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Operation Moonshot: rapid translation of a SARS-CoV-2 targeted peptide immunoaffinity liquid chromatography-tandem mass spectrometry test from research into routine clinical use

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Abstract

Objectives: During 2020, the UK's Department of Health and Social Care (DHSC) established the Moonshot programme to fund various diagnostic approaches for the detection of SARS-CoV-2, the pathogen behind the COVID-19 pandemic. Mass spectrometry was one of the technologies proposed to increase testing capacity.

Methods: Moonshot funded a multi-phase development programme, bringing together experts from academia, industry and the NHS to develop a state-of-the-art

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targeted protein assay utilising enrichment and liquid chromatography tandem mass spectrometry (LC-MS/MS) to capture and detect low levels of tryptic peptides derived from SARS-CoV-2 virus. The assay relies on detection of target peptides, ADETQALPQRK (ADE) and AYNVTQAFGR (AYN), derived from the nucleocapsid protein of SARS-CoV-2, measurement of which allowed the specific, sensitive, and robust detection of the virus from nasopharyngeal (NP) swabs. The diagnostic sensitivity and specificity of LC-MS/MS was compared with reverse transcription quantitative real-time polymerase chain reaction (RT-qPCR) via a prospective study.

Results: Analysis of NP swabs (n=361) with a median RT-qPCR quantification cycle (Cq) of 27 (range 16.7–39.1) demonstrated diagnostic sensitivity of 92.4% (87.4–95.5), specificity of 97.4% (94.0–98.9) and near total concordance with RT-qPCR (Cohen's Kappa 0.90). Excluding Cq>32 samples, sensitivity was 97.9% (94.1–99.3), specificity 97.4% (94.0–98.9) and Cohen's Kappa 0.95.

Conclusions: This unique collaboration between academia, industry and the NHS enabled development, translation, and validation of a SARS-CoV-2 method in NP swabs to be achieved in 5 months. This pilot provides a model and pipeline for future accelerated development and implementation of LC-MS/MS protein/peptide assays into the routine clinical laboratory.

Keywords: high performance liquid chromatography; laboratory methods & tools; mass spectrometry; proteins.

Introduction

Coronavirus disease 2019 (COVID-19) is highly prevalent and remains a global issue due to its seasonality and mutability [1]. Measurement of the causal agent (severe acute respiratory syndrome coronavirus 2; SARS-CoV-2) is predominantly achieved through measuring viral ribonucleic acid using reverse transcription quantitative real-time polymerase chain reaction (RT-qPCR) or antigen tests such as lateral flow tests. Due to its widespread use and sensitivity, RT-qPCR is accepted as the gold standard detection method [2]. A variety of other tests have been established that afford applicability to community testing but are less sensitive than RT-qPCR [3] and have variable reliability [4].

The UK Government's Moonshot Programme funded the development of a mass spectrometry (MS) test to detect SARS-CoV-2 in nasopharyngeal (NP) swab samples. Clinical laboratories use mass spectrometry (MS) to measure a range of analytes [5–7] with steroid hormones [8–10], toxicology [11–13] and newborn screening [14–16] being some of the commonest applications. MS can also provide quantitative measurements and although targeted protein analysis is not yet commonplace in clinical laboratories, MS was put forward as a candidate method for detection of COVID-19 [17, 18]. The programme was initially set up in three phases:

- P1 – Development of a harmonised LC-MS/MS method for the measurement of SARS-CoV-2 in NP swabs, with multiple research groups employing different approaches to detect the virus. The work of Van Puyvelde et al. [19] was used as a starting point to investigate a range of sample processing, chromatographic options and mass spectrometric end points. Identification of the candidate peptides from the nucleocapsid protein (NCAP), their evaluation in terms of enrichment affinity and LC-MS/MS behaviour, and the subsequent selection of the target peptides has been reported previously [20].
- P2 – Translation of the assay (combined tryptic peptide immunocapture/targeted LC-MS/MS) into NHS laboratories and modification for routine use.
- P3 – Potential upscaling of the assay into hub laboratories for population screening.

This article describes the translation from P1 to P2, and the results obtained after adaption and implementation of the assay in the NHS. In particular, the validation of the method to ISO15189:2012 standards and comparison with RT-qPCR are described.

Materials and methods

Chemicals and reagents

A full list of reagents, standards and internal quality control (IQC) materials are provided (see Supplementary Material, S1).

