**Title page**

**Title** A high throughput immuno-affinity mass spectrometry method for detection and quantitation of SARS-CoV-2 nucleoprotein in human saliva and its comparison with RT-PCR, RT-LAMP, and lateral flow rapid antigen test

**Running head** SISCAPA-LC-MS detection of SARS-CoV-2 nucleoprotein

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**Abbreviations**

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| --- | --- |
| **ADE** | ADETQALPQR |
| **AYN** | AYNVTQAFGR |
| **COVID-19** | Coronavirus disease 2019 |
| **Ct** | Cycle threshold |
| **DGI** | DGIIWVATEGALNTPK |
| **ESI** | Electrospray ionisation |
| **LC-MS** | Liquid chromatography mass spectrometry |
| **LOD** | Lower limit of detection |
| **MALDI-TOF** | Matrix assisted laser desorption ionization time of flight  |
| **MRM** | Multiple reaction monitoring |
| **MSI** | MassLynx Skyline interface |
| **NCAP** | Nucleocapsid protein |
| **TR** | Retention time |
| **RAT** | Rapid antigen test |
| **RT-PCR** | Reverse transcription polymerase chain reaction |
| **RT-LAMP** | Reverse transcription loop-mediated isothermal amplification |
| **S** | Spike protein |
| **SARS-CoV-2** | Severe acute respiratory syndrome coronavirus 2  |
| **SISCAPA** | Stable isotope standards and capture by anti-peptide antibodies  |
| **ss-RNA** | Single stranded ribonucleic acid |
| **TTP** | Time-to-positive |
| **UPLC** | Ultra-performance liquid chromatography |

**Key words:** SARS-CoV-2, immuno-affinity, mass spectrometry**,** RT-PCR, RT-LAMP, rapid antigen test

**Abstract (words 235)**

***BACKGROUND*** Many reverse transcription polymerase chain reaction (RT-PCR) methods exist that can detect SARS-CoV-2 RNA in different matrices. RT-PCR is highly sensitive, although viral RNA may be detected long after active infection has taken place. SARS-CoV-2 proteins have shorter detection windows hence their detection might be more meaningful. Given salivary droplets represent a main source of transmission, we explored the detection of viral RNA and protein using 4 different detection platforms including SISCAPA peptide immunoaffinity liquid chromatography-mass spectrometry (SISCAPA-LC-MS) using polyclonal capture antibodies.

***METHODS*** The SISCAPA-LC MS method was compared to RT-PCR, RT-loop-mediated isothermal amplification (RT-LAMP), and a lateral flow rapid antigen test (RAT) for the detection of virus material in the drool saliva of 102 patients hospitalised after infection with SARS-CoV-2. Cycle thresholds (Ct) of RT-PCR (*E* gene) were compared to RT-LAMP time-to-positive (TTP) (*NE* and *Orf1a* genes), RAT optical densitometry measurements (test line/control line ratio) and to SISCAPA-LC-MS for measurements of viral protein.

***RESULTS*** SISCAPA-LC-MS showed low sensitivity (37.7%) but high specificity (89.8%). RAT showed lower sensitivity (24.5%) and high specificity (100%). RT-LAMP had high sensitivity (83.0%) and specificity (100.0%). At high initial viral RNA loads (<20 Ct), results obtained using SISCAPA-LC-MS correlated with RT-PCR (R2 0.57, p value 0.002).

***CONCLUSIONS*** Detection of SARS-CoV-2 nucleoprotein in saliva was less frequent than the detection of viral RNA. The SISCAPA-LC-MS method allowed processing of multiple samples in <150 minutes and was scalable, enabling high throughput.

**Introduction**

Coronavirus disease 2019 (COVID-19) will likely remain a global issue for many years [1]. As of July 2023, deaths of more than 6.9 million people due to COVID-19 have been recorded [2]. The most widely employed test used to determine exposure to severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is the reverse transcription polymerase chain reaction (RT-PCR). This detects the presence or absence of SARS-CoV-2 RNA (**Figure 1**). Due to its great sensitivity [3], ease of sample collection, and the wide availability of PCR instrumentation, nasopharyngeal swab RT-PCR has been employed globally for widescale screening of infection [4].

