A Rapid RT-LAMP SARS-CoV-2 Screening Assay for Collapsing Asymptomatic COVID-19 Transmission.

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Abstract

Purpose: Todemonstrate the diagnostic performance of rapid SARS-CoV-2 RT-LAMP assays, comparing the performance of genomic versus sub-genomic sequence target with subsequent application in an asymptomatic screening population.

Methods: RT-LAMP diagnostic sensitivity (DSe) and specificity (DSp) was determined using 114 RT-PCR clinically positive and 88 RT-PCR clinically negative swab samples processed through the diagnostic RT-PCR service within the University Hospitals of Leicester NHS Trust. A swab-based RT-LAMP SARS-CoV-2 screening programme was subsequently made available to all staff and students at the University of Leicester (Autumn 2020), implemented to ISO 15189:2012 standardsusing NHS IT infrastructure and supported by University Hospital Leicester via confirmatory NHS diagnostic laboratory testing of RT-LAMP 'positive' samples.

Results: Validation samples reporting a Ct < 20 were detected at 100% DSe and DSp, reducing to 95% DSe (100% DSp) for all samples reporting a Ct < 30 (both genomicdual sub-genomic assays). Advisory screening identified nine positive cases in 1680 symptom free individuals (equivalent to 540 cases per 100,000) with results reported back to participants and feed into national statisticswithin 48 hours.

Conclusion: This work demonstrates the utility of a rapid RT-LAMP assay for collapsing transmission of SARS-CoV-2 in an asymptomatic screening population.

Background

According to Government guidance published by the Medicines and Healthcare Products Regulations Agency (Target Product Profile for Laboratory-Based SARS-CoV-2 Viral Detection tests), a dual (or more) target SARS-CoV-2 RNA format is desirable for diagnostic testing, but use of a single target is acceptable. Dual target assays protect against false-negative results caused by genome sequence mutations in the assay target sites and can offer improved certainty in results when the results of both targets are in agreement, but interpretation of results can be complicated where the results are discrepant.The interpretation and assessment of clinical significance ofpositive SARS-CoV-2 RT-PCR results can be equally challenging (PHE guide 'Understanding cycle threshold' (Ct) in SARS-CoV-2 RT-PCR'). A positive result with low viral load (high Ct) can be seen in both the early stages of infection (before the person becomes capable of transmission) or later in infection when transmission risk is low [1]. If therefore, the purpose of a public health utility test is to identify individuals who are currently infectious then data from highly sensitive RT-PCR needs careful interpretation.

Mass demand and delays associated with centralised RT-PCR testing and reagent availabilitywere soon recognised as major obstacles in effectively responding to the SARS-CoV-2 pandemic with mitigation strategies urgently required [2]. Use of an alternative rapid and cheaper isothermal reverse transcriptase loop mediated amplification (RT-LAMP) strategy [3] with its completely different design, equipment and reagent requirements could largely bypass some of the challenges detailed above. Reactions comprise of

4-6 primers targeting 6-8 template region, typically spanning in excess of 200 bp. Therefore, detection of slowly degrading RNA fragments, of historical no longer contagious infection, is less likely with RT-LAMP than with conventional RT-PCR designed to amplify significantly shorter sequence regions.

Fast amplification via LAMP can bedetected by a variety of endpoint readouts including fluorescence, turbidity and colorimetric change optimal for point of care LAMP-based diagnostics [4]. Several groups demonstrated the suitability of an RT-LAMP detection strategy early on in the pandemic. Rabe and Cepko[5] optimised primers directed toward a non-conserved region of the SARS-CoV-2 Orf1a gene, (assay termed Orf1a-Harvard Medical School enhanced or 'Orf1a-HMSe'). New England Biolabs (NEB) developed a dual sub-genomic assay targeting regions of the N and E gene, plus a separate internal control assay targeting the human beta actin gene (ACTB) for confirmation of total RNA indicative of appropriate sample collection[6]. Fowleret al.,[7]optimised and validatedOptiGene's COVID-19 RT-LAMP workflow, successfully establishing the first CE-IVD registered RT-LAMP kit, now employed nationally across several NHS Trusts.

The work presented herein compares the choice ofSARS-CoV-2 RT-LAMP targets(genomic versus sub-genomic) and end-point readouts (colorimetric versus fluorescent detection). Assay limit of detectionwas confirmed using synthetic SARS-CoV-2 positive control RNA of known concentration, and evaluation of diagnostic sensitivity (DSe) and specificity (DSp)was confirmed using residual RNA from UHL NHSinpatientoropharyngeal / nasopharyngeal (ON) swab sampleswith correspondingRT-PCR Ct value. A swab based fluorescent end-point rapid RNA to RT-LAMP reaction targeting the genomic Orf1a with paralleltotal RNA internal control reactionto mitigate reporting of false negative results subsequently provided the basis of an asymptomatic screening programme available to all staff and students at the University of Leicester.

Methods

Ethics Statement:UHL NHS patient ONswab samples used in this study were collected in the context of routine clinical patient care. RT-LAMP analyses performed on residualde-identified patient material served as post-RT-PCR assay validation.