Study design

NP samples were collected with informed consent from patients with symptoms of a coronavirus infection, via the Facilitating AcceLerated Clinical Validation Of Novel Diagnostics of COVID-19 (FALCON) research study (NCT04408170, https://www.condor-platform.org/condor_workstreams/falcon). Samples were collected prospectively

between May 2020 and February 2021 from patients recruited in hospital with either query COVID-19 or who had tested positive for COVID-19 and known COVID-19 positive and/or COVID-19 negative community testing. The samples were approved for use by Health Research Authority (HRA) and Health and Care Research Wales (HCRW) and sponsored by Manchester University NHS Foundation Trust (REC: 20/WA/0169). Respiratory samples were collected by swabbing the posterior pharynx and nasal cavity (mid-turbinate) with a flocked NP swab (Miraclean MC-96000). Three separate swabs were collected from each subject. The first two swabs were placed in viral transport medium (VTM) for analysis by RT-qPCR (ThermoFisher TaqPath™ COVID-19 CE-IVD RT-qPCR kit), (ThermoFisher Scientific, Loughborough, UK). The first swab was analysed at Francis Crick Institute and the second swab was analysed at Lighthouse Laboratory, Milton Keynes. The third swab was collected in ethanol deactivation solution for analysis by MS. The method comparison between LC-MS/MS and RT-qPCR was performed using the swabs analysed by the Francis Crick Institute. Comparison of two independently collected swab samples was performed using the two RT-qPCR assays. Samples were stored at −80 °C prior to analysis. The P2 laboratories performed the LC-MS/MS analysis blind.

Sample preparation

Acetone (0.5 mL) was added to a 2 mL 96 deep-well plate and cooled at −20 °C for 30 min. Samples and IQC were vortexed (5 s). Ethanol deactivation solution (500 µL) was removed from each swab collection tube and added to the acetone. The plate was sealed and cooled at −80 °C for 10 min, prior to centrifugation (3,500×*g*, 10 min). The supernatant was discarded and the protein pellet allowed to air dry. *Rapi*Gest™ SF (Waters Corporation, MA, USA), (0.1% in 200 mM ammonium bicarbonate, 200 µL) was added to each well and the plate shaken at room temperature on a thermomixer (1,500 *g*, 5 min). The swab was transferred from the collection tube into the corresponding well of the plate. The swab handle was removed, and the plate shaken at room temperature on the thermomixer (1,500 *g*, 5 min). Trypsin solution (3 mg/mL in 10 mM HCl, 20 µL) was added to each well prior to incubation for 1 h (37 °C, 500 *g*). The digested solution was removed from the swabs and transferred to a QuanRecovery 700 µL 96 well plate (Waters Corporation). Subsequent sample processing was performed using an Andrew Alliance™ Andrew+™ pipetting robot (Waters Corporation). The automated procedure quenched the tryptic digest by addition of TLCK (0.5 mg/mL in 10 mM HCl, 20 µL) to each well prior to vortex mixing and incubation at room temperature for five min. Stable isotope label (SIL) peptides (20 µL, 0.45 fmol/µL) were added to each well and mixed. SISCAPA beads (10 µL of each monoclonal antibody, ~0.01 pmol/mL) were added to each well, with agitation of the beads before addition to the sample after every three wells, and the plate shaken (1700 *g*) at room temperature for 1 h. Wash buffer (0.5 mM CHAPS in PBS, 150 µL) was added to each well and the plate shaken (1700 *g*) for 30 s. Wash buffer was removed from each well and discarded, and the wash step repeated a further two times. Elution buffer (0.5 mM CHAPS, 1% formic acid, 50 µL) was added to each well and the plate was shaken (1,500 *g*) at room temperature for 6 min. The supernatant was removed from each well and transferred into a QuanRecovery 700 µL 96-well plate. The plate was manually removed from the robot, sealed and placed in the autosampler on a magnetic base to prevent any spurious magnetic particles from being injected into the LC-MS/MS.

