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A fast and sensitive absolute quantification assay for the detection of SARS-CoV-2 peptides using parallel reaction monitoring mass spectrometry

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ABSTRACT

The on-going SARS-CoV-2 (COVID-19) pandemic has called for an urgent need for rapid and high-throughput methods for mass testing and early detection, prevention as well as surveillance of the disease. We investigated whether targeted parallel reaction monitoring (PRM) quantification using high resolution Orbitrap instruments can provide the sensitivity and speed required for a high-throughput method that could be used for clinical diagnosis. We developed a high-throughput and sensitive PRM-MS assay that enables absolute quantification of SARS-CoV-2 nucleocapsid peptides with short turn-around times by using isotopically labelled synthetic SARS-CoV-2 concatenated peptides. We established a fast and high-throughput S-trap-based sample preparation method and utilized it for testing 25 positive and 25 negative heat-inactivated clinical nasopharyngeal swab samples for SARS-CoV-2 detection. The method was able to differentiate between negative and some of the positive patients with high viral load. Moreover, based on the absolute quantification calculations, our data show that patients with Ct values as low as 17.8 correspond to NCAP protein amounts of around 7.5 pmol in swab samples. The present high-throughput method could potentially be utilized in specialized clinics as an alternative tool for detection of SARS-CoV-2 but will require enrichment of viral proteins in order to compete with RT-qPCR.

1. Introduction

The on-going human Coronavirus Disease 2019 (COVID-19) pandemic caused and still causes severe global health and economic problems in almost every country in the world. Since the beginning of the pandemic till May 2022, COVID-19 has affected >500 M people and caused >6 M deaths worldwide [1]. The disease is caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), a novel and more virulent strain of coronaviruses (CoVs) [2]. This strain of coronavirus is more virulent but similar to two other strains of betacoronaviruses SARS-CoV and Middle East respiratory syndrome (MERS-CoV) [3]. Like other CoVs, the SARS-CoV-2 genome comprises of linear, single-stranded positive-sense RNA which encodes 10 genes, responsible for production of total 26 proteins. Out of the 26 proteins, four structural proteins contribute to ~80% of the genome. These four structural proteins comprise of a spike glycoprotein (S), which enables viral entry into the mammalian cell by binding to the angiotensin-converting enzyme 2 (ACE2) receptor, a nucleoprotein (N) that provides stability to the viral genome by directly binding to the RNA, an envelope protein (E) and membrane protein (M) that forms the outer layer of the virus [4]. The N and S protein copies per virion are estimated to be approximately 1000 and 300, respectively. The remaining proteins of the genome are required for functions such as proofreading, RNA polymerase, proteases and other supporting proteins for replication of the genome.

The predominant method of testing for individuals infected with SARS-CoV-2 includes real-time quantitative polymerase chain reaction (RT-qPCR), which is usually done on nasopharyngeal (NP) swabs. The RNA is extracted from these swabs and amplified using specific primers, which makes it more specific, sensitive and relatively rapid, deeming it as the gold standard assay for SARS-CoV-2 detection by World Health Organization (WHO). Other rapid tests including Simple Amplification Based Assay (SAMBA) [5], which uses nucleic acid for detection, serological assays such as Lateral Flow Immunoassays (LFA) [6] and enzyme-linked immunosorbent assays (ELISA) have also been

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developed. These have been implemented owing to the global pressure for identifying the infected individuals with quick turnaround rates. The high demand for RT-qPCR testing has in recent times caused a global shortage of reagents as well as other rapid tests are prone to false-positive and false-negative reporting which might be caused due to inhibition of substances in clinical samples [7]. Thus, at times during the pandemic, complementary alternative assays for detection of SARS-CoV-2 became increasingly urgent to share the burden of immediate mass testing required for management of the pandemic.

In recent years mass spectrometry (MS)-based targeted proteomic approaches have increasingly been implemented in clinical labs due to advancements in the sensitivity and accuracy of instrumentation. An excellent example of this is successful application of Matrix-Assisted Laser Desorption/Ionization Time-Of-Flight mass spectrometry (MALDI-TOF-MS) for characterization of Nucleoprotein (NCAP) and Spike Glycoprotein (SPIKE) from the SARS virus, which caused an outbreak in 2003 [8]. Building on this, many MS-based methods have emerged for detection of the SARS-CoV-2 NCAP, and SPIKE proteins based on their tryptic peptides. Most of these methods either have been built directly from specimens infected with high viral loads of SARS-CoV-2; for instance, a study by Gouveia *et al* selected 14 peptides from proteomics of SARS-CoV-2 infected Vero cells [9], or from clinical specimens where enrichment was required for detection of these peptides. Ihling *et al* used gargle solution for detection of SARS-CoV-2 peptides from three COVID-19 positive individuals [10]. In another study by Nikolaev *et al*, peptides from NCAP protein were detected by tandem mass spectrometry using nasopharyngeal epithelial scrapings [11]. Similarly, Singh *et al* [12], Gouveia *et al* [13] and Saadi *et al* [14] utilized nasopharyngeal swabs to establish proof-of-principle studies for detection of SARS-CoV-2 peptides. More recently Renuse *et al* [15] present a LC-MS based method where peptides for NCAP and SPIKE were enriched using immunoaffinity beads. Puyvelde *et al* [16] have attempted to use a quantification concatemer (QconCAT) Stable Isotopic Labelled (SIL) internal standard for improving the efficiency of their MRM-MS assay. Importantly, most of these protocols include acetone precipitation-based methods for sample preparation and are either low-throughput or time consuming, thereby lacking the short turn-around time required for a clinical high-throughput assay.