University of Leicester Asymptomatic Screening Programme: Participants accessing the screening programme required registration at the primary care Leicester Victoria Park Health Centrefor generation of a pathology request form. Individuals self-swabbed (throat and lower nasal cavity) at a supervised screening venue using Miraclean swabs placed into PrimeStore Molecular Transport Medium for viral inactivation and RNA stabilisation at room temperature (Longhorn Vaccines and Diagnostics). Within a 24-hour period, total nucleic acid extraction was followed by RT-LAMP targeting the Orf1a plus an internal total RNA control reaction. Datawas uploaded to the pathology iLab system and NHS laboratory RT-PCR testing confirmed any positive RT-LAMP results, feeding into the national track and trace system. Results were reported back to participants (<48 hours post sample collection) via SMS(negative result) or phone call (positive results) from the Victoria Park Health Centre. Figure 1 details the programme workflow.

Control RNA: Synthetic SARS-CoV-2 RNA at a concentration of 1×10^6 RNA copies per microliter was purchased from Twist Bioscience and diluted appropriately in nuclease free water (Twist Synthetic SARS-CoV-2 RNA Control 1 [MT007544.1] - SKU: 102091 and Control 2 [MN908947.3] - SKU: 102024). Negative control RNA from related Betacoronavirus 1 (Strain OC43) and non-related Influenza A (H1N1) was purchased from ATCC. Total human RNA purchased from Invitrogen (4307281).

Swab sample collection and RNA extraction of RT-LAMP assay validation material: Standard ON swabs from hospital inpatients were collected using PHE-approved flocked swabs placed into viral transport media (Virocult / VTM-M4RT).RNA extraction (on 200 µl of inactivated sample mixed with 265 µl Binding Solution) was carried out using the MagMAX Viral/Pathogen Nucleic Acid Isolation kit (MVPII, Thermofisher) on the KingFisher Flex Purification System.Residual patient RNA samples used for validation purposes potentiallysuffered a degree of sample degradation during prolonged storage prior to RT-LAMP.

NHS real-time RT-PCR: Purified nucleic acid from ON swab samples was reverse transcribed into cDNA and amplified using a CE marked, locally validated commercially available kit targeting the E and S-gene sequence regions (RealStar® SARS-CoV-2 RT-PCR kit, Altona Diagnostics GmbH, Hamburg, Germany).

RT-LAMP primers: Primers (HPLC grade, Merck)targetingthe Orf1a (Orf1a-HMSe) were designed by Rabe and Cepko[5]. Primers targeting the nucleocapsid (N),envelope gene (E) and internal human beta-actin internal control (ACTB)were designed by NEB [6]. All primer sequencesare listed in Additional file 3. Individual RT-LAMP primer sets were prepared as 20 times final concentration stock, final assay concentrations of0.2 µM F3/B3, 1.6 µM FIP/BIP and 0.4 µM LoopF/Loop B.

Fluorescent RT-LAMP: Reactionscontained1 X WarmStart® LAMP Master Mix (E1700) supplemented with 1 X fluorescent dye (NEB dye provided with E1700 master mix), 0.02 U/µL Antarctic Thermolabile UDG (NEB), 700 µM Thermolabile dUTP (NEB) and 1 X standard concentration LAMP primers. Assay validation reactions were prepared to a final reaction volume of 25 µl with nuclease free water, subsequently miniaturised to 15 µl for the screening programme (data not shown). Reactions were incubated at 65°C(StepOnePlus Applied Biosystems or Rotor-Gene QQiagen thermoclycler)for 30 minutes (for validations runs) and 20 minutes for screening programme runs (equivalent to a Ct <30).

Colorimetric RT-LAMP: Reactions contained 1 X WarmStart® Colorimetric LAMP Master Mix (M1800; NEB) supplemented with 1 x EvaGreen (Biotium), 0.02 U/µL Antarctic Thermolabile UDG (NEB), 700 µM Thermolabile dUTP (NEB), 1 X standard concentration LAMP primers and 40 mM guanidine chloride solution (Sigma G3272, pH adjusted to pH ~8). Reactions were prepared to final volume of 25 µl using nuclease free water,incubated at 65°C for 30 minutes on a StepOnePlus thermocycler (Applied Biosystems). The colour of finished reactions wasrecorded using an office flatbed scanner.

Statistical analysis: Time to positive (TTP in minutes) served as a surrogate for RT-PCR Ct and a semi-quantitative measure of viral RNA concentration. Additional product specificity checkswere provided by melt curveanalysis within acceptance range (2 degrees either side of the mean Tm determined during

assay validation using patient samples). Regression calculations determining reaction efficiency incorporated data from concentrations where three or more values reported a TTP in under 25 minutes. Reactions were considered as negative with a TTP above 25 min. Reaction efficiency is calculated by $E=-1+10^{(-1/slope)}$. Validation data using synthetic material represent the average of two independent experiments, performed in quadruplicate, presented as mean TTP ± S.E.M. Validation data using residual patient RNA represent a single reaction performed in parallel reactions targeting the Orf1a, N+E and ACTB total RNA control sequence. MedCalc® Scientific Software was used for diagnostic test evaluation to determine the diagnostic sensitivity (DSe) referring to the proportion of known positive samples that tested positive in the assay and diagnostic specificity (DSp) referring to the proportion of samples from known negative reference samples that test negative in the assay.