However, although useful for determining whether a person has been exposed to the virus, it is unclear whether RT-PCR allows any meaningful information to be derived regarding infection status [5]. Such tests, that involve amplification of nucleic acid sequences, do not necessarily determine that an active, productive infection has occurred or is underway. RT-PCR tests are extremely sensitive, requiring <100 copies of viral RNA to yield positive results [3]. The results are reported as cycle thresholds (Ct), which are the number of successive amplification cycles required before PCR products become detectable. Ct values are therefore inversely related to the load of viral RNA. Ct values can remain low (*i.e.*, indicating high viral load) in infected individuals irrespective of symptom severity or duration of exposure [6]. One study found 79.2% of those with initial Ct values of <25 tested positive by RT-PCR 15-30 days after initial testing [7]. A case report noted positive RT-PCR detection in one patient 63-days after symptom onset [8]. Thus, there is no clearly defined role of RT-PCR for disease monitoring or determination of prognosis. RT-PCR based assays are suited for large scale screening of populations to determine whether a person has been exposed to SARS-CoV-2.

RT-loop-mediated isothermal amplification (RT-LAMP) shares a similar mechanism to RT-PCR (**Figure 1**) but can provide results more quickly (~30 mins versus ~120 mins) [9]. RT-LAMP operates with loop-mediators that can propagate amplification at a constant temperature (65 oC) – this reduces the cycling and thus time required for RT-PCR [9]. In addition, RT-LAMP detects larger genomic fragments, which may not persist in patients as long as the shorter RNA fragments used in RT-PCR [10], thus may better correlate to active disease.

Detection and quantitation of viral protein may better indicate the presence and course of active disease. Researchers have demonstrated a shorter detection window for viral proteins when compared to RNA detection [11], implying a shorter half-life for proteins. “Point of care” rapid antigen tests (RAT) were developed to provide fast, at-home diagnosis. These mostly target intact nucleocapsid (NCAP) proteins using a lateral flow immunocapture system (**Figure 2;** top). However, the US Food and Drug Administration (FDA) communicated a stop order over concerns of their performance in late 2021 [12]. These concerns were shared by the National Health Service (NHS) of Great Britain [13], since a recent systematic review found an average sensitivity of RATs was 68.9% (61.8-75.1%, 95%CI) [14] which was 11.1% below the World Health Organisation’s performance standards of >80% sensitivity.

Liquid chromatography mass spectrometry (LC-MS) is a highly sensitive and selective [15] analytical platform amenable to high throughput analysis [16]. LC-MS platforms are available in most large clinical laboratories [17] and are widely used for measurement of a multitude of small molecules ranging from vitamin D [18] to therapeutic drugs [19]. In addition, LC-MS analysis of peptides (as surrogates for proteins) has increasingly been adopted for protein measurement in biological fluids [20,21]. LC-MS was proposed as an alternative method to RT-PCR for SARS-CoV-2 detection in mid-2020 [22]. In this work, detection of multiple SARS-CoV-2 peptides derived from the NCAP and spike (S) proteins was achievedusing material from nasopharyngeal swabs [22]. Developing from this, an arm of the UK Department of Health and Social Care initiative, *Operation Moonshot*, utilised stable isotope standards and capture by anti-peptide antibodies (SISCAPA [23]) for peptide enrichment and analysis by LC-MS in nasopharyngeal swabs, achieving 92.4% sensitivity and 97.4% specificity when compared to RT-PCR [24]. Other methods using SISCAPA-LC-MS for NCAP detection have also been published, where some have used monoclonal antibodies [25–27] and others have used polyclonal antibodies [25,28,29]. In the context of future pandemic preparation, a capture workflow using polyclonal antibodies may be useful as these can be produced faster and at less cost [30].

SISCAPA-LC-MS has primarily been used in nasopharyngeal swabs, though salivary droplets represent the main source of human-to-human transmission of the SARS-CoV-2 infection [31]. Furthermore, saliva can be collected by non-invasive means and is thus an ideal matrix for screening of virus presence.

Given the usefulness of a test that may differentiate active infections, we sought to explore how common RNA tests compare to protein tests for SARS-CoV-2 detection in saliva.

We report the adaption of the SISCAPA-LC-MS workflow using polyclonal antibodies for drool saliva (**Figure 2**; bottom) and its comparison with RT-PCR, RT-LAMP and RAT from a cohort of 102 patients hospitalised with COVID-19. Different Ct and time-to-positive (TTP) cut-offs were tested against SISCAPA-LC-MS to examine how RNA tests correlate with protein tests.

