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Short communication

Preliminary optimisation of a simplified sample preparation method to permit direct detection of SARS-CoV-2 within saliva samples using reverse-transcription loop-mediated isothermal amplification (RT-LAMP)

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ABSTRACT

We describe the optimisation of a simplified sample preparation method which permits rapid and direct detection of SARS-CoV-2 RNA within saliva, using reverse-transcription loop-mediated isothermal amplification (RT-LAMP). Treatment of saliva samples prior to RT-LAMP by dilution 1:1 in Mucolyse™, followed by dilution in 10 % (w/v) Chelex® Resin and a 98 °C heat step for 2 min enabled detection of SARS-CoV-2 RNA in positive saliva samples. Using RT-LAMP, SARS-CoV-2 RNA was detected in as little as 05:43 min, with no amplification detected in 3097 real-time reverse transcription PCR (rRT-PCR) negative saliva samples from staff tested within a service evaluation study, or for other respiratory pathogens tested (n = 22). Saliva samples can be collected non-invasively, without the need for skilled staff and can be obtained from both healthcare and home settings. Critically, this approach overcomes the requirement for, and validation of, different swabs and the global bottleneck in obtaining access to extraction robots and reagents to enable molecular testing by rRT-PCR. Such testing opens the possibility of public health approaches for effective intervention during the COVID-19 pandemic through regular SARS-CoV-2 testing at a population scale, combined with isolation and contact tracing.

The COVID-19 pandemic, caused by the SARS-CoV-2 virus, remains a significant burden to global communities, economic activity and healthcare systems. Although studies have reported the development of safe and efficacious vaccines (Mulligan et al., 2020; Folegatti et al., 2020; Anderson et al., 2020), uncertainty remains as to when these may become generally available. One public health approach that has been advocated for suppression of the COVID-19 pandemic is regular SARS-CoV-2 testing at a population scale, combined with isolation and contact tracing.
contact tracing for positive cases (Peto et al., 2020). Such an approach requires a rapid, inexpensive diagnostic, based on non-invasive samples that can be collected in both healthcare and non-healthcare settings (Sax, 2020).

The current diagnostic standard for SARS-CoV-2 is viral RNA detection by real-time reverse transcriptase polymerase chain reaction (rRT-PCR) from nasopharyngeal/oropharyngeal swabs (World Health Organization, 2020). However, the procedure for collecting good quality swab samples requires training, potentially exposes the health-care worker to infectious droplets during sample collection, and can be uncomfortable for the patient, especially if undertaken frequently. Critically, supply issues during the pandemic have led to bottlenecks in availability of reagents for molecular assays. Furthermore, the demand for swabs (Azzi et al., 2020a) has resulted in laboratories having to undertake frequent validation on different swab types. Exploring alternative sample types and detection methods is an attractive solution.

Saliva shows promise as an alternative sample type for diagnostic detection of coronaviruses and has been shown as a matrix where SARS-CoV-2 is found in early infection (Wei et al., 2020; Sri Santosh et al., 2020). Furthermore, collection is straightforward and can be self-collected by drooling into a universal plastic container.

Reverse-Transcription Loop-merged isothermal AMPlification (RT-LAMP) is a sensitive, isothermal nucleic acid amplification technology (Notomi et al., 2000) which is more resistant to inhibitors than rRT-PCR, enabling simplification and even removal of the sample extraction procedure (Francois et al., 2011). LAMP has been applied for detection of a wide range of pathogens in both the veterinary (Fowler et al., 2016; Armson et al., 2019) and medical sector (Yan et al., 2020; De Paz et al., 2020).

At the height of the SARS-CoV-2 epidemic in the UK, Hampshire Hospitals NHS Foundation Trust (HHFT) rapidly validated a novel RT-LAMP assay for SARS-CoV-2 RNA detection from nasopharyngeal/oropharyngeal swabs either directly, or following RNA extraction (Fowler et al., 2020). For direct detection of SARS-CoV-2 RNA from swabs, diluting the viral transport media 1 in 20 in nuclease free water (NFW) overcame inhibition. When this sample preparation method was trialed on paired swab and saliva samples, inhibition was still evident for SARS-CoV-2 RNA detection from saliva. Herein we describe the further development of a simple preparation method for direct detection of SARS-CoV-2 RNA within saliva samples using Direct RT-LAMP.