Results

Genomic versus sub-genomic RT-LAMP assaytarget validation. The performance of fluorescent and colorimetric end-pointRT-LAMP reactionstargeting the genomicOrf1a andsub-genomic N and E generegions were tested solo, in duplex (N+E) and in multiplex combination (Orf1a+N+E) against a single concentration oftwo synthetic positive control RNAs (Figure2). Fluorescent end-point data presented in Figure 2A demonstrates equivalent amplification of theTwist positive controls (T1 and T2)with no statistically significant difference. Reactions targeting the Orf1a were fastest to exceed the threshold(17 ± 0.17 and 16.7 ± 0.17 minutes,T1 andT2 respectively), followed by reactions targeting the E gene (12.1 ± 0.09 and 12.4 ± 0.10 minutes) and finally the N gene (17 ± 0.17 and 16.7 ± 0.17 minutes). Dual N+E reactions exceeded the amplification threshold at a mid-point between N and E alone (14.3 ± 0.10 and 14.2 ± 0.08 minutes). Finally, addition of the Orf1a primer set to the dual N+E reaction further enhanced dual velocity (13.4 ± 0.22 and 13.0 ± 0.21 minutes). A lack of cross-reactivity of all SARS-CoV-2 targets was confirmed by a lack of amplification in negative control wells (related human coronavirus OC43, non-related influenza A and total human RNA). Presence of viable RNA within these samples was confirmed by control ACTB amplification (8.9 ± 0.25, 13.0 ± 0.19 and 9.5 ± 0.09respectively).The ACTB total RNA control reaction failed to amplify the positive RNA controls consistent with these being synthetic material. Finally, no template controls (NTC) confirmed the absence of non-specific amplification in any reactions.Fluorescent data is summarised in Table 1. Summary melt curve data was also collated for each primer set (Orf1a 82.1 ± 0.058, N+E 89.4 ± 0.060, ACTB 92.7 ± 0.050), and used as an amplification product specificity check in subsequent analyses of patient samples. End-point colorimetric detection (Figure 2B) consistent with fluorescent findings, demonstrated clear amplification in under 30 minutes with a colour change from pink to yellow for all primers (Orf1a, N and E) and primer combinations (N+E & Orf1a+N+E) against positive RNA controls T1 andT2. SARS-CoV-2 targeting reactionsfailed to amplify negative controls (OC43,influenzaA and total human RNA), whilst the ACTB internal control reaction confirmed the presence of viable template RNA. ACTBinternal control reaction failed to amplify synthetic material (as expected) and a NTC confirmed the absence of non-specific amplification in any reactions.

Table 1. RT-LAMP primer investigation

SARS-CoV-2 target:	TTP (minutes):					
	Twist 1 (SKU 102019)	Twist 2 (SKU 102024)	OC43	Influenza A	Human RNA	NTC
Orf1a	9.1 ± 0.07	8.7 ± 0.16	na	na	na	na
N	17 ± 0.17	16.7 ± 0.17	na	na	na	na
E	12.1 ± 0.09	12.4 ± 0.10	na	na	na	na
N+E	14.3 ± 0.10	14.2 ± 0.08	na	na	na	na
Orf1a+N+E	13.4 ± 0.22	13.0 ± 0.21	na	na	na	na
ACTB	na	na	13.0 ± 0.19	9.5 ± 0.09	8.9 ± 0.25	na

Data summary of an average of 2 independent experiments each performed in quadruplicate, presented as mean TTP ± S.E.M. No amplification is noted as 'na'.

Limit of detection of fluorescent and colorimetric end-point RT-LAMP reactions targeting the Orf1a, dual N+E gene and multiplex Orf1a+N+E genes of SARS-CoV-2: Parallel fluorescent and colorimetric end-point reactions were performed against synthetic RNA (Twist control 2)serially diluted to 10,000, 1,000, 500, 100, 50 and 10 copies of viral sequence. Representative fluorescent end-point amplification curves and linear regression analysis of primer setsOrf1a, N+E duplex and multiplex Orf1a+N+E targetare presented in Figure 3 with summary data presented in Table 2.RT-LAMP targeting Orf1a was the fastest to exceed amplification threshold at 8.9 ± 0.12 minutes (10,000 viral RNA copies),capable of reproducibly detecting500 copies synthetic viral RNA. Lower viral loads down to 10 copies were detectable within 20 minutes although not reproducibly. RT-LAMP dual targetingN+E and multiplex Orf1a+N+E were~5 minutes slower to exceed amplification threshold for equivalent viral loads,analogously capable of reproducibly detecting 500 copies of synthetic viral RNA. All assays demonstrated good linearity and efficiency, 102.1%, 99.5% and 105.5% respectively.Colorimetric reactions (Additional file 1) augmented with EvaGreen intercalating dye and guanidine hydrochloride were equally able to reproducibly detect 500 viral RNA copies, exceeding the amplification threshold slightly faster that their fluorescent counterpart (2.7, 5.6 and 4.5 minutes faster for Orf1a, N+E and Orf1a+N+E respectively for 500 viral copies). All reactions that exceeded the amplification threshold (indicated byintercalating dye associated TTP) also showed a clear visual colorimetric change from pink to yellow in under 30 minutes.