In this study, we report development of a high-throughput liquid chromatography mass spectrometry based parallel reaction monitoring (PRM) assay for detection and absolute quantification of SARS-CoV-2 peptides, which attempts viral detection directly from patient samples. We could detect viral proteins in patients with high viral load and Ct values up to 17.8 in a cohort of 50 patient samples. This method allows detection of SARS-CoV-2 peptides with a turnaround time of ~2 h for each patient starting from sample preparation to reporting the results and up to 200 samples per day.

2. Materials and methods

2.1. Chemicals and reagents

Sodium dodecyl sulfate (SDS), >98% pure was purchased from VWR International Limited. Triethylammonium bicarbonate (TEAB) 1 M, and ortho-phosphoric acid 85%, Tris(2-carboxyethyl) phosphine (TCEP) and Iodoacetamide ≥98% were purchased from Sigma. Proteomics Grade Trypsin was purchased from Pierce Thermo Fisher Scientific. Acetonitrile, Methanol (LC-MS grade) and Formic Acid (>99.0%, LC-MS, UHPLC-MS) were purchased from Fisher Scientific. Formic acid was obtained from Merck. 96-well plate (Cat#: C02-96well-1) and micro format (Cat#: C02-micro-80) S-traps were purchased from Protifi. Evotip disposable C18 columns (Cat#: EV2001) were purchased from EVOSEP. SARS-CoV-2 Spike Glycoprotein (Full-Length) expressed in CHO cells from Native Antigen, Synthetic Sputum from LGC and PolyQuant Cov-MS QconCAT from PolyQuant were received as part of the COVID Moonshot Consortium in purified form, for sequence information see (Supplementary Data 4 and Supplementary Fig. 1). SARS-CoV-2 Nucleoprotein was received from the University of Sheffield.

2.2. Preparation of SARS-CoV-2 protein standards for LC-MS analysis

The SARS-CoV-2 recombinant protein standards were processed for peptide identification, targeted MS-based proteomic method development, and to determine their limits of detection with the targeted method. Each protein standard was processed separately by suspension trapping (S-Trap micro format, Cat#: C02-micro-80) [23], with minor modifications as previously described [17,18], for method development and peptide identification. Modifications included a single step reduction and alkylation with 10 mM TCEP and IAA, respectively, and digestion at 47 °C for 1 h using a 1:10 trypsin: protein ratio. The method was further optimized to exclude the reduction and alkylation step, as well as sample elution, to achieve a faster and high-throughput method discussed further in the results section. The final method for S-Trap digestion of protein standards was as follows; 1:10 (v:v) addition of 12% phosphoric acid for a final concentration of 1.2% followed by dilution in S-Trap binding buffer (100 mM TEAB in 90% MeOH, pH 7.1) for a final volume of 260 μL after which the samples were loaded onto S-Trap micro digestion column by centrifugation at 4000 x*g* for 2 min. The loaded samples were then washed 5 times with S-Trap binding buffer followed by centrifugation at 4000 x*g* for 2 min after each wash. Samples were digested by directly adding Trypsin (1:10, wt:wt of sample protein amount) in 50 mM TEAB buffered to pH 8.0 and incubating for 1 h at 47 °C. After digestion, peptides are eluted with 50 μL of 50 mM TEAB followed by 50 μL of 0.2% formic acid. The eluates were loaded into Evotip C18 columns to be analysed by LC-MS.

2.3. LC-MS Method and data analysis

LC-MS/MS analysis was performed on a Thermo Scientific QExactive-HF (QE-HF) hybrid mass spectrometer coupled to an Evosep One liquid chromatography instrument [27]. The Evosep One was operated using the standard 200 samples per day method at a flow rate of 2 μl/min with a 7.2-min total run time per sample [19]. The Evosep One was equipped with a 40 mm × 150 μm column from Evosep packed with Dr. Maisch C18 AQ, 1.9 μm beads. The QE HF was operated in either DDA or PRM-mode using a resolution of 60,000 AGC target of 2×10^5 for DDA and 30,000 for PRM, maximum injection time of 50 ms, and a quadrupole isolation width of 1.2 *m/z*. Peptides were selected for MS/MS data acquisition using an un-scheduled method and fragmented using collision energies optimized for each peptide. An electrospray voltage of 2.1 kV and capillary temperature of 300 °C, with no sheath and auxiliary gas flow, were used. MaxQuant 1.6.10.43 was used for peptide and protein identification [20] using UniprotKB/Swissprot databases for *Homo Sapiens* containing 42,437 sequences downloaded on September 2020 and a combined viral database SARS and influenza viruses including the SARS-Cov-2 virus sequences. Targeted proteomic method refinement and data analysis was performed in Skyline-daily 20.2.1.286 [21]. The recombinant protein standards and the QconCAT construct were identified by LC-MS analysis performed in DDA mode and the peptides belonging to the recombinant proteins as well as to the QconCAT construct were identified by a MaxQuant search against the database mentioned above. The MaxQuant output files from this DDA search were used to build a spectral library in Skyline. Further optimization runs and patient sample results were imported to Skyline and identified with this spectral library, default peptide and transition settings were used. In the final PRM method, the peptides were identified by matching their elution time with a 1-min window with a dot product (dotp) value of larger than 0.9 in the case of strong positive samples and heavy labelled QconCAT peptides, and for tentatively identified positive cases with a dotp value of larger than 0.5. Ion match tolerance was set to 0.05 *m/z* and method match tolerance was set to 0.055 m/z.