**Materials and methods**

Sample collection, ethics, and demographics

Neat saliva samples were obtained from 102 in-patients with suspected COVID-19. The participants gave informed consent as part of a prior study performed between January and July 2021 in the University Hospitals of Leicester, UK (SARS-CoV-2 RT-LAMP saliva study; REC approval number 20/EE/0212). Saliva sampling was performed at least 30 min after ingestion of meals or drug prescriptions or after teeth brushing. Participants rinsed their mouths with water 10 minutes before collection, then were asked to drool (without contaminating this with sputum) between 2 mL and 5 mL of saliva into a plain, sterile 30 mL universal container (Elkay Laboratory Products (UK) Ltd, Basingstoke, UK). The saliva samples were aliquoted and separated for RT-PCR, RT-LAMP, RAT, and SISCAPA-LC-MS experiments. These samples were stored at 4 oC for less than 3 days before RNA extraction and parallel analysis by RT-PCR and RT-LAMP. The saliva samples for RAT and SISCAPA-LC-MS analysis were stored at -80 oC prior to use.

Materials, reagents, and method development

SISCAPA-LC-MS and RAT

The methods are summarised in **Figure 2** and described below. Full details of buffers and reagents are provided in the **Supplementary data**.

Sample preparation

The generic SISCAPA protocol [28] was modified to accommodate 100 µL of neat saliva samples. Briefly, neat saliva samples were initially treated with 500 µL ethanol to inactivate any viruses, thus allowing use in a biosafety level 2 laboratory. Proteins in the ethanol treated saliva (500 µL) were precipitated by addition of 1000 µL of ice-cold acetone followed by centrifugation at 13,000 *g* for 10 minutes at 4 oC. The precipitated pellets were reconstituted in 100 µL of 200 mM ammonium bicarbonate buffer and split for use in SISCAPA-LC-MS (80 µL) and RAT (20 µL).

RAT

Part of the reconstituted pellet (20 µL) was transferred to extraction tubes along with 180 µL of extraction buffer provided in the Innova SARS-CoV-2 Antigen Rapid Qualitative Test kit (Innova Medical Group; Pasadena, CA, USA). The tubes were mixed and 100 µL were loaded onto the RAT test strip. NCAP was allowed to bind to the bound antibodies over 30 min. Photos were taken and uploaded into ImageJ (version 1.51o) where optical densitometry values were calculated over the entire test strip. Values were then normalised using the control strip lines to create a ratio.

SISCAPA surrogate peptide enrichment for LC-MS analysis

An “addition only” protocol was employed for analysis of viral NCAP by SISCAPA-LC-MS [28]. Reconstituted salivary protein pellets (80 µL) were transferred to a 96-deepwell plate and the proteins were denatured by addition of 73 µL denaturant solution [28]. Tryptic digestion of proteins was performed by addition of 10 µL of digestion solution followed by mixing and incubation for 30 minutes at 37 oC. Digestion was terminated with 5 µL stop solution [28]. Stable isotope labelled forms of the SARS-CoV-2 NCAP peptides (AYNVTQAFGR (AYN), DGIIWVATEGALNTPK (DGI), and ADETQALPQR (ADE)) were added as standards for quantitation. Thirty microliters of a suspension of protein G magnetic beads cross-linked with polyclonal, affinity-purified SISCAPA anti-peptide antibodies specific for each of the three proteotypic, surrogate target peptides from the SARS-CoV-2 nucleoprotein were added to the mixture and the plate was incubated for 1 hour at room temperature on a microplate shaker (Stuart SSM5) at 180 RPM. The microplate was loaded onto a custom-made magnetic plate for sample preparation (SISCAPA Assay Technologies Inc.) designed to immobilize the magnetic beads and allow the unbound digest fraction to be removed before washing the beads thrice with 150 µL wash buffer [28]. Bead-bound peptides were acid-eluted from their specific antibody with 45 µL elution buffer before analysis by LC-MS [28]. In this study the SISCAPA protocol was performed by manual pipetting.