Table 2. Limit of detection of fluorescent end-point RT-LAMP reactions

Synthetic positive control RNA	Fluorescent RT-LAMP Mean TTP (min) ± SEM (N)		
Copies / reaction	Orf1a	N+E	Orf1a+N+E
10,000	8.9 ± 0.12 (8/8)	14.4 ± 0.16 (8/8)	14.1 ± 0.12 (8/8)
1,000	11.6 ± 0.49 (8/8)	17.1 ± 0.77 (7/8)	17.2 ± 0.50 (8/8)
500	13.3 ± 0.65 (8/8)	21.4 ± 2.51 (8/8)	17.2 ± 0.29 (8/8)
100	14.1 ± 0.0 (1/8)	22.0 ± 1.44 (5/8)	23.2 ± 1.66 (5/8)
50	16.4 ± 3.91 (3/8)	na	29.2 ± 0.0 (1/8)
10	11.6 ± 0.0 (1/8)	na	21.1 ± 0.85 (2/8)
NTC	na	na	na

Reactions targeting the Orf1a, dual N+E gene and multiplex Orf1a+N+E of SARS-CoV-2 using a serially diluted Twist Bioscience synthetic positive control RNA.Data summary is an average of 2 independent experiments, performed in quadruplicate and presented as mean TTP ± S.E.M. Numeration in parentheses indicates the number of repeat reactions achieving the amplification threshold required to report a TTP. No amplification ('na').

Diagnostic validation of fluorescent end-point RT-LAMP assays targeting Orf1a and dual N+E gene regions. Residual RNA extracted from patient ON swab samples originally processed through the diagnosticRT-PCR service in theUHL Trustwere processedthrough parallel RT-LAMP reactions targeting the Orf1a, dual N+E gene and ACTB internal control.Two PCR platforms wereassessed:the ABI StepOnePlus PCR platform (114 UHL positive samples & 88 UHL negative samples) andthe QiagenRotor-Gene Q(39 UHL positive samples & 41 UHL negative samples).RT-LAMP reactions performed on the StepOnePlus platformwithpatient samples reporting an original RT-PCR of Ct<20 were equally detected by Orf1a and N+E RT-LAMP at 100% / 100% (DSe/DSp). DSe / DSp decreased slightly to 93.6% / 100% and 92.6% / 100% for theOrf1a and N+E targets respectively for patient samples reporting an RT-PCR Ct <30 and then to79.8%/100% and 78.1%/100% DSe / DSp respectively upon processing all patient samples reporting an RT-PCR Ct<40 (Table 3A).For the Rotor-Gene Q platform,RT-LAMP reactions of samples reporting an original RT-PCR of Ct<20 were detected equivalently by Orf1a and dual N+E RT-LAMP,reporting 100% / 100 % DSe / DSp respectively. This decreased to 93.9 % / 100% and97.0% / 100 % DSe / DSp for samples reporting an RT-PCR Ct<30 and finally to 84.3 % / 100% and 82.4% / 100 % DSe / DSp for samples reporting an RT-PCR Ct< 40 (Table 3B).Additional product specificity check provided by the melt curve (Tm) confirmed selective amplification of the product.

Table 3:DSe and DSp of single genomic versus dual sub-genomic SARS-CoV-2 fluorescent end-point RT-LAMP assays.

A)StepOnePlus; Applied Biosystems:

RT-LAMP target:	SARS-CoV-2 Orf1a	SARS-CoV-2 Dual N+E
Dse (95% CI)	100.0% (91.6% to 100.0%)	100.00% (91.6% to 100.0%)
DSp (95% CI)**Ct < 20** (N42)	100.0% (95.9% to 100.0%)	100.00% (95.9% to 100.0%)
Dse (95% CI)	93.6% (86.6% to 97.6%)	92.6% (85.3% to 97.0%)
DSp (95% CI) **Ct < 30** (N94)	100.0% (95.9% to 100.0%)	100.0% (95.9% to 100.0%)
Dse (95% CI)	79.8% (71.3% to 86.8%)	78.1% (69.4% to 85.3%)
DSp (95% CI) **Ct < 40**(N114)	100.0% (95.9% to 100.0%)	100.0% (95.9% to 100.0%)

B) Rotor-Gene Q; Qiagen

RT-LAMP target:	SARS-CoV-2 Orf1a	SARS-CoV-2 Dual N+E
Dse (95% CI)	100.0% (76.8% to 100.0%)	100.0% (76.8% to 100.0%)
DSp (95% CI) **Ct < 20** (N14)	100.0% (91.4% to 100.0%)	100.0% (91.4% to 100.0%)
Dse (95% CI)	93.9% (79.8% to 99.3%)	97.0% (84.2% to 99.9%)
DSp (95% CI) **Ct < 30** (N33)	100.0% (91.4% to 100.0%)	100.0% (91.4% to 100.0%)
Dse (95% CI)	84.4% (76.0% to 90.6%)	82.4% (73.9% to 89.1%)
DSp (95% CI)**Ct < 40** (N39)	100.0% (95.9% to 100.0%)	100.00% (95.9% to 100.0%)

RT-LAMP reactions, performed using residual RNA extracted from clinical patient ON swab samples with comparator RT-PCR Ct values, were run at 65°C for 30 minutes on **A)** StepOnePlus PCR platform (114 UHL positive samples & 88 UHL negative samples) and**B)** Rotor-Gene Q Qiagen PCR platform (39 UHL positive samples & 41 UHL negative samples). Sensitivity and specificity with 95% CI are shown for samples with a corresponding RT-PCR Ct <20, Ct <30 and Ct < 40. Calculations performed using the MedCalc Scientific Software.