2.4. Determination of LOD and LOQ

Calibration curves for LOD and LOQ determination were obtained from SARS-CoV-2 NCAP peptides in 0.1% formic acid, synthetic sputum, swab and saliva samples. To generate the calibration curves in different matrices, proteotypic peptides for NCAP were spiked into each matrix at 10 fmol/μL followed by serial dilution (2-fold) until 10 attomoles/μL was reached. For each concentration, 5 μL sample was injected on the LC-MS system in triplicates A calibration curve was created using the raw intensity values for each peptide in each matrix and a regression value (R^2) was calculated. Limits of detection and quantitation were determined using the standard deviation of the points from the curve and the slope values and were defined as $LOD = 3.3\sigma/S$ and $LOQ = 10\sigma/S$ where σ is the standard deviation of the calibration curve and S is the slope.

2.5. Patient samples for method validation

Fifty nasopharyngeal swab samples including both positive and negative for COVID-19, were received from the Newcastle Upon Tyne Hospitals NHS Foundation Trust, in 2.5 mL viral transfer medium (VTM). The samples were stored at −20 °C until further processing.

2.6. Patient sample -preparation for LC-MS analysis

Nasopharyngeal swab samples were received in 2.5 mL of Viral Transport Medium(VTM), comprising of Anderson's modified Hanks Balanced Salt Solution (8.0 g/L NaCl, 0.4 g/L KCl, 0.05 g/L Na_2HPO_4, 0.06 g/L KH_2PO_4, 1.0 g/L Glucose, 0.7 g/L $NaHCO_3$, 0.2 g/L $MgSO_4.7H_2O$, 0.14 g/L $CaCl_2.2H_2O$) with 2% (v:v) heat-inactivated fetal bovine serum, 100 μg/mL gentamicin and 0.5 μg/mL amphotericin B, as recommended by the CDC [22].The virus was inactivated by heating at 80 °C for 5 min and 1 mL of 20% SDS was added to the VTM containing the swab for a final concentration of ~5% which is compatible with the S-trap method of sample digestion. The samples were vortexed for 15 min followed by centrifugation at 1500 xg and the entire solution was collected, aliquoted and stored at −80 °C for further use. From these aliquots 50 μL of each sample were transferred to LoBind Eppendorf tubes and 40 fmol of SARS-CoV-2 QconCAT protein was spiked in. Clinical patient samples were further processed for LC-MS analysis using 96-well plate S-Trap digestion columns with the same protocol as mentioned above for the protein standards. After elution, the samples were loaded into Evotip C18 columns to be analysed by LC-MS. (Fig. 1).

3. Results

A high-throughput LC-MS method should be reproducible, robust and sensitive. Towards our goal of developing a high-throughput PRM method for detection of SARS-Cov-2 peptides, we developed a fast and reproducible sample preparation method which can be used in conjunction with the PRM method. The details of the of the sample preparation and LC-MS method can be found above in the methods section.

3.1. Sample preparation for high-throughput analysis of SARS-CoV-2 samples

The sample preparation was designed to accommodate nasopharyngeal swabs which are the preferred sample collection methods in use in the clinic. In order to return clinical data quickly, we aimed to design the sample preparation method for high-throughput sample preparation, efficient virus inactivation and analysis within 2 h (Fig. 1). To this end, we have investigated ways to decrease the sample preparation time significantly.

As none of the previously reported proteotypic peptides of NCAP or SPIKE contain a cysteine, we tested if reduction with TCEP and