LC-MS/MS conditions

Samples (20 µL) were analysed using a Xevo™ TQ-XS MS with electrospray ionisation source coupled to an ACQUITY™ UPLC™ I-Class chromatography system with autosampler (Waters Corporation). Chromatographic separation was achieved on an ACQUITY™ Premier Peptide BEH C18 Column (1.7 µm, 2.1 × 50 mm) with in-line filter (Waters Corporation.). The mobile phase consisted of a water (A) and acetonitrile (B) both containing 0.1% formic acid v/v. Initial conditions were 5% B, changing to 40% B between 0.25 and 2.20 min and then switching to 85% B by 2.30 min and holding for 0.3 min prior to reverting to 95% A by 2.61 min and re-equilibrating for 0.39 min. The flow rate was 0.6 mL/min, the column was held at 40 °C and the autosampler at 10 °C. The weak needle wash was water containing 0.1% formic acid v/v, the strong needle wash was acetonitrile containing 0.1% formic acid v/v, and the seal wash was water and acetonitrile in the ratio 90:10 v/v. Analysis time was 3.0 min. Mass spectrometer settings were capillary voltage, 0.5 kV, desolvation temperature, 600 °C, desolvation gas flow, 1000 L/h, and cone gas, 150 L/h. Cone voltage and collision energy were optimised for each analyte. Data were acquired by Multiple Reaction Monitoring (MRM) in positive-ionisation mode. One quantifier and two qualifier ions were monitored for the target peptides and their respective SILs (see Supplementary Material, Table S1). Dwell times were 17 ms for each transition. Data were processed using MassLynx™ 4.1 and TargetLynx™ software (Waters Corporation). Results were assessed numerically and visually for each peptide. Numerical assessment included review of peak area intensity for each SIL and analyte; concentration; signal:noise ratio; quantifier:qualifier ratio. Visual assessment included review of the individual extracted ion current chromatograms.

Method validation

Acceptable analytical performance was based on the FDA Guidance for Industry Bioanalytical Method Validation criteria [21]. Validation parameters and criterion are described (see Supplementary Material, S2).

Results

Chromatographic separation of the target peptides was acheived with ADE, AYN, and DGI eluting at t_R 0.92, 1.34 and 1.83 min respectively, in a total run time of 3.0 min. Replicate injections of extracted samples demonstrated reproducible retention times with %RSDs of ≤1.1. The method showed good selectivity, with no significant interfering peaks detected at the t_R of the analytes/SILs. Typical extracted ion current chromatograms are show in Figure 1A–C.

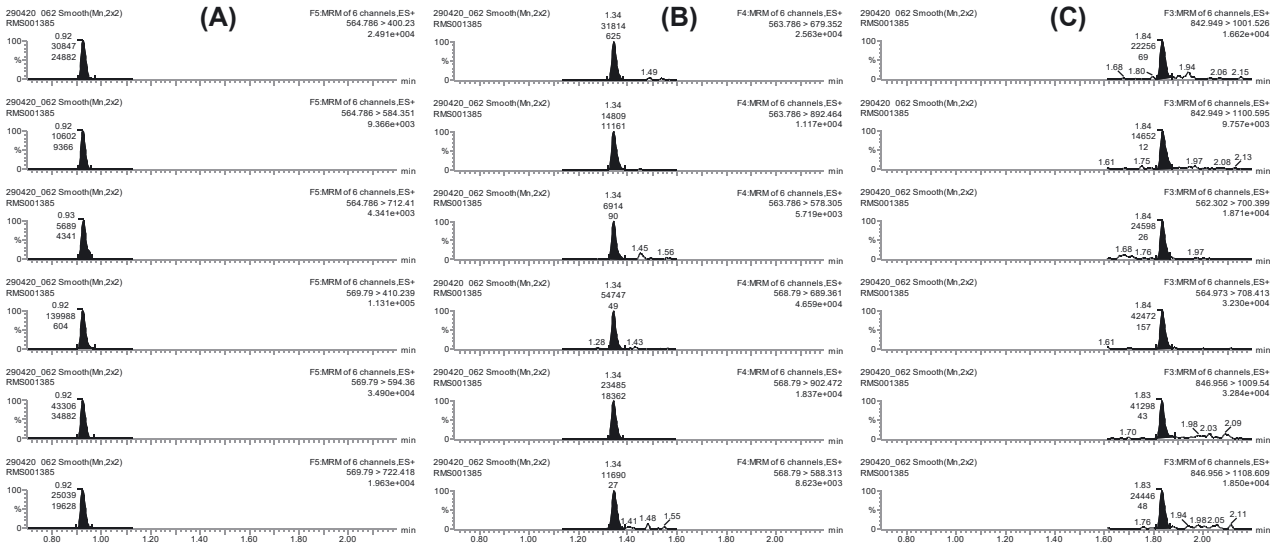

Figure 1: Extracted ion current chromatograms for the three target peptides (A) ADE, (B) AYN, and (C) DGI for a SARS-CoV-2 positive sample with a RT-qPCR cycle threshold value of 23.