Diagnostic validation of Colorimetric RT-LAMP assays targeting Orf1a and dual N+E gene regions:
Residual RNA extracted from patient ON swab samples were also processed through colorimetric end-point RT-LAMP reactions targeting the Orf1a, dual N+E geneplusACTB internal control reaction. Samples reporting an original RT-PCR of Ct<30 were detected equally by Orf1a and N+E RT-LAMP assays(21 of 21 RT-PCR positive samples and 40 of 40 negative samples) demonstrating 100% DSe and DSp. Assay performance decreased to 85.7%/ 100 % DSe / DSp for patient samples reporting an RT-PCR Ct<40 (18 of 21 RT-PCR positive samples).Additional performance of a triple target (Orf1a+N+E) assay demonstrated 100% DSe and DSp (41 of 41 RT-PCR positive samples and 40 of 40 negative samples) decreasing to 90.2% / 100% DSe / DSp for samples reporting an RT-PCR Ct<40 (37 of 41 RT-PCR positive samples). (Additional file 2).

University of Leicester RT-LAMP asymptomatic screening Programme: Screening was performed to ISO 15189:2012 standards, guided by the Leicester Molecular Diagnostic Lab. RNA extracted from throat and lower nasal cavity swabs from 1,673 symptom free individuals attending campus (autumn 2020) were processed through RT-LAMP targeting the Orf1a and internal control ACTB.During this period, a single repeat swab was requested due to inefficient sampling as identified by failure of amplification within the ACTB control reaction indicating absence of total RNA.In total, 9 RT-PCR confirmed RT-LAMP positive resultsfrom a total of 1,673 tests demonstrated a prevalence of asymptomatic infection of 0.54% (540 cases per 100,000) demonstrating the value and usability of RT-LAMP molecular diagnostic tool for the detection of SARS-CoV-2 in an asymptomatic population.

Conclusions

This report demonstrates selective amplification of SARS-CoV-2 viral RNAby rapid and cheap RT-LAMP assays targeting genomic (Orf1a) anddual sub-genomic (N+E) RNA sequence regions via fluorescentor colorimetric determination. Equivalent sensitivity was observed for genomic (Orf1a) and dual sub-genomic (N+E) targetswith assays capable of reproducibly detecting 500 copies of Twist Bioscience synthetic positive control RNA.RT-LAMP targeting the Orf1a was significantly faster to exceed amplification threshold due to inclusion of a poly T linker within the FIP and BIP primer pairs, facilitating faster loop formation[5]. Inclusion of guanidine chloride within colorimetric reactionsalso slightly enhanced amplification velocity compared to the fluorescent end-point counterparts [6].

Diagnostic validation ofSARS-CoV-2 RT-LAMPreactions usingRNA extracted from hospital inpatient ON swabs,demonstratedequivalentDSe and DSp (100% / 100%) of the genomic and dual sub-genomic target assays, concordant with comparator RT-PCR for Ct<20. A small, comparative dropin DSe was observed when including all samples reportingan RT-PCR Ct< 30. DSe acrossdifferent PCR platforms decreased within range of 92.6%and 97.0% along with a slight reduction in DSp (96.83%) for RNA-RT-LAMP when running the N+E assay on the StepOnePlus.

Upon inclusion of all samples reporting a comparator RT-PCR Ct < 40,the Orf1a and N+E RT-LAMP assays performed on the Rotor-Gene Q PCR platform,remained comparable with a DSe between82.4% and85.9% (100% DSp). For assays performed on the StepOnePlus platform, DSe dipped just below 80% (100% DSp).Interestingly, colorimetric triple target (Orf1a+N+E) RT-LAMP performed on the StepOnePlus platform achieved the highest sensitivity, reporting DSe / DSpof 90.2% / 100% forall patient samples with Ct < 40. Equivalent triple target fluorescent end-point assays were not assessed due to lack of template RNA.

Ultimately, the superior sensitivity harnessed by RT-PCR presentsa well-documented drawback ubiquitous for inferring infectiousness from RT-PCR detection, with slowly degrading SARS-CoV, MERS, Influenza, Ebola and Zika viral RNA all detected long after the disappearance of the infectious virus [8]. An RT-PCR positive therefore reflectsan assay’s ability to detect viral RNA and not necessarily the presence of viable virus. The only robust way to detect viable virus is by cell culture, however this method is labour-intensive,

slow and not amenable to high-throughput processing so is not suited to large-scale diagnostics.In comparison to RT-PCR, RT-LAMP assays are designed over a larger RNA template (>200 bp) reducing the likelihood of detecting residual fragments of viral RNA. Pertinently, the anonymised patientswab samples used to validate this study were not paired with clinical data detailing the duration from onset of symptoms, however,patientspresent to hospital a median of 7 to 10 days from onset of symptoms [9]at which point infectious virus may no longer be found despite ongoing detection of viral load by RT-PCR[8, 10]. Word by La Scola's group [11, 12] conducted RT-PCR testing and virus culture on positive samples with known Ct valuesshowing that virus could not be isolated from samples collected after day eight of symptom onset, despite ongoing high viral loads. Only 70% of 3790 positive samples with Ct < 25 could be cultured, compared with less than 3% of the cases with Ct values above 35. In agreement, Bullard *et al.,*[13] took 90 SARS-CoV-2 RT-PCR–confirmed positive samples and demonstrated no viral growth in samples with a Ct > 24 or symptom onset to test time > 8 days suggesting the infectivity of patients with Ct > 24 and duration of symptoms > 8 days may be low. Current guidelines from the Centers for Disease Control and Prevention and World Health Organization also call for patients to isolate for 10 days after onset of symptoms, recognising that individuals are not likely to be infectious after that period. For these reasons (and assuming good sampling and sample handling) use of an assay of lower analytical sensitivity (or application of a cut-off in an assay of higher analytical sensitivity) may be preferable for an asymptomatic screening programme designed to detect potentially infectious individuals, to avoid 'false-positive' detection of residual RNA in individuals who have recovered from COVID-19.