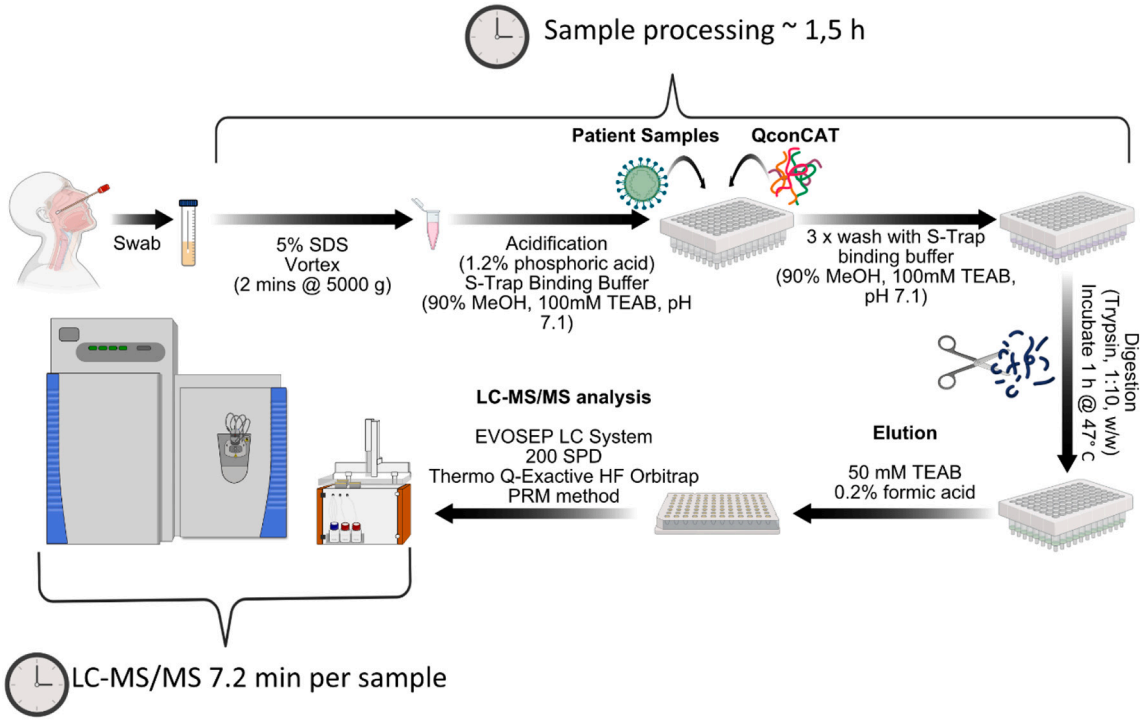

Fig. 1. A stepwise workflow for high-throughput sample processing for LC-MS based detection of SARS-CoV-2 peptides. 20% SDS was added to nasopharyngeal swab samples from patients for a final concentration of 5%. Fifty μL of the 3 mL sample were added onto 96-well S-Trap plates as well as the QconCAT protein, where the samples were washed, digested, and eluted in a mass spectrometry compatible buffer. The eluates were directly separated on an EvoSep LC system and analysed on a QExactive HF mass spectrometer. The present method allows efficient digestion of multiple samples with various matrices under 2 h, permitting the LC-MS and data analysis in approximately 15 mins.

alkylation with IAA were necessary for the efficient detection of the target peptides. Digestion efficiency was assessed by LC-MS/MS analysis of tryptic peptides and was found to be unaffected even when samples were not reduced and alkylated before digestion. (Supplementary Fig. 2A).

In the standard S-Trap protocol peptides are eluted with two aqueous (50 mM TEAB and 0.2% formic acid) and an organic (50% MeCN, 0.2% formic acid) step. Due to the organic solvent, the samples require vacuum drying before LC-MS analysis. In order to save time, we tested if we could omit the elution in MeCN, thereby allowing straight injection of the eluted digests into the mass spectrometer. Indeed, omission of the organic elution did not affect overall intensities of SPIKE and NCAP peptides (Supplementary Fig. 2B).

3.2. Identification of SARS-CoV-2 proteotypic peptides for targeted analysis

Selection of appropriate, proteotypic peptides is a key step towards developing a robust and reproducible targeted LC-MS assay. In order to identify the proteotypic peptides of SARS-CoV-2 SPIKE and NCAP proteins, recombinant proteins were digested, redissolved in 0.1% formic acid and analysed by LC-MS. The DDA-based analysis of neat SPIKE and NCAP recombinant proteins revealed a list of tryptic peptides (Supplementary Data 1), which provided the basis for initial screening of the target peptides. The recombinant peptide standards were then spiked into artificial saliva, as well as saliva and swab samples collected from healthy volunteers. Digests were analysed on a Q-Exactive HF in data-dependent analysis (DDA) mode coupled to an Evosep LC -MS system, using the 200 sample per day (200 SPD) method (Fig. 1). Furthermore, a QconCAT for stable isotope labelling based absolute quantification that includes SARS-CoV-2 specific SPIKE and NCAP peptide sequences as described by Puyvelde et al. was used [16]. Finally, DDA analysis of saliva samples revealed a list of consistently identified proteotypic peptides from high-abundant human proteins such as Lysozyme C (LysC) which we used as an internal control to check for sample preparation and acquisition efficiency (Supplementary Data 2).

In order to select optimal proteotypic peptides for the targeted PRM analysis, we followed published selection criteria for a targeted-based LC-MS assays such as tryptic peptide length of 8 to 25 amino acid and excluded peptides with possible modifications such as oxidation on Methionine, deamidation on Asparagine followed by Glycine, and any other possible post-translational modifications [24]. Following these criteria, an initial list of tryptic peptides was generated which were further analysed in matrices such as saliva. Of the initial 19 peptides (Supplementary Data 3), only five could reproducibly be detected when analysed in a background matrix of artificial or volunteer saliva. As peptides from SPIKE were inconsistently observed and their retention times varied across runs, SPIKE peptides were omitted from future analysis. SPIKE peptides are known to be heavily glycosylated, making them notoriously harder to detect [25]. Moreover, NCAP is considered to be about 3-fold more abundant in SARS-CoV-2 than SPIKE [4], increasing the sensitivity. Therefore, it was not surprising that the highest sensitivity was achieved for the NCAP peptides AYNVTQAFGR and ADETQALPQR. Additionally, as KADETQALPAR was consistently detected in all the DDA runs, as well as in the QconCAT runs, it was included in the list even though it contains a missed cleavage. Human Lysozyme C, as mentioned earlier, was used as an internal control for sample preparation and to check for digestion and sample injection efficiency. The final list consisted of total seven peptides including the stable isotopically labelled peptides from QconCAT which were further used for MS method development and optimization (Table 1).

3.3. PRM-MS method development and optimization

A PRM method was created on the QE-HF using the 200 samples per day (200 SPD) short gradient LC method integrated on the Evosep One.

Table 1
Diagnostic peptides selected for the development of PRM-MS assay for detection of SARS-CoV-2 peptides.

No	Peptide Sequence	Source	m/z, charge	Norm. Collision Energy	Type
1	AYNVTQAFGR	NCAP(SARS-CoV-2)	563.785^{2+}	24	light
2	ADETQALPQR	NCAP(SARS-CoV-2)	564.785^{2+}	23	light
3	KADETQALPQR	NCAP(SARS-CoV-2)	419.557^{3+}	20	light
4	AYNVTQAFGR	QconCAT	571.263^{2+}	24	heavy
5	ADETQALPQR	QconCAT	572.263^{2+}	23	heavy
6	KADETQALPQR	QconCAT	425.207^{3+}	20	heavy
7	STDYGIFQINS	LYS-C (*Homo sapiens*)	700.843^{2+}	25	light

Evosep One is specifically designed to run samples back-to-back without carryover due to the tip-based sample injection system, which serves as a single-use trap column thereby increasing the overall life of the separation column, making the system well-suited for high-throughput methods [26]. The PRM method was further optimized for collision energies (Supplementary Fig. 3) for the individually selected peptides along with their corresponding SIL peptides (referred to as heavy) emerging from the QconCAT. The normalized collision energies (NCE) for the targeted peptides were optimized within the range of 20–29 for all the peptides. The optimal collision energies for individual peptides were selected based on the intensities of their product ions using Skyline. Details of the seven peptides chosen for diagnosis and collision energies used for the final PRM assay are listed in Table 1. A comprehensive list of transition ions resulting from fragmentation of precursor ions given in Table 1 can be found in Supplementary Data 5.