Targeted LC-MS analysis

Immuno-enriched proteotypic, surrogate peptides were resolved, separated and analysed using an ACQUITY ultra performance liquid chromatography (UPLC) I-Class PLUS system coupled to a Xevo TQ-XS Tandem Quadrupole mass spectrometer (Waters Corporation, Manchester, UK), equipped with a Z-spray electrospray ionisation (ESI) source set to positive mode. An ACQUITY UPLC Peptide BEH C18 1.7 µm, 2.1 mm x 50 mm column was fitted to the LC system.

Peptide eluates were analysed as 10 µL injections and chromatographic separation was performed at a constant flow rate of 0.6 mL/min with water/0.1% formic acid (A) and acetonitrile/0.1% formic acid (B) mobile phases. A 3.5-minute gradient was used: 95% mobile phase A initial to 0.25-minutes, to 60% A at 2.2-minutes, to 5% A at 2.3-minutes until 2.8-minutes and to 95% A at 3.15-minutes. Column temperature was set to 40 oC. For the source parameters; capillary voltage (0.6 kV), desolvation temperature (650 oC), desolvation flow (1000 L/hour) and cone flow (150 L/hour) were set accordingly. Simultaneous multiple reaction monitoring (MRM) of SARS-CoV-2 peptides AYN, DGI, and ADE and their stable isotope labelled analogues, was performed (**Supplemental Table I**).

LC-MS data processing, calibration, and interpretation

Data were imported into TargetLynx v4.2 (Waters Corporation, Manchester, UK) for transition review and into RStudio (version 1.4.1106) for calibration modelling and quantitative analysis. Peaks were integrated using Savitzky-Golay smoothing, and each integration was reviewed. Peaks were included for analysis only if >2 transitions were present for qualification of the signal. Calibration models were determined through triplicate analysis of saliva fortified with the NCAP peptides (AYN, DGI, ADE) over the range 0.01 to 25 fmol/µL. Models with the highest R2 values were selected and applied to all data for quantitative analysis (**Supplemental Figure 2**).

A positive sample was defined as the presence of AYN or the presence of both ADE and DGI peptides. The AYN peptide sequence has so far not been significantly affected by SARS-CoV-2 virus mutations [28] and its signal characteristics (noise, peak shape) were the most consistent of the peptides examined (data not shown). AYN and DGI have not mutated in known variants, although the Delta variant contains a D377Y mutation so that the ADE peptide becomes AYE [32]. All concentrations reported used the maximum value obtained for each peptide, where positive.

RT-PCR and RT-LAMP

RNA extraction

RNA was extracted from 200 µL of neat saliva as previously described [33]. Briefly, saliva was mixed with a binding solution (265 µL) using the MagMAX Viral/Pathogen Nucleic Acid Isolation Kit on a KingFisher Flex Purification system (Thermofisher, Loughborough, UK). The nucleic acid eluates were stored at -20 oC prior to RT-PCR analysis.

 RT-PCR analysis

cDNA was produced by reverse transcribing the purified viral RNA using a RealStar® SARS-CoV-2 RT-PCR kit (Altona Diagnostics, Hamburg, Germany). The SARS-CoV-2 *E* gene sequence region was targeted, using 10 µL of nucleic acid eluate. All Ct values were reported below Ct 40.

 RT-LAMP analysis

*Orf1a* and *NE*-gene sequences were targeted using a fluorescence-based assay with reagents from New England Biolabs (Hitchin, UK) as described previously [10]. Reagents included WarmStart® RT-LAMP Master Mix (1 X), fluorescent dye (1 X), thermolabile UDG (0.02 U/µL), thermolabile dUTP 700 µM), RT-LAMP primers (1 X, standard concentration) and 5 µL of nucleic acid eluate. The final reaction solution (25 µL in nuclease free water) was incubated at constant 65°C using a thermocycler (StepOnePlus; Thermo Fisher, Loughborough, UK) with signal acquisition every 60 secs. Time to positive (TTP) was measured in minutes.

Statistical analysis

Specificity, sensitivity, and accuracy analysis were performed for each of the methods against RT-PCR. Ct thresholds defined by the 95th and 75th percentiles were also tested. Peptide concentration (SISCAPA-LC-MS), Ct (RT-PCR), TTP (LAMP), and normalised optical densitometry data (RAT), results were imported into GraphPad Prism (version 4.07) for regression analysis and for F-tests comparing each method. Slopes that were below a p value of 0.05 were deemed significantly different to zero. Regression analysis was performed only on positive samples.