On the basis of these findings and in-line with best documented practices at the time, implementation of a University of LeicesterAsymptomatic COVID-19 RT-LAMP Screening Programmefollowed a swab based RNA-extraction with rapid RT-LAMP assay targeting theOrf1awith a reaction cut off time equivalent to Ct<30. Importantly, the cost per test of this assay was about half that of standard RT-PCR, widely in use for COVID-19 diagnostics. In any asymptomatic populationwhen the infection prevalence is low, test specificity must be sufficiently high to ensure an acceptable positive predictive value[14]. In contrast to other RT-LAMP COVID-19 diagnostic workflows, an internal total RNA control reaction was included for each sample, minimizing the chance of false negative reporting and consequently improving the negative predictive value of this assay. Carry-over contamination prevention reagents (dUTP and UGD from NEB) were also included as standard, minimizing a serious and known challenge associated with isothermal amplification strategies. Targeting of the genomic sequence, combined with product melt curve specify check and confirmation by NHS laboratory RT-PCRfound a SARS-CoV-2 asymptomatic infection incidence of 0.54 %within this population. Given that at least 50% of new SARS-CoV-2 infections are estimated to originate from exposure to asymptomatic individuals [15]able to transmit the virus for an extended period [16](perhaps longer than 14 days),this value is relatively low, suggesting that social distancing behaviors and the prolonged restrictions imposed on this population have been successful. Due to the potential of significant silent spread by asymptomatic persons[17-19] it is crucial that screening efforts such as the work described here are directed at those without symptoms in a targeted manner. In conclusion,this work demonstrates the utility of a rapid and cost effective RT-LAMP assay for collapsing transmission of SARS-CoV-2 in an asymptomatic screening population.

Abbreviations

SARS-CoV-2: *Severe acute* respiratory syndrome coronavirus 2.

RT-LAMP: reverse transcriptase loop-mediated isothermal amplification.

RT-PCR: reverse transcriptase polymerase chain reaction.

DSe: Diagnostic sensitivity.

DSp: Diagnostic specificity.

Declarations

Ethics approval and consent to participate: Not applicable. Samples

Consent for publication: Not applicable.

Availability of data and material: All data generated or analysed during this study are included in this published article and its additional information file.