3.4. Assessing the sensitivity and robustness of the PRM method

The next step in establishing the high throughput PRM method involved its assessment for sensitivity and specificity. Calibration curves of the target peptides in neat, artificial saliva as well as real saliva and swab samples were generated using the tryptic digests for recombinant protein standards for NCAP. Recombinant tryptic peptides in 0.1% formic acid ("neat") (Fig. 2A), spiked swab (Fig. 2B), spiked oral fluid (Fig. 2C) and spiked saliva (Fig. 2D) were injected in a dilution series from 50 femtomoles down to 50 attomoles on column. The limit of detection (LOD) for NCAP peptides was calculated to be as low as 170 amol on column, while the limit of quantitation (LOQ) was found to be 850 amol in neat samples. (Table 2). However, when spiked into saliva and swab biological matrices, the LOD and LOQ decreased to 0.63 fmol and 3.24 fmol on column, respectively. The drop in sensitivity was expected when peptide standards were injected with saliva sample due to the matrix effect contributed by highly abundant endogenous salivary proteins such as Lysozyme C and Albumin..

The existing PRM assay method needed to not only be sensitive but also robust enough to carry out high-throughput analysis of many samples injected back-to-back. To test the robustness of the LC-MS system a continuous 200 sample test was performed with a saliva sample spiked in with recombinant NCAP protein standard. The variability of peak area and retention time of the NCAP peptide AYNVTQAFGR revealed that the system performed consistently without a noticeable drop in sensitivity across all 200 injections. (Fig. 3A and B).

3.5. Validation of PRM Assay using 50 patient samples

The final PRM method was validated using 25 positive and 25 negative patient samples. The samples had been analysed by either RT-PCR, SAMBA II or LumiraDx. RT-PCR provided a Cycle Time (Ct) value and any Ct <40 was considered as positive. The Simple AMplification-