The calibration curves for AYN and ADE exhibited a linear response over the concentration range 1.8–114 amol/µL. For DGI the range was 14.0–1,136 amol/µL. Correlation coefficients (R^2) of ≥0.99 were achieved for all curves apart from one AYN calibration (R^2=0.9816).

The method had acceptable sensitivity for both AYN and ADE. The LOD was 0.45 amol/µL (9 amol on-column) for AYN and 3.6 amol/µL (72 amol on-column) for ADE. The LLOQ for both analytes was 3.6 amol/µL. The LOD and LLOQ for DGI were not determined due to significant carry over and this peptide was subsequently excluded from the validation.

Imprecision was satisfactory for both AYN and ADE. The intra-well %RSD was <5% for both analytes. The intra-batch %RSD for the positive control material was 8.8 and 4.7 for AYN and ADE at concentrations of 14.7 and 8.2 amol/µL, respectively. The inter-batch %RSD for the positive control material was 7.0 and 4.5 for AYN and ADE at concentrations of 14.9 and 8.2 amol/µL respectively. Quantifier to both qualifier ion ratios were reproducible for both peptides with %RSDs of 4.5 and 5.4 for AYN and 2.5 and 3.6 for ADE. Neither AYN nor ADE were detected in the negative control material (25/25).

There was negligible carryover for ADE (peak area of blank was 3% of peak area at LLOQ) whereas some carryover was evident for AYN (peak area of blank was 57% of peak area at LLOQ).

The method demonstrated good selectivity with no interference from Influenza A, B and Rhinovirus (see Supplementary Material, S3 and Figure S3). Background signals were all <20% of AYN/ADE peak areas at LLOQ and <5% of the area of the SIL. The mean AYN ion ratio in true positive patient samples was 3.1 (median 2.9, range 1.0–42.2). The mean ADE ion ratio in true positive patient samples was 3.6 (median 3.0, range 0.76–23.7).

Swab extracts remained stable for all analytes when stored at 10 or −80 °C for up to 72 h, with accuracies within ±15% of freshly prepared samples. If necessary, samples can be reconstituted in 0.1% formic acid (20 µL) prior to analysis.

Of the 396 swab samples received, 35 were excluded from the method comparison; 20 due to an inconclusive RT-qPCR result; 11 due to poor sample quality or sampling issues; three due to the absence of a RT-qPCR result; one due to analytical failure. Of the samples in the method comparison, 22% (88/396) were self-collected and 72% (286/396) were collected by a healthcare professional (HCP). Results are summarised in Figure 2A, B and Tables 1 and 2.

Cycle-threshold (Cq) values of the n=361 samples in the comparison ranged from 16.7 to 39.1 (median 27). Testing showed near total concordance with the Taqpath RT-qPCR (Cohen's Kappa 0.90) [22]. The LC-MS/MS method had a diagnostic sensitivity of 92.4% (87.4–95.5) and a diagnostic specificity of 97.4% (94.0–98.9). If samples with Cq>27 were excluded (n=68), the method had a diagnostic sensitivity of 100% (95.9–100) and a diagnostic specificity of 97.4% (96.0–100.0) thus exceeding the target performance criteria set by DHSC and giving Cohen's Kappa=0.96. As the significance of a positive result with Cq>32 is generally considered to be unclear when interpreted in isolation, it is pertinent to note that excluding these samples (n=26) gives a