**Results**

SISCAPA enrichment of SARS-CoV-2 nucleoprotein peptides in trypsin-digested saliva from hospitalised patients

Representative LC-MS measurements of enriched, surrogate NCAP peptides in saliva samples from a COVID positive patient, a COVID negative patient and a matrix free (blank) sample are shown in **Figure 3.** The left-hand, middle, and right-hand panels display the MRM chromatograms for peptides DGI (retention time [T*R*] 2.0 min), AYN (T*R* 1.5 min), and ADE (T*R* 1.0 min). Each peptide contains multiple fragment ion transitions in the COVID-19 positive patient (top panels). These transitions were absent in samples from both the COVID-19 negative patient (middle panels) and matrix free (blank) samples (bottom panels). The top panels in **Figure 3** demonstrate that signals for each peptide fragment were strong enough to confidently identify the peptides. Unequivocal interpretation of the peptide peaks was facilitated by the SISCAPA immuno-affinity enrichment of the peptide targets, which effectively selectively, and specifically bound the target surrogate peptide while eliminating the non-specific peptides comprising the matrix background, thus significantly improving the signal to noise ratio. For quantitation, calibration models were fitted (**Supplemental Figure 2**). ADE, AYN, and DGI ADE peptides were all fit using a quadratic model (R2 0.99, 0.98, and 0.99, respectively).

Virus protein and nucleic acid detection in saliva using SISCAPA-LC-MS, RAT, RT-PCR, and RT-LAMP

Analysis of 102 saliva samples was performed using each of the protein detection or nucleic acid detection platforms. Virus peptides were detected in 25 patients (SISCAPA-LC-MS; median concentration 2.03 fmol/µL; S.D. 2.93). RAT showed a positive result in 13 patients (median optical density ratio 0.11; S.D. 0.42). Whereas a higher number of positives was seen using nucleic acid amplification methods. Virus RNA was detected by nucleic acid amplification in 44 patients (LAMP *NE* gene; median TTP 11.8-mins; S.D. 2.9), 44 patients (LAMP *Orf1a* gene; median TTP 9.2-mins; S.D. 2.5), and 53 patients (RT-PCR; median Ct 23.2; S.D. 6.0). A comparison of the results is shown in **Figure 4**. Of the cohort, 22.4% were analytically positive across SISCAPA-LC-MS, RAT, RT-LAMP, and RT-PCR methods; 8.6% of cases were positive only by SISCAPA-LC-MS; 15.5% of cases were positive only by RT-PCR. No unique cases were identified by RAT. All samples positive by RT-LAMP were also positive when tested by RT-PCR.

Given that the stoichiometry of a proteotypic, surrogate peptide to its protein is 1:1, the median NCAP protein concentration in saliva was determined by the SISCAPA-LC-MS method to be 2.03 fmol/µL.

Specificity, sensitivity, and accuracy

Detection of the virus nucleoprotein in patients’ saliva by SISCAPA-LC-MS showed sensitivity of 37.7%, specificity of 89.8%) and accuracy of 62.7% based on comparisons with RT-PCR (**Table I**). RAT had lower sensitivity and accuracy across all samples (24.5% and 60.8%, respectively), and higher specificity (100%). Setting Ct thresholds by the 95th (Ct <27.5) and 75th (Ct <20.0) percentiles increased the sensitivity and accuracy between SISCAPA-LC-MS and RT-PCR. At <20 Ct, the SISCAPA-LC-MS sensitivity was 78.9% when compared with RT-PCR. In addition, RT-LAMP sensitivity and specificity increased to 100% at Ct values <27.5 the 75th percentile. Virus protein was only detected in 5 samples (SISCAPA-LC-MS) and 1 sample (RAT) when the RT-PCR Ct values were above 20. One case was detected by SISCAPA-LC-MS when the Ct was above 27.5. Over a quarter (26.6%) of RT-PCR cases showed Ct values above 27.5.