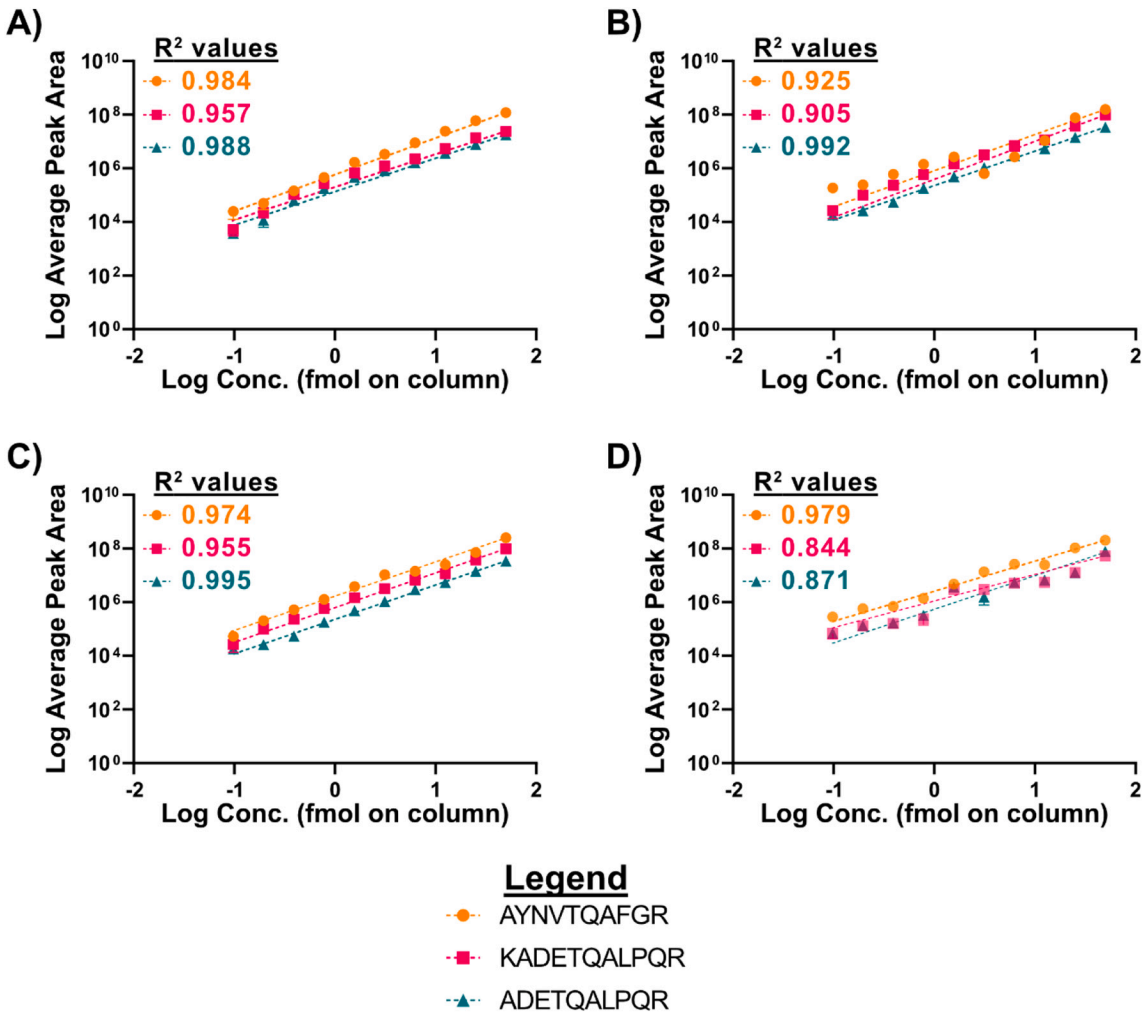

Fig. 2. Calibration curves for estimating the limits of detection (LOD) and quantitation (LOQ). Diagnostic peptides generated from recombinant SARS-CoV-2 NCAP standard protein were injected on the LC-MS system to generate calibration curves starting from 50 fmol on column and diluted 2-fold until 48 amol was achieved either in 0.1% formic acid as depicted in fig. A. Figs. B, C and D represent the drop in sensitivity of the LOD and LOQ when injected as a spike-in in a swab, oral fluid or saliva sample respectively. The sensitivity of the system was assessed based on the three peptides AYNVTQAFGR, ADETQALPQR and KADETQALPQR selected for final PRM method. The figures are in base-10 logarithmic scale.

Table 2
Limits of detection and quantitation calculated for the diagnostic NCAP peptides on the existing LC-MS system spiked into various matrices are represented below.

Protein	Matrix	Peptide	LOD (fmol on column)	LOQ (fmol on column)
NCAP	0.1% Formic acid	AYNVTQAFGR	0.17	0.85
		ADETQALPQR	0.37	1.1
		KADETQALPQR	0.26	1.3
	Artificial sputum	AYNVTQAFGR	1.6	8.0
		ADETQALPQR	0.66	3.3
		KADETQALPQR	6.3	32.1
	Saliva	AYNVTQAFGR	2.6	12.8
		ADETQALPQR	4.7	23.6
		KADETQALPQR	7.3	36.4
	Swab	AYNVTQAFGR	1.5	7.2
		ADETQALPQR	3.0	14.8
		KADETQALPQR	4.4	21.9

Based Assay (SAMBA) II nucleic acid testing system (SAMBA II) finds traces of viral genetic material and amplifies it a billion times [28] and LumiraDx is a microfluidic immunofluorescence assay for qualitative detection of nucleocapsid protein antigen [29]. The latter two methods do not provide a quantitation of viral load. A full list of patients and information about COVID-19 detection method employed as well as patient viral load (when available) can be found in (Supplementary Data 6).

The samples were attributed as tentatively positive according to the following criteria: (i) At least two out of maximum six fragment ion transitions were detectable and (ii) The retention times matched that of the heavy QconCAT counterpart of the same peptide. However, in most cases of tentatively identified patients (see Fig. 4C and Supplementary Fig. 5) not all peptides belonging to endogenous NCAP protein were present compared to their heavy labelled QconCAT counterparts. In fact, only the AYNVTQAFGR peptide was identified with more than two transition ions being present. Out of the 25 positive patients, AYNVTQAFGR peptide could be identified reliably with >0.9 dotp value only in two cases (see Fig. 4A and B). These two strongly positive samples were then used for calculation of the absolute concentration from the total H/L ratios calculated using Skyline software.

Our PRM method was able to detect two strongly positive cases (Fig. 4A and 4B), the other seven tentatively detected cases are presented in (Fig. 4C and Supplementary Fig. 5). LUMIRA, qPCR and SAMBA II results for all 50 patient samples are given in (Supplementary Data 6).