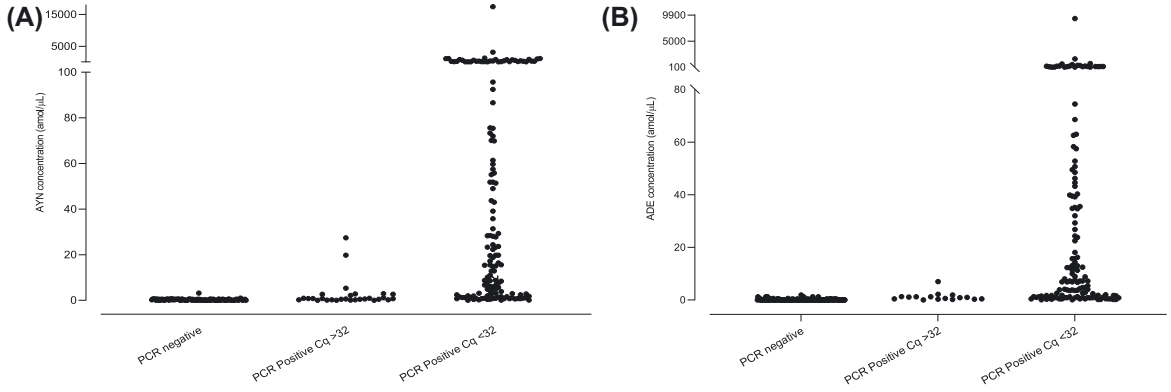

Figure 2: Method comparison of RT-qPCR with LC-MS/MS for (A) the AYN peptide and (B) the ADE peptide. Results are categorised according to RT-qPCR classification; negative; positive with Cq value >32; positive with Cq value ≤32.

Table 1: Diagnostic sensitivity and specificity of the peptide immunoaffinity LC-MS/MS method when compared with the ThermoFisher Taqpath RT-qPCR assay.

	All samples	Samples with Cq≤32	Samples with Cq≤27
Total samples	361	335	282
False negative (FN)	13	3	0
False positive (FP)	5	5	5
True negative (TN)	185	184	184
True positive (TP)	158	143	93
Sensitivity (95% CI)	92.4% (87.4–95.5)	97.9% (94.1–99.3)	100% (95.9–100)
Specificity (95% CI)	97.4% (94.0–98.9)	97.4% (94.0–98.9)	97.4% (96.0–100)
Cohen's kappa	0.8998	0.9515	0.9604
Positive predictive value	96.9%	96.6%	94.9%
Negative predictive value	93.4%	98.4%	100%

Table 2: Breakdown of nasopharyngeal swab results by source of collection.

Source of sample	Total number	False negative	False positive	True positive	True negative	False	Total
RMS2[c]	88	12 (13.6%)	1 (1.1%)	54 (61.4%)	16 (18.2%)	5 (5.7%)	88
RMS0[a]	286	12 (4.2%)	4 (1.4%)	98 (34.3%)	157 (54.9%)	15 (5.2%)	286
RMS1[b]	22	0	0	6 (27.3%)	12 (54.6%)	4 (18.2%)	22
Total no.	396						

[a]RMS0=sampling by HCP. [b]RMS1=unknown. [c]RMS2=self sampling.

diagnostic sensitivity of 97.9% (94.1–99.3), a specificity of 97.4% (94.0–98.9) and near perfect concordance with RT-qPCR (Cohen's Kappa=0.95), a performance directly comparable with many commercial RT-qPCR assays.

For the swab samples sequentially collected and analysed by the same RT-qPCR method at two different laboratories, 87.5% of results agreed and 12.5% were discrepant. Of those that were discrepant, 77.8% were void/inconclusive results and 22.2% differed on final classification. Overall, 2.8% (14/502) of samples were classified differently by the two PCR methods (Table 3). This discrepancy could reasonably be attributed to inconsistency in swabbing efficacy and/or analytical performance; these samples had a mean Cq=33.5 (range 31.0–35.8).

Discussion

This unique collaboration between academia, industry and the NHS resulted in the successful development of

Table 3: Comparison of the Crick Taqpath RT-qPCR assay with the Milton Keynes Taqpath RT-qPCR assay on sequentially collected nasopharyngeal swabs.

	Crick to Milton Keynes *ORF1ab* gene comparison	Crick to Milton Keynes *N* gene comparison	Crick to Milton Keynes *S* gene comparison	Crick to Milton Keynes *MS2* gene comparison
Match[a]	439	446	106	388
Crick void, MK positive	1	1	0	57
Crick void, MK negative	7	6	55	0
Crick void, MK inconclusive	49	50	2	0
Crick negative, MK positive	6	0	340	0
Crick positive, MK negative	0	0	0	58
FALSE (no MK result)	11	10	10	10
Total	513	513	513	513

[a]Match based on final classification of Crick & Milton Keynes results for *ORF1ab* gene.

a targeted protein assay for the detection of SARS-CoV-2. Furthermore, the test was developed, validated to ISO15189:2012 standard and translated into the NHS within 5 months. This manuscript demonstrates the power of collaboration across this triplex of sectors and highlights the benefits of this approach to clinical diagnostics. Continued communication during the P2 phase was key to success and an invaluable lesson in bridging the gap between research test development and clinical implementation. Currently in the UK there are no defined positions for translational research scientists. This is likely a key factor in why so few biomarkers are successfully translated into clinical use.