Optimisation of the sample preparation method was initially performed using (i) spiked saliva, in which a pool of five SARS-CoV-2 negative saliva samples (University Hospital Southampton [UHS] staff) was spiked with whole beta-propiolactone inactivated virus (SARS-CoV-2 at \( \sim 1 \times 10^{5} \text{TCID}_{50}/\text{mL} \)), (ii) three SARS-CoV-2 positive saliva samples collected from HHFT (n = 1) and UHS (n = 2) (confirmed SARS-CoV-2 positive by rRT-PCR) and (iii) a pool of fifteen SARS-CoV-2 negative saliva samples (UHS).

To further refine optimum sample preparation methods, a rRT-PCR characterised panel of SARS-CoV-2 positives collected at HHFT (n = 5) and service evaluation studies from Southampton (n = 10) was used, alongside negative saliva samples from HHFT (n = 5) and healthcare and university staff in Southampton (n = 3097). Analytical sensitivity was determined using a titration of a synthetic DNA fragment (Integrated DNA Technologies, Coralville, United States) containing the SARS-CoV-2 RT-LAMP target in nuclease free water. Analytical specificity was determined using the NATcert™ Respiratory Verification Panel 2 (Zetaplex Technologies, Corporation, New York, United States). All saliva was collected into a universal collection container, with UHS saliva collection analysis conducted with informed written consent following institutional review board approval (ENACT – Enabling New Approaches for COVID-19 Treatment).

For comparator rRT-PCR, saliva samples processed at HHFT were extracted using the Maxwell® RSC Viral Total Nucleic Acid Purification Kit (Promega UK Ltd., Southampton, UK) according to manufacturer’s instructions. 200 μL sample was added to 223 μL prepared lysis solution (including 5 μL of genesig® Easy RNA Internal extraction control (Primedesign Ltd, Chandler’s Ford, UK)). Samples were inactivated for 10 min at room temperature followed by 10 min at 56 °C on a heat block before automated RNA extraction using a Maxwell® RSC 48 Instrument (Promega UK Ltd.). RNA was eluted in 50 μL NFW. rRT-PCR was performed in single replicates (using 5 μL RNA) using the COVID-19 genesig® Real-Time PCR assay (Primedesign Ltd) according to manufacturer’s guidelines, on a Mic qPCR Cycler (Bio Molecular Systems, London, UK). Cycling conditions were adjusted to 10 min at 55 °C, 2 min at 95 °C, then 45 cycles of 95 °C for 10 s and 60 °C for 30 s.

The saliva samples processed at the Animal and Plant Health Agency (APHA) were extracted using the MagMAX™CORE Nucleic acid purification kit (Thermofisher). 200 μL sample was added to 700 μL prepared lysis solution. Samples were inactivated for 10 min at room temperature before automated RNA extraction using a KingfisherFlex Thermofisher. RNA was eluted in 90 μL NFW and tested using the E gene RT-PCR as described previously (Corman et al., 2020) using the AgPath-ID™ PCR kit (Thermofisher). Samples were run on an Aria qPCR Cycler (Agilent) and results analysed using the Agilent AriaMX 1.5 software. Cycling conditions were adjusted to 10 min at 45 °C, 10 min at 95 °C, then 45 cycles of 95 °C for 15 s and 60 °C for 45 s. For both rRT-PCRs, a positive control, a negative extraction control, and a no template control were included on each run.