 Regression analysis

Results obtained with RT-PCR did not show significant correlation with SISCAPA-LC-MS using all data. The positive correlation between SISCAPA-LC-MS and RT-LAMP data (O*rf1a*: R2 0.34, p value 0.007; *NE*: R2 0.28, p value 0.018) was stronger than SISCAPA-LC-MS and RT-PCR (R2 0.05, p value 0.32; **Figure 5**). On exploration of Ct cut-offs, regression on 75th percentile (Ct <20.0) demonstrated significant correlation between SISCAPA-LC-MS and RT-PCR (R2 0.57, p value 0.002; **Figure 5A**). Setting cut-offs for RT-LAMP did not significantly improve correlation to SISCAPA-LC-MS: R2 was similar across each threshold (**Figure 5C-D**). On regression analysis, SISCAPA-LC-MS and RAT positively correlated (R2 0.47; **Figure 5B**). RT-LAMP and RT-PCR were linearly predictive of each other across most of the dataset, except Ct <18 where RT-LAMP followed a similar saturation effect as seen in the SISCAPA-LC-MS and RT-PCR regression analyses (**Supplemental Figure 3**).

**Discussion**

This study describes 1) a high throughput and scalable, SISCAPA immuno-affinity LC-MS method capable of detecting and measuring SARS-CoV-2 proteins in saliva samples 2) a comparison of the SISCAPA-LC-MS protein (peptide) detection method with widely used virus RNA detection methods 3) a discussion of the suitability of saliva testing using RT-PCR, RT-LAMP, SISCAPA-LC-MS, and RAT for diagnosis of COVID-19 4) a description of discrepant results between RNA and protein detection methods 5) an indication that at different Ct thresholds nucleic acid tests may confer with protein tests.

It has been widely demonstrated by RT-PCR [33–36] and RT-LAMP [10,37–40] that saliva is an appropriate sample for detection of SARS-CoV-2 RNA. Since there is evidence that virus nucleic acid can persist in patients [7,11], it is questionable whether methods such as RT-PCR can be used to determine infection or treatment status. The use of Ct as a surrogate for infectious status has drawn major criticism given the lack of standardisation between RT-PCR methods and differences seen between laboratories [5]. Detection of virus protein may be more indicative of active infection given their shorter detection window [11], although more research is required on this subject. We therefore sought to compare viral protein detection against RNA detection.

A few studies have used mass spectrometry platform for detection of SARS-CoV-2 proteins in saliva. Matrix assisted laser desorption ionization time of flight (MALDI-TOF) MS has been used for protein detection [27,41–43], and nucleic acid detection [44]. A targeted metabolomics approach for identification of COVID-19 biomarkers demonstrated difficulty regarding sampling variability [45]. Reports have described methods for SARS-CoV-2 virus protein detection in triple point throat/nasopharynx/saliva swabs [28] and spiked saliva [25], although to the best of our knowledge, no study exists that uses SISCAPA-LC-MS in saliva samples within a clinical cohort. An LC-MS method that detects NCAP peptides in saliva and gargle fluid showed similar limits of detection (LOD) (0.5 fmol/µL) to that shown in this study (0.01 fmol/ µL for AYN) and led us to believe that the protein detection assay may be suitable for the clinical samples used herein (median viral load was 2.03 fmol/µL) [42]. Interestingly, Kollhoff *et al*., 2023 has recently demonstrated a similar NCAP concentration in clinical nasopharyngeal swabs with SISCAPA-LC-MS [27].

We have recently shown 95% sensitivity and specificity in a cohort of nasopharyngeal swabs when comparing RT-LAMP against RT-PCR [10]. In saliva, Amaral *et al.,* (2021) demonstrated RT-LAMP sensitivity of 67.2% compared to RT-PCR using nasopharyngeal swabs over the Ct range of 0-40, increasing to 85% sensitivity below 28 Ct [37]. One study that used saliva found the sensitivity of RT-LAMP to be slightly worse than that of RT-PCR (70.9% vs 81.6%) in comparison to a nasopharyngeal RT-PCR test [40]. Our results align with those elsewhere, indicating that saliva RT-LAMP may be a suitable alternative to RT-PCR given the quicker turnaround and equivalent cost. In addition, detection of larger genomic fragments may better reflect active infection in comparison with RT-PCR.

We did not compare our methods against highly sensitive, automated antigen detection methods such as the LIAISON® SARS-CoV-2 Antigen chemiluminescent immunoassay assay [46]. Such tests are capable of processing reasonable numbers of samples (136 per hour) and are less expensive when compared to both RT-PCR and SISCAPA-LC-MS (approx. £3 vs. £20) [47]. However, it is not clear how antibodies to viral proteins would interfere with such immunoassays. Antibodies produced by patients upon infection or by immunization could be problematic in this regard, yielding lower or false-negative values. SISCAPA technology avoids this problem since antibodies are inactivated by trypsin digestion as part of the standard protocol.