Whilst the analytical performance of the AYN and ADE peptides was acceptable, that of DGI was not. It is postulated that this reflects its hydrophobicity (GRAVY score=0.59) [23] and absorption to surfaces during the analytical process. This highlights the importance of investigating the binding properties and surface reactivity when developing methods and of including multiple target peptides during initial validation [24]. The DGI peptide was subsequently removed from the validated method.

Following translation of the assay into a clinical laboratory, several modifications and refinements were made to facilitate larger scale preparation in a routine environment. The original protocol specified the addition of ethanol storage solution to a 96-well plate prior to precipitation of the protein in ice-cold ethanol. This was a manual process, prone to error and cross contamination of samples due to the difficulties associated with transferring solvents by pipette. To negate these risks, an acetone precipitation step was evaluated by directly adding the ethanol storage solution to 96-well plates containing pre-cooled acetone (–20 °C). The benefit of using acetone as a precipitant was an increase in signal of approximately 20–40%.

Other improvements introduced during the P2 phase included refinement of calibrator concentrations; introduction of matrix matched IQC materials to demonstrate control of the entire analytical process; optimisation of MS conditions to increase sensitivity; automation of the SISCAPA capture using liquid-handling robots; development of a standardised classification algorithm to ensure consistency in interpretation of results. The algorithm is under further development to ensure manual interpretation of the data will not be necessary in future.

Automation of the sample preparation process was essential for such a complex, manual method to be feasible for use in a routine laboratory. With support from industry, the process was automated, reducing batch processing time by 2 h and increasing sample throughput by ~40%. The validation has also demonstrated the ease with which the SISCAPA workflow could be adapted for future applications.

There are several acknowledged limitations of this study, perhaps the most important being that RT-qPCR and MS methods are not directly comparable. One is direct and measures the amplified signal from viral RNA, the other is in-direct and measures peptides derived from NCAP protein. As RT-qPCR is the accepted gold-standard method of analysis for SARS-CoV-2, it is understandably the point of reference to which novel methods must be compared, however these differences do pose a challenge. RT-qPCR is very sensitive at targeting and detecting one or more gene fragments and can detect non-viable virus thus the prolonged RNA shedding and subsequent positive detection may not correlate to persistence of infectious virus. It is therefore possible that some of the infected people in the validation may have been identified after the infectious period had passed i.e., RT-qPCR testing has poor specificity when used during this phase. Furthermore, RT-qPCR itself

is not a perfect test, with drop-out of the *S* gene an issue for both Alpha and Omicron variants [25]. Conversely, the methodology of the MS test means that to date, it has proved robust to the presence of emerging COVID-19 variants. The decision to use target peptides derived from the NCAP protein rather than the S-protein reflected the S-proteins proclivity to mutate at a faster rate than NCAP protein and the higher abundance of NCAP protein in the virus compared with S. This has proved advantageous and as new variants appear, the nature of the methodology and the multiplexing capability of MS means that, with minor modifications, the assay can quickly and easily be adapted to include new variants, thus enabling the simultaneous monitoring of multiple variants. To date, the LC-MS/MS assay has proved robust to all but one variant. The B1.617.2 variant, corresponding to D337Y mutation, altered peptide ADE is the only variant in which the mutation affected one of the target peptides. The D377Y mutation altered the target peptide A**D**ETQALPQR to A**Y**ETQALPQR. However, experiments demonstrated that the target peptide was still captured by SISCAPA with high affinity hence with minor modification to the MRMs, the Delta variant could be added to the portfolio of variants detected.

The assay was semi-quantitative, a reflection of the time constraints of the pandemic and the pace at which the method was developed. Inclusion of a normalisation peptide from a protein specific for the NP area would have allowed full quantitation and reflected the efficiency and quality of the sampling process. Subsequent protein analyses of different areas of the mouth, nose and nasopharyngeal area identified a candidate marker, BPI fold-containing family B member 1 (BPIB1) that was highly concentrated in the NP area. Retrospective analysis of the first SISCAPA wash in the validation sample set demonstrated the potential utility of this protein for normalisation. Three samples classified as false negatives had BPIB1 concentrations <7.5th centile and a further 22 samples classed as either true negative or inconclusive by RT-qPCR also had BPIB1 concentrations <7.5th centile, indicating that these samples could potentially have been 'missed' due to poor swabbing. Thus, there would be merit in developing a SISCAPA method against the BPIB1 target peptide, to quality control sample collection and provide a truer reflection of viral load. Although SISCAPA methodology significantly improved the assay, the lead time to create an antibody (~6 months) prevented the inclusion of the BPIB1 normalisation step.