RT-LAMP was performed using OptiGene Ltd. (Horsham, UK) COVID-19 Direct RT-LAMP KIT-500, which targets the SARS-CoV-2 ORF1ab region. Each reaction consisted of: 17.5 μL rRT-PCR Isothermal Mastermix (containing 8 units of GspSSD2.0 DNA Polymerase, 7.5 units Opti-RT reverse transcriptase, a proprietary fluorescent dsDNA intercalating dye and a proprietary enhancing enzyme), 2.5 μL 10X COVID-19 Primer Mix and 5 μL sample. Reactions were performed in duplicate at 65 °C for 20 min on a Genie® HT (OptiGene Ltd.). An exponential increase in fluorescence (ΔF) indicated a positive reaction, which was quantified by the time to positivity (Tp). To confirm amplification specificity, an annel curve was performed: RT-LAMP products were heated to 98 °C for 1 min, then cooled to 80 °C decreasing the temperature by 0.05 °C/s. Genie® embedded software was utilised to analyse results.

For RT-LAMP optimisation, saliva was initially diluted 1:1 in Mucolyse® (active ingredient: dithiothreitol, Pro-Lab Diagnostics, UK) and then a dilution series (1 in 5 to 1 in 640) was prepared in either NFW or 10 % (w/v) Chelex® 100 Resin, with and without heat treatment (70 °C for 4 min or 98 °C for 2 min). Heating of samples was performed on a dry heat block. After addition of the sample to Direct RT-LAMP, treatments were pooled according to dilution and extracted for rRT-PCR. 10 % (w/v) Chelex® 100 Resin was prepared by resuspending Chelex® 100 Resin (200–400 mesh) (Bio-Rad Laboratories, catalogue number #1421253) in Milli-Q® water. The Chelex® 100 Resin solution was heated at 70 °C for 30 min and following two washes, Milli-Q® water was added to give 10 % (w/v) Chelex® 100 Resin.

The rRT-PCR C\textsubscript{T} value for the spiked saliva sample used to optimise the sample preparation methods was 22.86 (Table 1). When this sample was diluted in NFW, SARS-CoV-2 RNA was detected by Direct RT-LAMP in duplicate at one dilution (1 in 80) without heat, in three dilutions (1 in 5, 1 in 10 and 1 in 80) following 70 °C for 4 min and in three dilutions (1 in 5, 1 in 10 and 1 in 40) following 98 °C for 2 min (Table 1). When this sample was diluted in 10 % (w/v) Chelex® 100 Resin, SARS-CoV-2 RNA was detected in duplicate in three dilutions (1 in 20, 1 in 40 and 1 in 80) without heat treatment, in six dilutions (1 in 5 to 1 in 160) following 70 °C for 4 min and in six dilutions (1 in 5 to 1 in 160) following 98 °C for 2 min (Table 1). The pool of SARS-CoV-2 positive saliva samples was negative on Direct RT-LAMP for all assay conditions (data not shown).

The rRT-PCR C\textsubscript{T} values for the three SARS-CoV-2 positive clinical saliva samples used to optimise the sample preparation methods were 21.08, 24.47 and 25.27 (Table 2). The saliva sample with the highest viral load (C\textsubscript{T} 21.08) when diluted in water, SARS-CoV-2 RNA was detected by Direct RT-LAMP in duplicate in five dilutions (1 in 40 to 1 in
640) without heat treatment, in all eight dilutions (1 in 5 to 1 in 640) following 70°C for 4 min and in seven dilutions (1 in 5 to 1 in 320) following 98°C for 2 min (Table 1, Panel A). When diluted in 10% (w/v) Chelex® 100 Resin SARS-CoV-2 RNA was detected in duplicate in seven dilutions (1 in 10 to 1 in 640) without heat treatment and in all eight dilutions (1 in 5 to 1 in 640) following either 70°C for 4 min or 98°C for 4 min.