We demonstrated that SISCAPA-LC-MS may detect NCAP in saliva of almost twice the number of people when compared to RAT, with the caveat that the RAT was used outside of its stipulated remit *i.e.,* the manufacturer validated their tests using nasopharyngeal samples. Also, less neat saliva (10 µL, final equivalent) was loaded onto the RAT test strips versus SISCAPA-LC-MS (approx. 17.8 µL injected), though one SISCAPA-LC-MS positive at 0.7 fmol/µL was also detected by RAT, demonstrating comparable absolute sensitivities. Again, interference with the point-of care immunoassays by anti-virus antibodies may be problematic.

SISCAPA-LC-MS detected viral protein (peptide) in only a fraction of the samples that were deemed positive based on the saliva RT-PCR test. This observation may be explained by the great sensitivity achieved by PCR-amplification assays whereas protein detection by the non-amplifying SISCAPA-LC-MS was less sensitive. Thus, PCR methods present excellent population screening assays for determination of virus RNA. Indeed, specificity was high against RT-PCR (89.8%). In only a few instances was viral protein detected in the absence of viral RNA, which could be explained by differential clearance.

In contrast to virus nucleic acid, viral proteins are cleared rapidly [11,48] in correlation with the viral loadand thus may represent a more current picture of infection status and likelihood of transmission. It may be possible to use Ct cut-off values from PCR data to act as a surrogate for virus protein detection, but this will require studies comparing to viral cultures [5]. Our investigation of saliva Ct thresholds seen in RT-PCR showed that Ct 20 appears to be a possible cut-off level where SISCAPA-LC-MS and RT-PCR results become more congruous. The R2 between the measures was still low at this point (R2 0.57), however the use of more selective monoclonal capture antibodies rather than the polyclonal antibodies may have seen greater correlation. Van puyvelde *et al*., (2022) compared the two antibodies in spiked universal transport media and found enhanced performance with monoclonal antibodies at lower concentrations [25].

Hällqvist *et al.,* (2023) recently used SISCAPA-LC-MS with monoclonal antibodies on nasopharyngeal swabs, demonstrating 92.4% sensitivity and 97.4% specificity when compared to RT-PCR [24]. This indicated that the protein detection platforms were congruous with genetic material detection platforms. The salivary method we used for the work herein was less comparable to RT-PCR. However, as our study was done before the availability of the monoclonal antibodies (*circa* early 2021), polyclonal antibodies were used. Mangalaparthi *et al.,* (2021) similarly used polyclonal antibodies and compared to RT-PCR with nasopharyngeal swabs and established an R2 of 0.63 for the DGI peptide with 100% specificity and 93% sensitivity [29], implying nasopharyngeal swabs are more suitable than saliva when comparing SISCAPA-LC-MS to RNA tests.

The manual implementation of the SISCAPA-LC-MS protocol described here currently can provide results in less than 2.5 hours of sampling (2.25 hours for sample processing, 3.5-min LC-MS run time). Using four 96-well plates, approximately 400 samples can be manually processed per day using a single, at capacity, LC-MS system. Full automation of the SISCAPA workflow has been shown to drastically increase the throughput capabilities of this approach by a) increasing the number of samples that can be processed in a day [21] and b) reducing the need for chromatography by eliminating the nonspecific peptide background [40]. Given the state of automation of this workflow (versions available for most laboratory robotic platforms) and the fact that the LC format used here (UPLC) is also utilised in routine clinical laboratories, we foresee few challenges for large scale implementation of this method. It is also possible that dried blood spot sampling of patients can be used for longitudinal COVID-19 monitoring [49]. Currently, the cost per SISCAPA-LC-MS test is approximately equivalent to that of RT-PCR, depending on location.