Of the samples in the method comparison, 22% (88/396) were self-collected and 72% (286/396) were collected by a healthcare professional (HCP). The percentage of false negatives seen in the self-collected swab group is significantly higher (13.6%) than that seen when sampling is performed by an HCP (4.2%) (Table 2).

It is important to note that the swab used for the MS test was the last of three samples collected from the patient. As swab collection is generally viewed as an unpleasant procedure, it is possible that the efficacy of collection deteriorated, again highlighting the benefit of including a marker of swab integrity in the assay in future.

The design of the FALCON study meant that the validation was performed on separately collected specimens, so in addition to differences in analytical performance, the results of the comparison also reflect any differences in sampling efficacy. To benchmark the likely error rate associated with a method comparison based on separate swab samples, a comparison of the Crick Taqpath RT-qPCR assay with the Milton Keynes Taqpath RT-qPCR assay (n=502) showed a 2.8% error rate, which could reasonably be attributed to inconsistency in sampling efficacy rather than analytical performance (Table 3). The error rate seen when RT-qPCR is compared with RT-qPCR for the measurement of paired, sequentially collected NP swab samples can be used as an estimate of the error rate that sampling efficiency alone could reasonably be expected to contribute to a comparison of MS/MS with RT-qPCR, i.e, over and above the analytical agreement of the two methods. It should also be noted that for five samples, the MS classification was positive and the RT-qPCR negative. This observation could again highlight sampling inconsistencies.

The MS test has comparable performance to other tests used routinely for the detection of COVID-19. It has been demonstrated that 2.8% of results are discordant when a RT-qPCR method is compared with itself and using this as an indicator of the 'allowable' error, a test with a diagnostic sensitivity of 92.4% (87.4–95.5) and 97.4% (94.0–98.9) could be considered to have broadly comparable performance to that reported in the literature for many commercially available RT-qPCR tests.

This study provides an intriguing insight and valuable evidence of the speed with which a complex, multiplexed targeted proteomic assay can be translated into a routine clinical laboratory and validated to ISO15189:2012 standards. In many respects, for those working in the field of MS and clinical diagnostics, the true legacy of the Moonshot project is not the development and validation of the SARS-CoV-2 test itself, it is the understanding that a collaborative approach, access to state-of-the-art technology and automation and ring-fenced time for development and translation have been identified as the key components to success.

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Author contributions: All authors have accepted responsibility for the entire content of this manuscript and approved its submission.

Competing interests: LC, DF, TM, JV, RP, AB, SF and RW are employed by Waters Corporation. LA, MR, JP and MP are employed by SISCAPA Assay Technologies. SH and RND were advisors to the DHSC for the duration of this project. None of the other authors declared any potential conflicts of interest.

Informed consent: Informed consent was obtained from all individuals included in this study.

Ethical approval: Research involving human subjects complied with all relevant national regulations, institutional policies and is in accordance with the tenets of the Helsinki Declaration (as revised in 2013), and has been approved by the authors' Institutional Review Board or equivalent committee (NCT04408170, https://www.condor-platform.org/condor_workstreams/falcon).