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Figures

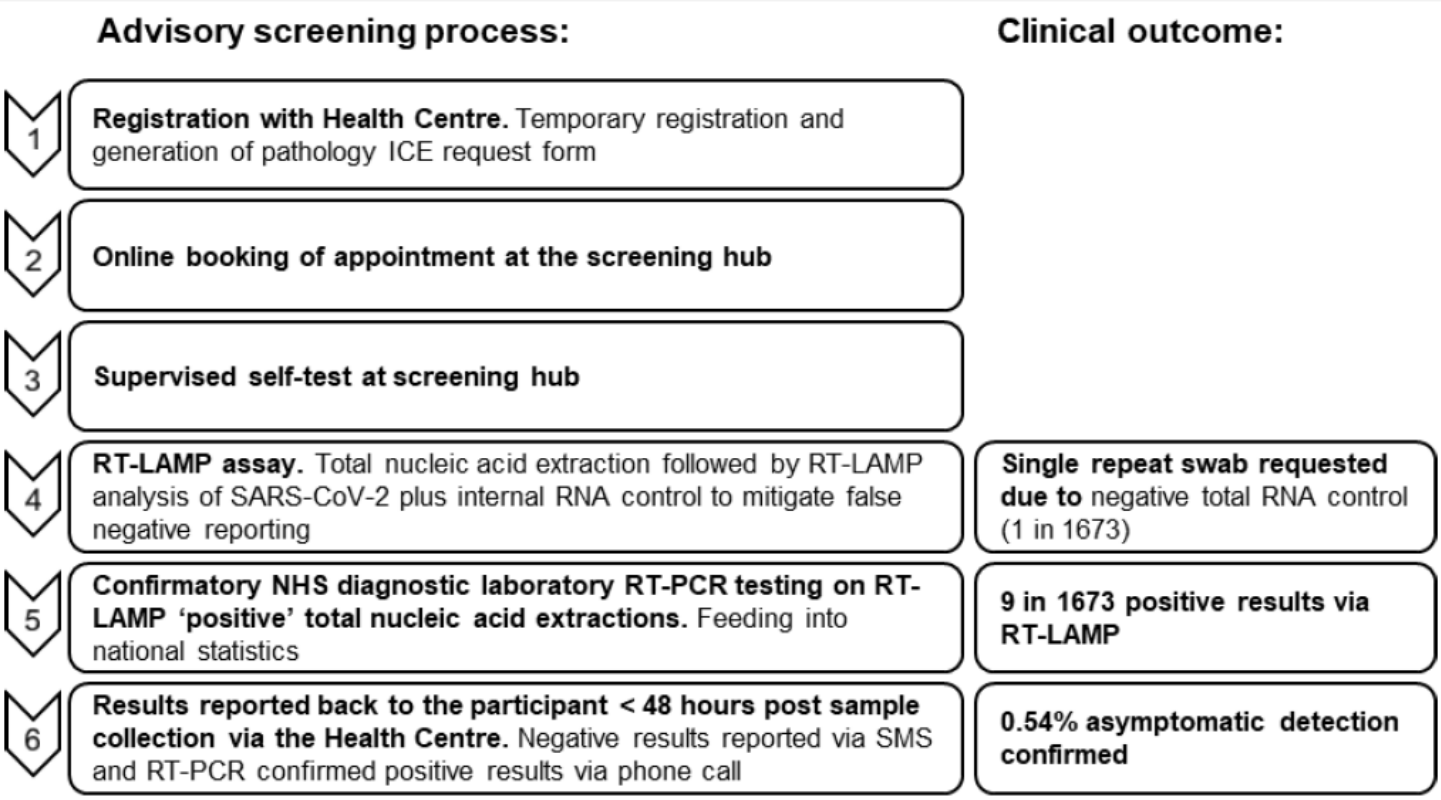

Figure 1

University of Leicester SARS-CoV-2 Advisory Screening Programme. Available to all students and staff without symptoms for a period of twelve weeks (October 2020 – December 2020) to allow rapid isolation and reduce outbreaks.

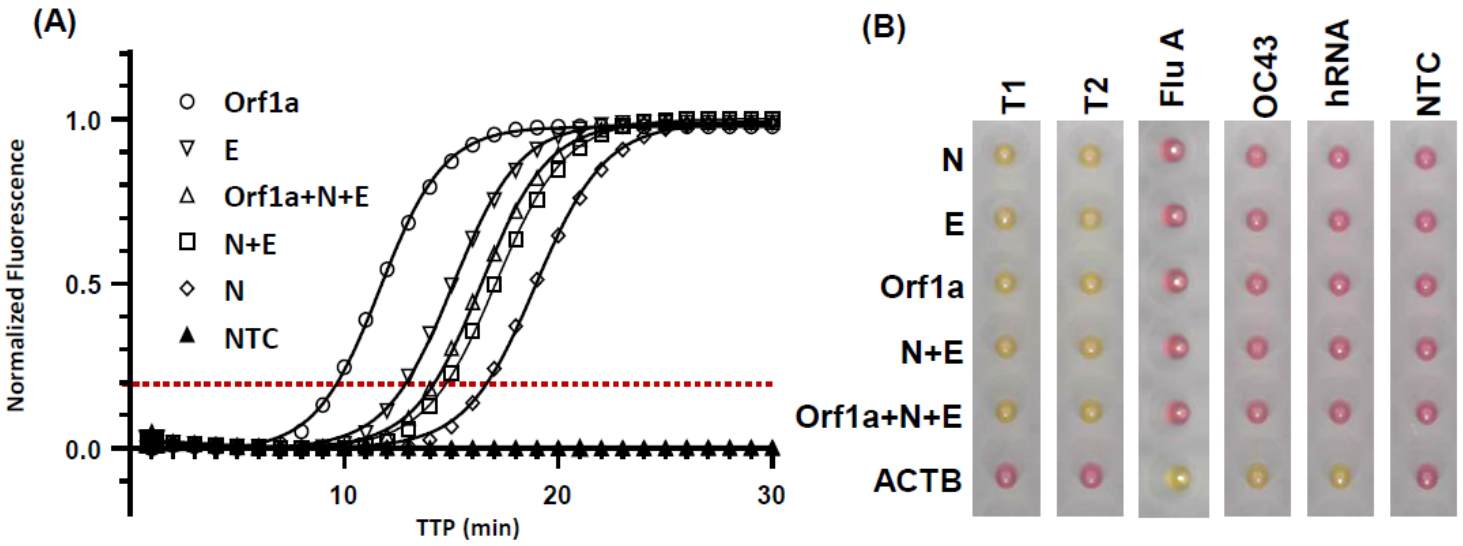

Figure 2

RT-LAMP primer investigation: SARS-CoV-2 RNA primer sets targeting the nucleopcapsid (N), envelope gene (E) (NEB design) and Orf1a (Rabe and Cepko Harvard Medical School) were tested independently

and in combination (dual N+E reaction and multiplex Orf1a+N+E reaction) against $1x10^4$ copies of Twist synthetic SARS-CoV-2 control RNA (T1 and T2). Negative control RNA from Betacoronavirus 1 strain OC43 and Influenza A (H1N1) at a single concentration ($1x10^5$ copies per well) plus a water no template control (NTC). A total RNA control primer set (NEB) targeting human beta actin (ACTB) was also included and tested against 5 ng total human RNA (hRNA). Both RT-LAMP fluorescent end-point and colorimetric 25 µl reactions were performed at 65°C for 30 minutes on a StepOnePlus thermocyler. (A) Representative fluorescent amplification curves where time to positive (TTP) is the time at which amplification exceeds the manually set, reaction consistent threshold (red dotted line). (B) Representative colorimetric reactions whereby yellow indicates positive amplification and pink no amplification.