Table 1
Optimisation of sample preparation methods using a dilution series of inactivated whole virus spiked into pooled saliva.

| Treatment | No Heat (1:1 in Mucolyse™) | 70°C 4 mins (1:1 in Mucolyse™) | 98°C 2 mins (1:1 in Mucolyse™) |
|-----------|----------------------------|--------------------------------|--------------------------------|
| 1 in 5    | RRT-PCR Ct | Tp | Annual | RRT-PCR Ct | Tp | Annual | RRT-PCR Ct | Tp | Annual |
| 1 in 5    | 21.97       | 11.31 | 0.03  | 21.98       | 10.30 | 0.48  | 21.97       | 09.01 | 83.41  |
| 1 in 10   | 22.32       | 13.36 | 0.42  | 22.35       | 12.11 | 0.39  | 22.32       | 09.37 | 83.36  |
| 1 in 20   | 23.67       | 10.14 | 0.20  | 23.61       | 11.15 | 0.22  | 23.67       | 11.52 | 83.23  |
| 1 in 40   | 24.32       | 10.15 | 0.60  | 24.32       | 10.30 | 0.60  | 24.32       | 08.44 | 83.44  |
| 1 in 80   | 25.30       | 09.30 | 0.35  | 25.30       | 09.42 | 0.35  |
| 1 in 160  | 26.81       | -     | -     | 26.81       | -     | -     |
| 1 in 320  | 28.77       | -     | -     | 28.77       | -     | -     |
| 1 in 640  | 20.72       | 10.10 | 83.63 | 20.72       | -     | -     |
| 1 in 1280 | 29.85       | -     | -     | 29.85       | -     | -     |

| Treatment | No Heat (1:1 in Chelex® 100 Resin) | 70°C 4 mins (1:1 in Chelex® 100 Resin) | 98°C 2 mins (1:1 in Chelex® 100 Resin) |
|-----------|--------------------------------|--------------------------------|--------------------------------|
| 1 in 5    | RRT-PCR Ct | Tp | Annual | RRT-PCR Ct | Tp | Annual | RRT-PCR Ct | Tp | Annual |
| 1 in 5    | 22.49       | 07.21 | 83.68 | 22.49       | 07.11 | 83.50 | 22.49       | 07.43 | 83.41 |
| 1 in 10   | 23.17       | 08.16 | 83.45 | 23.17       | 08.16 | 83.45 | 23.17       | 08.16 | 83.45 |
| 1 in 20   | 24.33       | 07.41 | 83.43 | 24.33       | 08.42 | 83.35 | 24.33       | 08.42 | 83.35 |
| 1 in 40   | 25.80       | 08.05 | 83.46 | 25.80       | 08.38 | 83.41 | 25.80       | 08.38 | 83.41 |
| 1 in 80   | 26.63       | 07.56 | 83.37 | 26.63       | 07.56 | 83.37 | 26.63       | 07.56 | 83.37 |
| 1 in 160  | 26.43       | 07.14 | 83.54 | 26.43       | 07.14 | 83.54 | 26.43       | 07.14 | 83.54 |
| 1 in 320  | 27.31       | 08.00 | 83.50 | 27.31       | 08.00 | 83.50 | 27.31       | 08.00 | 83.50 |
| 1 in 640  | 28.56       | -     | -     | 28.56       | -     | -     |
| 1 in 1280 | 29.26       | -     | -     | 29.26       | -     | -     |

Table 2
Sample preparation optimisation for direct detection of SARS-CoV-2 in crude saliva.

| Panel A: Saliva C: 21.08 (1:1 in Mucolyse™) | Panel B: Saliva C: 24.47 (1:1 in Mucolyse™) | Panel C: Saliva C: 25.67 (1:1 in Mucolyse™) |
|--------------------------------|--------------------------------|--------------------------------|
| RRT-PCR | No heat | 70°C 4 mins | 98°C 2 mins | RRT-PCR | No heat | 70°C 4 mins | 98°C 2 mins | RRT-PCR | No heat | 70°C 4 mins | 98°C 2 mins |
| Ct       | Tp      | Annual      | Tp      | Annual      | Tp      | Annual      | Tp      | Annual      | Tp      | Annual      |
| 25.81    | -       | -           | -       | -           | -       | -           | -       | -           |
| 26.29    | -       | -           | -       | -           | -       | -           | -       | -           |
| 27.92    | -       | -           | -       | -           | -       | -           | -       | -           |
| 30.36    | -       | -           | -       | -           | -       | -           | -       | -           |
| 32.17    | -       | -           | -       | -           | -       | -           | -       | -           |