Data availability: The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

References

1. Kissler SM, Tedijanto C, Goldstein E, Grad YH, Lipsitch M. Projecting the transmission dynamics of SARS-CoV-2 through the post-pandemic period. Science 2020;368:860–8.
2. Holborow A, Asad H, Porter L, Tidswell P, Johnston C, Blyth I, et al. The clinical sensitivity of a single SARS-CoV-2 upper respiratory tract RT-PCR test for diagnosing COVID-19 using convalescent antibody as a comparator. Clin Med 2020;20: e209–11.
3. COVID-19: Rapid antigen detection for SARS-CoV-2 by lateral flow assay: a national systematic evaluation of sensitivity and specificity for mass-testing. EClinicalMedicine 2021;36. https://doi.org/10.1016/j.eclinm.2021.100924.
4. US Food & Drug Administration Safety Communication. Stop using innova medical group SARS-CoV-2 antigen rapid qualitative test: FDA safety communication; 2021. Available from: https://www.fda.gov/medical-devices/safety-communications/stop-using-innova-medical-group-sars-cov-2-antigen-rapid-qualitative-test-fda-safety-communication. [Accessed 9 Nov 2022].
5. Jannetto PJ, Fitzgerald RL. Effective use of mass spectrometry in the clinical laboratory. Clin Chem 2016;62:92–8.
6. Strathmann FG, Hoofnagle AN. Current and future applications of mass spectrometry to the clinical laboratory. Am J Clin Pathol 2011;136:609–16.
7. Adaway JE, Keevil BG, Owen LJ. Liquid chromatography tandem mass spectrometry in the clinical laboratory. Ann Clin Biochem 2015;52:18–38.
8. Sandhu JK, Auluck J, Ng LL, Jones DJL. Improved analysis of vitamin D metabolites in plasma using liquid chromatography tandem mass spectrometry, and its application to cardiovascular research. Biomed Chromatogr 2014;28:913–7.
9. Keevil B. Steroid mass spectrometry for the diagnosis of PCOS. Med Sci 2019;7:78.
10. Keevil BG. LC–MS/MS analysis of steroids in the clinical laboratory. Clin Biochem 2016;49:989–97.
11. Zhang Y, Wei B, Zhu U, Zhang Y, Bluth MH. Liquid chromatography-tandem mass spectrometry: an emerging technology in the toxicology laboratory. Clin Lab Med 2016;36: 635–61.
12. Remane D, Wissenbach DK, Peters FT. Recent advances of liquid chromatography-(tandem) mass spectrometry in clinical and forensic toxicology – an update. Clin Biochem 2016;49:1051–71.
13. Peters FT. Recent advances of liquid chromatography-(tandem) mass spectrometry in clinical and forensic toxicology. Clin Biochem 2011;44:54–65.
14. Johnson A, Mills K, Clayton P. The use of automated electrospray ionization tandem MS for the diagnosis of inborn errors of metabolism from dried blood spots. Biochem Soc Trans 1196;24: 932–8.
15. Carling RS, John C, Goddard P, Griffith C, Cowen S, Hopley C, et al. Evaluation of a common internal standard material to reduce inter-laboratory variation and ensure the quality, safety and efficacy of expanded newborn screening results when using flow injection analysis tandem mass spectrometry with internal calibration. Int J Neonatal Screen 2020;6:92.
16. Moat S, George R, Carling RS. Use of dried blood spot specimens to monitor patients with inherited metabolic disorders. Int J Neonatal Screen 2020;6:26.
17. Rappold BA. Clinical protein analysis by mass spectrometry: a new higher order. Clin Chem 2021;67:461–2.
18. Marcovina SM, Clouet-Foraison N, Koschinsky ML, Lowenthal MS, Orquillas A, Boffa MB, et al. Development of an LC-MS/MS proposed candidate reference method for the standardization of analytical methods to measure lipoprotein(a). Clin Chem 2021; 67:490–9.
19. Van Puyvelde B, Van Uytfanghe K, Tytgat O, Van Oudenhove L, Gabriels R, Bouwmeester R, et al. Cov-MS: a community-based template assay for mass spectrometry-based protein detection in SARS-CoV-2 patients. JACS Au 2021;1:750–65.
20. Hober A, Tran-Minh KH, Foley D, McDonald T, Vissers JP, Pattison R, et al. Rapid and sensitive detection of SARS-CoV-2 infection using quantitative peptide enrichment LC-MS analysis. Elife 2021;10:e70843.
21. The Food and Drug Administration Agency. Bioanalytical method validation guidance for industry; 2018. Available from: https://www.fda.gov/regulatory-information/search-fda-

guidance-documents/bioanalytical-method-validation-guidance-industry [Accessed 8 Aug 2022].
22. Landis JR, Kock GG. The measurement of observer agreement for categorical data. Biometrics 1977;35:159–74.
23. Kyte J, Doolittle RF. A simple method for displaying the hydropathic character of a protein. J Mol Biol 1982;157:105–32.
24. Goebel-Stengel M, Stengel A, Taché Y, Reeve JR. The importance of using the optimal plasticware and glassware in studies involving peptides. Anal Biochem 2011;414:38–46.
25. Lippi G, Adeli K, Plebani M. Commercial immunoassays for detection of anti-SARS-CoV-2 spike and RBD antibodies: urgent call for validation against new and highly mutated variants. Clin Chem Lab Med 2022;60: 338–42.

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