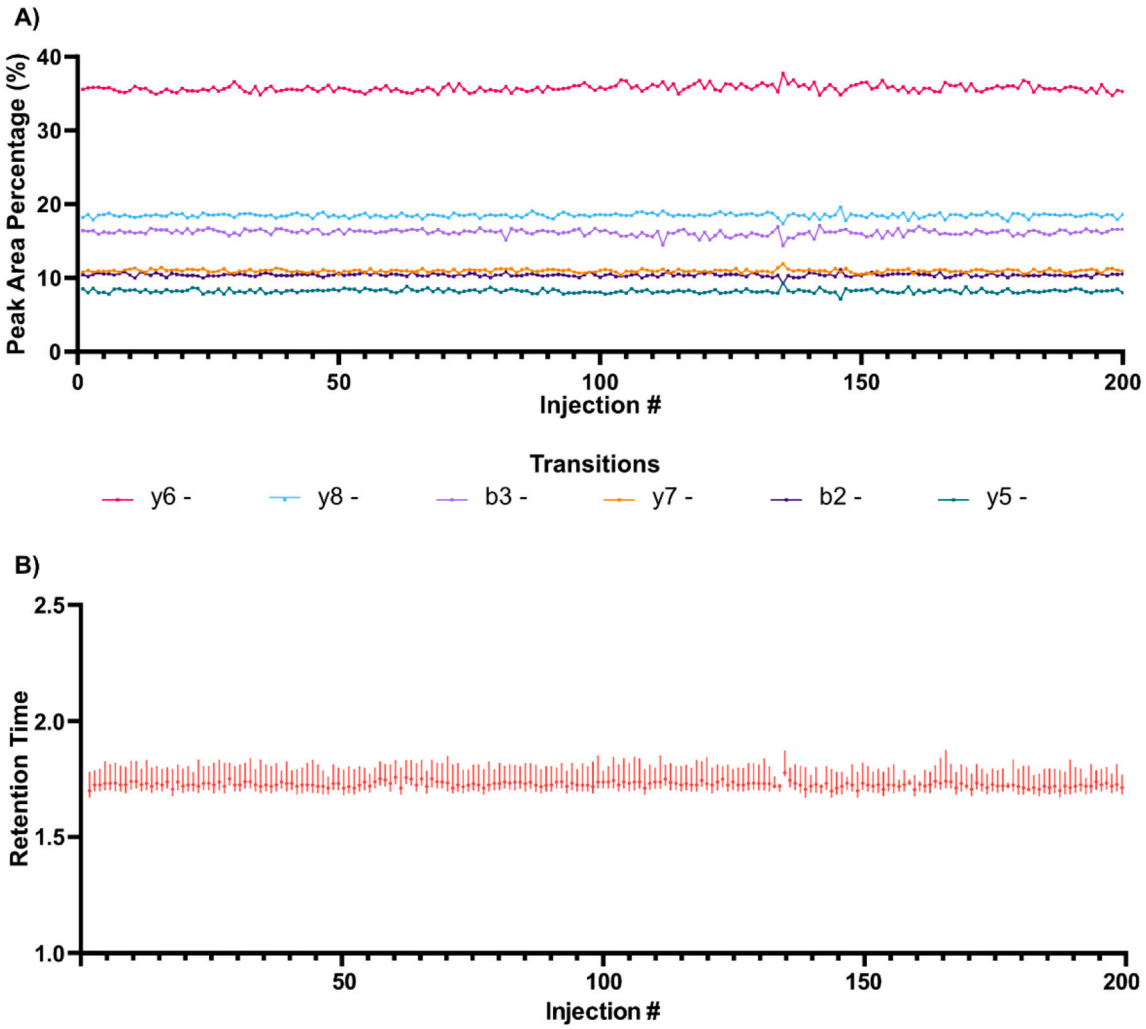

Fig. 3. Testing the robustness of the LC-MS assay. To test the stability and robustness of the existing PRM method, a stress test with 200 consecutive injections of saliva spiked with neat NCAP peptides was conducted where fig. A depicts overall consistent peak area percentage for all the 6 transitions for the NCAP peptide AYNTQAFGR. Fig. B depicts the intensity and retention time variation of the same peptide over 200 injections.

4. Discussion

The presented MS-based assay aims to directly detect viral peptides in nasopharyngeal swab samples with faster processing times in a high-throughput manner. The detection is pursued from miniscule amounts of total samples unlike the nucleic acid-based strategies which utilise amplification. This is one of the major reasons attributing to the lower sensitivity of the present MS-based targeted assay compared to nucleic acid amplification-based methods such as PCR and SAMBA. While Lumira does not require amplification, it requires antibodies which can be non-specific and can lead to false positives. In order to achieve more sensitivity higher sample volume needs to be injected onto the system, which poses a challenge when using a nano-flow LC system.

Additionally, to provide a boost in sensitivity pre-enrichment methods such as SISCAPA [30], aptamers [31] or bead-based enrichments as utilized by Renuse et.al [15] could be incorporated in the sample preparation workflow, as the viral peptides have very low abundance when measured against a background of human saliva samples rich with high-abundant proteins like Lys C and Amylase. The other reason that contributes towards lowered detection sensitivity of the PRM assay could be assigned to the way the samples were collected and stored. In many of the samples, the traces of food particles and phlegm from individual patients were found, which made sample processing difficult. This highlights the fact that sample collection and storage for MS-based assays should be standardized, such as making sure the nasopharyngeal swab samples are taken by qualified staff, making sure the swabs touch only the back of the throat and the base of the tonsils avoiding any contamination. Also, efforts should be taken towards immediate processing of the samples after collection or stored at −20 °C to reduce any protein degradation. The relatively low LOD values of diagnostic peptides in saliva and swab samples spiked with recombinant NCAP protein indicate that even at analytically low concentrations they can be detected with the presented LC-MS analysis workflow. However, the biological differences between positive patients and the variations in the clinical conditions that the samples are collected in strongly contribute to inaccuracies in the detection of diagnostic peptides.

Stable isotope standards help in achieving absolute quantification [32]. One such standard is QconCAT, which is an artificially synthesized protein, generated by concatenation of proteotypic peptides. A heavy labelled QConCAT internal standard enables assessing sampling quality, sample preparation efficacy, instrument robustness, and absolute quantification [16]. Among the patient samples, one sample had been analysed by RT-PCR with a Ct value of 17.8, which indicates a severe viral load. We calculated the amount for the viral NCAP peptide (AYNTQAFGR) to be 25 fmol on column. Assuming that each viral particle contains 300–350 NCAP molecules [33], this indicates that the swab contained approximately 5.25×10^8 viruses. This is in the right