NFW: Nuclease free water; Ct: Cycle Threshold; Tp: Time to positivity. Green shading indicates samples were positive in duplicates by Direct RT-LAMP, orange shading indicates samples were positive in single replicates, grey shading indicates samples were negative in both replicates. "-" represents no amplification detected (negative) in Direct RT-LAMP. For Tp 00:00 represents minutes:seconds.
2 min (Table 2, Panel A). The saliva sample with a Ct of 24.47 when diluted in water, SARS-CoV-2 RNA was not detected by Direct RT-LAMP in duplicate in any dilution without heat or following 70 °C for 4 min (Table 2, Panel B). This sample was positive in duplicate in three dilutions (1 in 5 to 1 in 20) following 98 °C for 2 min (Table 2, Panel B). When diluted in 10 % (w/v) Chelex® 100 Resin, SARS-CoV-2 RNA was detected in duplicate in one dilution (1 in 20) without heat treatment, in four dilutions (1 in 10 to 1 in 80) following 70 °C for 4 min and in five dilutions (1 in 5 to 1 in 40 and 1 in 160) following 98 °C for 2 min (Table 2, Panel B). For the saliva sample with the lowest viral load (Ct 25.27) when diluted in water, SARS-CoV-2 was not detected by Direct RT-LAMP in duplicate in any dilution without heat or following 70 °C for 4 min (Table 2, Panel C) and in one dilution (1 in 5) only following 98 °C for 2 min (Table 2, Panel C). When diluted in 10 % (w/v) Chelex® 100 Resin, SARS-CoV-2 RNA was detected in duplicate in two dilutions (1 in 40 and 1 in 80) without heat treatment, in no dilutions following 70 °C for 4 min and in four dilutions (1 in 5 to 1 in 40) following 98 °C for 2 min (Table 2, Panel C).

The three best performing sample preparation protocols (1:1 in Mucolyse™ then [i] 1 in 5 dilution in Chelex® plus 98 °C heat step; [ii] 1 in 10 dilution in Chelex® plus 98 °C heat step; [iii] 1 in 20 dilution in Chelex® plus 98 °C heat step) were then tested on a further 20 saliva samples (15 rRT-PCR positive and 5 rRT-PCR negative for SARS-CoV-2 RNA). All three sample preparation protocols detected SARS-CoV-2 RNA in both duplicates in the ten positive samples with rRT-PCR Ct values between 18.73 and 24.07 (Fig. 1). For the remaining five positive samples (rRT-PCR Ct values between 27.73 and 34.36), SARS-CoV-2 RNA was detected in [i] two samples in single replicates using a 1 in 5 dilution in 10 % (w/v) Chelex® 100 Resin plus 98 °C heat step, [ii] one sample in a single replicate using a 1 in 10 dilution plus 98 °C heat step, [iii] no samples using a 1 in 20 dilution plus 98 °C heat step, and the five saliva samples that were negative by rRT-PCR were negative by RT-LAMP for all three protocols (Fig. 1). Using a synthetic DNA template spiked into saliva, the detection limit for these three protocols gave comparable results with a previous publication (Fowler et al., 2020), with a detection limit of between 1 × 10^4 and 1 × 10^5 copies / μL of sample determined (data not shown).

To evaluate diagnostic and analytical specificity, a single protocol was selected (1:1 in Mucolyse™, 1 in 10 dilution in Chelex® 100 Resin, plus 98 °C heat step for 2 min). Using this protocol, all 3097 rRT-PCR negative saliva samples from healthcare and university staff were negative by RT-LAMP, with negative results also achieved against samples within the NATrol™ Respiratory Verification Panel (data not shown).

