design. It is known that primers are strongly influenced by their stability, melting temperature, secondary structure and interference with one another [16]. In addition, optimization of the primer length will improve RT-LAMP efficiency. New guidelines regarding length and melting temperature of the primer stem regions will increase assay efficiency and further optimize RT-LAMP. Finally, the diagnostic arena will move to saliva testing, particularly for diagnosis, as it is a noninvasive method for sample collection, nonmedical personnel can collect saliva and saliva is relatively stable at a broad range of storage temperatures [17]. FQ-LAMP is more amenable to a POC or home testing environment than standard RT-LAMP since test results can be determined with the unaided eye or a mobile phone camera.

Executive summary

Background

- Fluorescence-quenched reverse transcription loop-mediated isothermal amplification (FQ-LAMP) assay results are attained in 30 min (while eliminating the RNA purification step).

Methods

- FQ-LAMP uses labeled primers and a quencher oligonucleotide to achieve sensitive (1000 plaque-forming units/ml) fluorescence detection of SARS coronavirus 2 variants in clinical nasal samples.
- The FQ-LAMP end point fluorescent signal can be determined outside of the real-time thermocycler and directly in the assay tube using a simple excitation source for visual discrimination of positive/negative assay results.

Results & discussion

- FQ-LAMP does not detect irrelevant viruses even at high concentrations.
- FQ-LAMP assay has good specificity and can detect SARS coronavirus 2 variants of concern (i.e., Alpha, Delta and Omicron).

Supplementary data

To view the supplementary data that accompany this paper please visit the journal website at: www.future-science.com/doi/suppl/10.2144/btn-2022-0037

Author contributions

L Jones and RA Tripp were responsible for the design and implementation of the study, analysis of the results and writing of the manuscript. HK Naikare and Y-YC Mosley supplied and verified clinical samples by real-time reverse transcription quantitative PCR and reviewed the final manuscript.

Disclosure

The funders had no role in study design, data collection, analysis, decision to publish or preparation of the manuscript.

Financial & competing interests disclosure

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