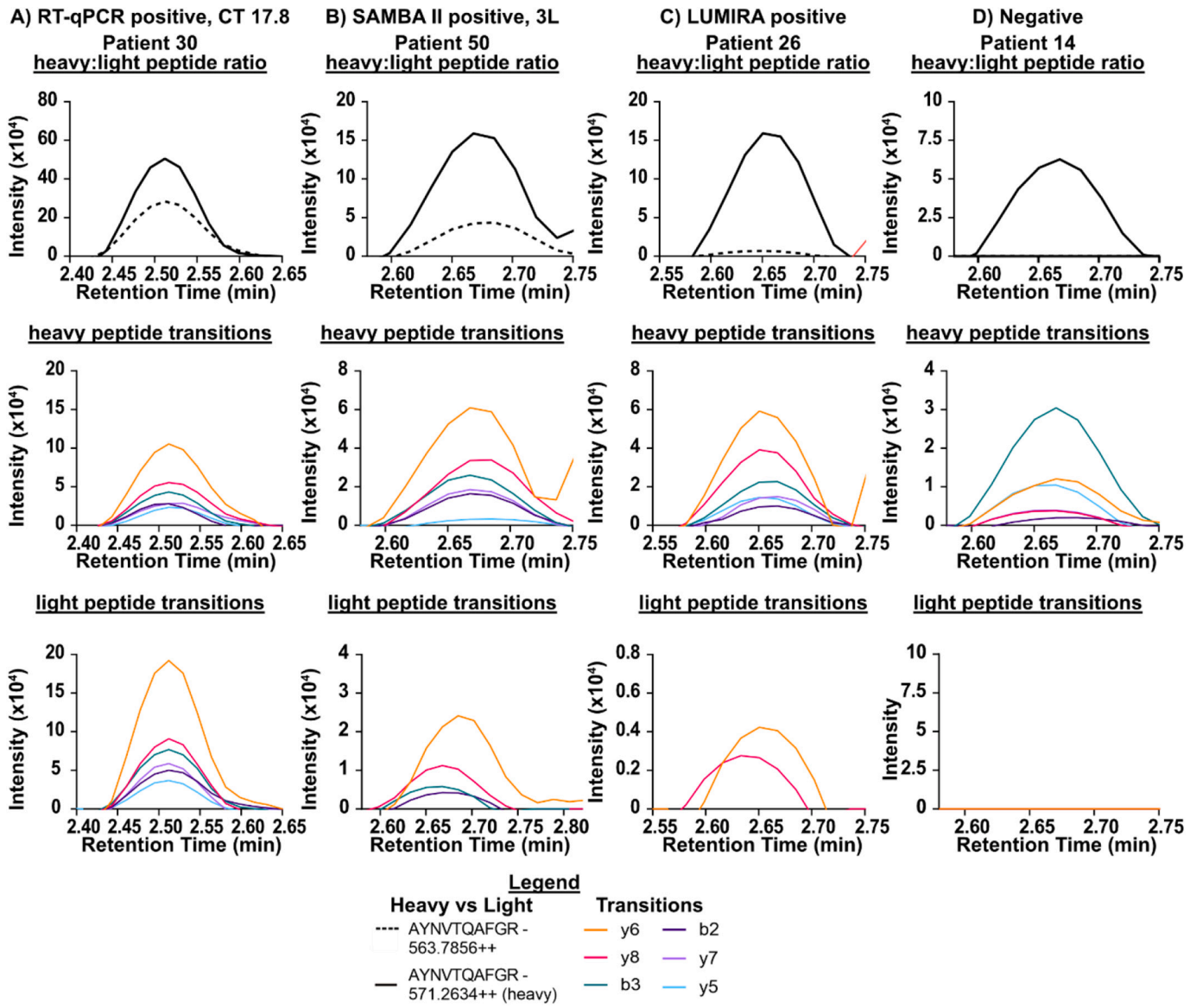

Fig. 4. PRM transitions detected in patient samples. A) Sample of a positive patient where the viral particles were detected by RT-qPCR with a CT value of 17.8. The concentration of the viral peptide AYNTQAFGR was estimated to be ~25 fmol (corresponds to 7.5 pmol in the whole swab sample) by calculating the heavy to light ratio (H/L). B) and C) are patient samples deemed positive with SAMBA II and Lumira which were identified with our method as positive respectively. D) is a negative patient sample.

order of magnitude with the theoretical calculations conducted by Puyvelde *et al* [16], where a correlation of number of viral particles from measured Ct values of plasmids was attempted. They estimated that a Ct value of 16 would amount to ~20 fmol NCAP/10 μL sample corresponding to 1.26×10^{10} NCAP copies or 4.2×10^{8} viral particles (one RNA per virus) per 10 μL sample. This shows good correlation of our PRM method with existing methods, as well as enables calculation of viral particles from absolute concentrations of viral proteins. Indeed, more rigorous studies with a greater number of patient samples with standardized sampling protocols are needed to establish a strong peptide concentration-viral particles correlation baseline. Nonetheless, the quantification of these peptides in the nasopharyngeal samples is a step in the right direction to assess not only the prognostic potential of the method but also determining the concentration range of the protein in correlation with the severity of the SARS-CoV-2 disease, which may prove helpful in designing treatments for disease management and thereby reducing complications and hopefully fatalities.

5. Future perspectives

The global response to the COVID-19 pandemic has come a long way since its inception in late 2019. Lately, the death toll and the number of SARS-CoV-2 variants arising with the on-going pandemic seem to be under control with the introduction of several vaccines, yet complete global immunization against COVID-19 is still far away. With the world starting to turn slowly towards normality, rigorous testing remains of prime importance until COVID-19 is completely eradicated which puts a burden on existing molecular testing methods. The present method could serve as an alternative for fast detection, where such mass spectrometric and chromatographic capabilities are available. With the little requirements for development of an MS-based testing method, it presents with a relative possibility of adapting the existing LC-MS method for detection of the existing and newly emerging SARS-CoV-2 variants as well as multiplexing with other respiratory viruses such as influenza.

The sensitivity of the present method has room for improvement by incorporating enrichment methods such as SISCAPA and

immunoaffinity in the sample preparation. Additionally, applying automated sample handling to the sample preparation workflow may further reduce the variability and improve the turnover times.

Author contributions

AG, AN, TH, FS, AP performed experiments and data analysis. YT provided access to patient samples. AG, AN and MT wrote the manuscript with input from all authors.

Declaration of Competing Interest

None.

Data availability

LC-MS data is publicly available at Panorama Web repository (Dataset: SARS-CoV-2 PRM) and on ProteomeXchange Database (Dataset: PXD033790). Additional data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jprot.2022.104664.

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