This study describes the rapid optimisation of a method for direct detection of SARS-CoV-2 RNA within saliva samples using RT-LAMP, without the need for RNA extraction. We show for the first time an optimised sample preparation method for SARS-CoV-2 RNA detection within crude saliva samples. Using this approach, SARS-CoV-2 RNA was reliably detected from positive samples in duplicates over a wide range of dilutions, successfully overcoming matrix inhibition and/or matrix protection of viral capsid nucleic acid release (observed in the samples that did not receive this protocol). Importantly, using this method, no amplification was detected in rRT-PCR negative saliva or against other respiratory pathogens, confirming the specificity of this approach.

Following optimisation of this protocol, a new kit has been launched by OptiGene Ltd. (COVID-19 Direct Plus RT-LAMP KIT-500), which includes an alternative sample preparation method combining a lysis (RapiLyze Sample Buffer) and heat step. Using this kit, samples (neat saliva or oropharyngeal/nasopharyngeal swabs) are diluted 1:1 in RapiLyze Sample Buffer and heated to 98 °C for 2 min, decreasing the dilution factor and number of pipetting stages required for sample preparation. Preliminary analysis of this kit was performed at APHA in triplicate on the ten SARS-CoV-2 positive saliva samples from service evaluation studies in Southampton and compared to rRT-PCR results using the E gene rRT-PCR as described above (Corman et al., 2020). Results were in agreement with this study, with all triplicates positive for all samples (Supplement 1), however, further work is required to validate this new kit format.

Studies in macaque monkeys demonstrated that salivary glands are the first site to be infected by SARS-CoV infection (Liu et al., 2011) and several groups have reported high sensitivity and specificity of rRT-PCR on saliva for SARS-CoV-2 in COVID-19 patients (Zhu et al., 2020; Azzi et al., 2020b). As such, population screening of saliva could be an effective strategy to detect individuals who are infectious (pre-symptomatic, symptomatic or asymptomatic). There is also evidence that SARS-CoV-2 may be present in saliva during the recovery phase, after upper respiratory samples have become negative (Azzi et al., 2020c), making saliva an attractive sample for prolonged identification of SARS-CoV-2 from infectious individuals (Azzi et al., 2020a).

These findings support saliva as a reliable sample in which to detect SARS-CoV-2 RNA. Using saliva collected in a simple container, we present a rapid diagnostic solution based on samples that can be collected at home or in non-healthcare settings. This approach overcomes the requirement for, and validation of, different swabs and the bottleneck observed in obtaining access to extraction platforms and reagents for rRT-PCR testing. Contributing to disease mitigation management, this opens the possibility of rapid public health testing to determine virus circulation through regular population-scale SARS-CoV-2 testing at relatively low cost, combined with isolation and contact tracing.

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CRedit authorship contribution statement

Emma L.A. Howson: Conceptualization, Methodology, Investigation, Writing - original draft. Stephen P. Kidd: Investigation, Writing - review & editing. Bryony Arrows: Investigation, Writing - review & editing. Alice Goring: Investigation. Jason Sawyer: Investigation. Claire Cassar: Investigation. David Cross: Investigation. Tom Lewis: Investigation. Jess Hockey: Investigation. Samantha Rivers: Investigation. Saira Gawthraw: Investigation. Ashley Banyard: Investigation, Writing - review & editing. Paul Anderson: Investigation. Sahab Rahou: Investigation. Michael Andreou: Methodology. Nick Morant: Methodology. Duncan Clark: Methodology. Charlotte Walsh: Methodology. Shaileen Laxman: Methodology. Rebecca Houghton: Writing - review & editing. Joanne Slater-Jefferies: Funding acquisition. Paula Costello: Funding acquisition. Ian Brown: Writing - review & editing. Nicholas Corte: Writing - review & editing. Keith M. Godfrey: Writing - original draft, Funding acquisition. Veronica L. Fowler: Conceptualization, Methodology, Investigation, Formal analysis, Writing - original draft, Writing - review & editing.

Declaration of Competing Interest

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jviromet.2020.114048.

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