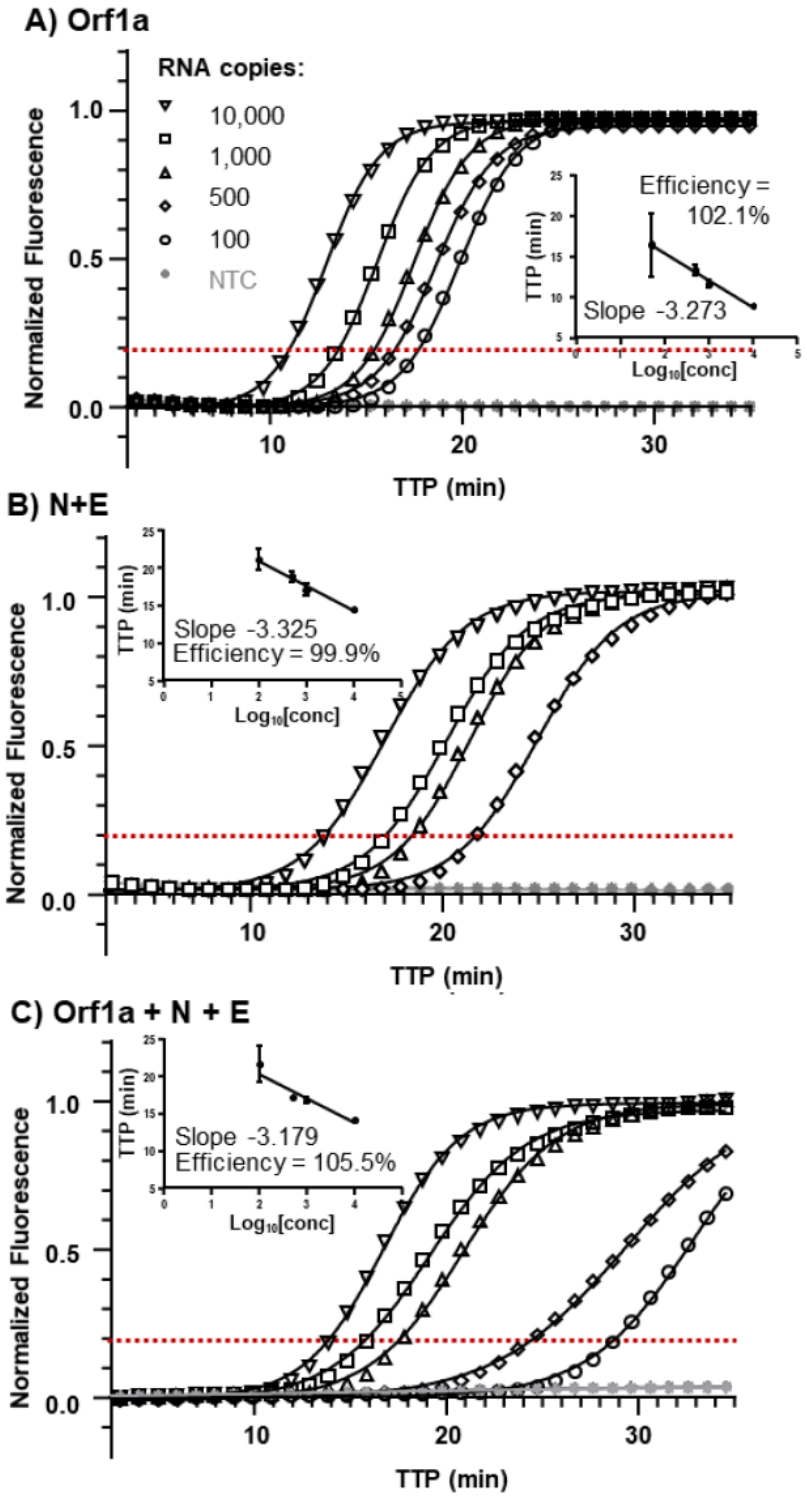

Figure 3

Limit of detection of fluorescent end-point RT-LAMP reactions targeting genomic and sub-genomic regions of SARS-CoV-2. Twist Bioscience synthetic positive control RNA (control 2 GenBank ID MN908947.3, GISAID Wuhan-Hu-1) was serially diluted to 10,000, 1,000, 500, 100, 50 and 10 copies of viral sequence per 25 µl reaction. Water no template control (NTC) were included in each reaction. Reactions were performed at 65°C for 40 minutes on the Qiagen Rotor-Gene Q Thermocycler platform.

Representative amplification and linear regression analysis for each primer set are shown. (A) RT-LAMP targeting the Orf1a. (B) RT-LAMP targeting N+E duplex. (C) RT-LAMP triple Orf1a+N+E target. Time to positive (TTP) is the time at which amplification exceeds the manually set, reaction consistent threshold (red dotted line) when amplification enters the rapid linear, exponential phase. Data represents the average of two experiments each performed in quadruplicate. Linear regression calculations incorporate data from concentrations whereby three of more values reported a TTP under 25 minutes. Reactions were considered as negative with a TTP above 25 min. Slope of linear regression and reaction efficiency (figure insets) whereby $E=-1+10^{(-1/slope)}$ are shown for each target reaction. No amplification ('na').

Supplementary Files

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- AdditionalFile1.pdf
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