**Limitations of the study**

Saliva was stored for up to 3 days prior to analysis at 4 oC prior to RT-PCR and RT-LAMP analysis, although others have demonstrated non-significant change in Ct after 26 days at 4 oC.[50]. No study was done on the preanalytical differences and reproducibility of drool saliva, where issues have been discussed in previous studies [45]. Data on patient demographics were not sought, neither were data on initial onset, symptom severity and disease outcomes. Such data would have enabled assessment of viral load *vs.* disease status determined by protein and RNA assays. Future work on the comparison of the protein and genomic material would benefit by contextual information at the time of sample collection (*i.e.,* which measurement had a better diagnostic/prognostic value). The comparison to viral culture methods would have also been beneficial in order to establish a transmission/infectiousness cut-off against the tests presented herein. Furthermore, the use of monoclonal SISCAPA antibodies may have increased assay sensitivity, although these were not available at the time of the study. The validity of the SISCAPA-LC-MS method could be supported with future studies exploring these types of experimental and clinical data.

RAT was used on a matrix that the tests were not explicitly produced for, although saliva was investigated across all detection platforms and may therefore be compared.

In the work reported here, a saliva based RT-PCR kit was used outside its intended remit *i.e.,* use in nasopharyngeal swabs. Furthermore, the SISCAPA-LC-MS test and the PCR-based tests compared dissimilar viral components (genetic material vs. proteins). Their direct comparison may not be meaningful without context of what each component is a marker for *i.e.,* exposure*,* active infection *etc*.

**Conclusion**

We report a high throughput and scalable SISCAPA immuno-affinity LC-MS method that can detect and measure SARS-CoV-2 protein in saliva samples using polyclonal antibodies. We also report detection of viral nucleic acid by RT-LAMP and RT-PCR. A RAT was also tested for the detection of viral protein. The relationship between RT-PCR Ct values and protein load as determined by the SISCAPA-LC-MS method requires further study. SISCAPA-LC-MS can be a useful tool for studying viral peptides, although we detected SARS-CoV-2 protein less frequently than viral RNA in saliva.

**Disclosures/Conflict of Interest**

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**Tables legends**

**Table I**. Sensitivity, specificity, and accuracy analysis of saliva RT-PCR compared to SISCAPA-LC-MS, RAT, and RT-LAMP. Ct cut-offs at 27.5 and 20 represent the 95th and 75th percentiles of the RT-PCR saliva data, respectively.

**Figure legends**

**Figure 1.** Reverse transcription polymerase chain reaction (top) and reverse transcription loop-mediated isothermal amplification (bottom) workflows for the amplification and detection of SARS-CoV-2 nucleic acid (ss-RNA). For RT-PCR, a sample is inactivated and RNA extracted followed by amplification through successive heating cycles with primers and polymerase. For RT-LAMP, a sample is inactivated, nucleic acid is extracted, and the ss-RNA is amplified by DNA polymerase in a single step with multiple primers, forming amplified products through mediated loop complexes. Both may be detected via fluorescence.

**Figure 2.** Rapid antigen test workflow for the detection of SARS-CoV-2 proteins is shown (top), where a sample is loaded onto the device and intact SARS-COV-2 protein conjugates with antibodies, forming a complex which binds to the test strip. SISCAPA-LC-MS workflow for the capture and detection of SARS-CoV-2 nucleocapsid proteotypic peptide surrogate (top). Viral proteins are subject to tryptic digestion and nucleocapsid (NCAP) peptides (AYNVTQAFGR, DGIIWVATEGALNTPK and ADETQALPQR) are captured by SISCAPA-high affinity anti-peptide antibodies before LC separation and analysis by mass spectrometry.

**Figure 3.** LC-MS chromatogram of the three SISCAPA-enriched, targeted NCAP peptides from COVID positive saliva, COVID negative saliva and a blank sample (to show baseline noise).

**Figure 4.** Venn diagram showing the overlap of COVID-19 positive patients diagnosed using saliva by SISCAPA-LC-MS, RAT, RT-LAMP and RT-PCR.

**Figure 5**. Regression analysis of SISCAPA-LC-MS against different percentile cut-offs (95th and 75th) for RT-PCR (**A**) and RT-LAMP (**C, D**) in saliva samples from COVID-19 positive patients. The value at each cut off (Ct or TTP) is annotated within each graph. Linear regression was performed only for samples positive in both comparators. An insert of the 75th percentile is shown in **A**. Ct and TTP axes are scaled by Log2. Regression analysis of SISCAPA-LC-MS against RAT is also shown (**B**). Photos of RAT were uploaded into densitometry software to calculate optical density across the test and control strips, using the control strips to normalise the data.