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Communication

Mass-Spectrometric Detection of SARS-CoV-2 Virus in Scrapings of the Epithelium of the Nasopharynx of Infected Patients via Nucleocapsid N Protein

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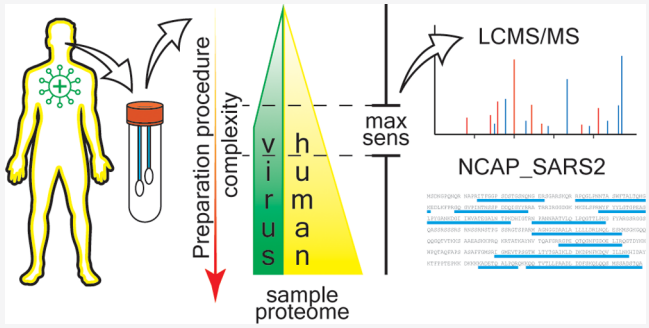
ABSTRACT: The detection of viral RNA by polymerase chain reaction (PCR) is currently the main diagnostic tool for COVID-19 (*Eurosurveillance* 2019, *25* (3), 1). The PCR-based test, however, shows limited sensitivity, especially in the early and late stages of disease development (*Nature* 2020, *581*, 465−469; *J. Formosan Med. Assoc.* 2020, *119* (6) 1123), and is relatively time-consuming. Fast and reliable complementary methods for detecting the viral infection would be of help in the current pandemic conditions. Mass spectrometry is one of such possibilities. We have developed a mass-spectrometry-based method for the detection of the SARS CoV-2 virus in nasopharynx epithelial swabs based on the detection of the viral nucleocapsid N protein. Our approach shows confident identification of the N protein in patient samples, even those with the lowest viral loads, and a much simpler preparation procedure. Our main protocol consists of virus inactivation by heating and the addition of isopropanol and tryptic digestion of the proteins sedimented from the swabs followed by MS analysis. A set of unique peptides, produced as a result of proteolysis of the nucleocapsid phosphoprotein of SARS-CoV-2, is detected. The obtained results can further be used to create fast parallel mass-spectrometric approaches for the detection of the virus in the nasopharyngeal mucosa, saliva, sputum and other physiological fluids.



KEYWORDS: *proteomics, mass-spectrometry, TIMS-TOF PRO, SARS-COV-2, COVID-19*

INTRODUCTION

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is the causative agent of coronavirus disease 2019 (COVID-19). The virus unit consists of a single-stranded RNA with nucleoproteins enclosed within a capsid containing matrix proteins. The genome of the SARS-CoV-2 virus has been sequenced,[4] and the polymerase chain reaction (PCR) method is currently the main diagnostics approach for COVID-19 diagnostics. However, it has shown limited accuracy and sensitivity, giving a high frequency of false-positive and false-negative results,[1−3] and is relatively time-consuming. The detection of viral proteins in body fluids by mass-spectrometry-based methods could serve as a complementary diagnostic tool. In addition, alternative testing assays would allow us to better understand the biological activity of the virus and to suggest potential drug targets for patient treatment.

SARS-CoV-2 proteins can be grouped into two major classes, structural and nonstructural proteins. Nonstructural proteins are encoded by the virus but are present only in the infected host cells and include the various enzymes and transcription factors necessary for virus replication. These proteins are not incorporated into the virion and are not as highly expressed and thus are less likely to be detected. The four structural proteins incorporated in the virion particle of SARS-CoV-2 virus are known as the S (spike), E (envelope), M (membrane), and N (nucleocapsid) proteins. The N protein encapsulates and protects the RNA genome, and the S, E, and M proteins together create the viral envelope. From the data previously obtained for SARS-CoV,[5] which has a structure similar to the SARS-CoV-2 structure, it can be concluded that the number of copies of E, M, and N proteins is much larger than that of S, but the E and M are relatively short and tightly membrane-bound proteins, what makes them difficult to extract, detect, and

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identify. Thus the main target for the mass-spectrometry-based detection of SARS-CoV-2 is the N protein. The possibility of developing such methods using gargle solution samples of COVID-19 patients has been reported.[6] We have performed a pilot study on nasopharynx epithelial swabs already collected from patients with COVID-19 for RT-qPCR and showed confident identification of the N protein of the SARS CoV-2 virus by mass spectrometry with the use of a very basic sample preparation procedure.

Also, results of the unique easily detectable peptides characteristic of the infection can further be used as targets for creating highly specific and sensitive parallel reaction monitoring (PRM)-based detection methods or fast parallel mass-spectrometric approaches based on immunoprecipitation and matrix-assisted laser desorption/ionization analysis (iMALDI), allowing very fast testing for the presence of the virus in nasopharyngeal mucosa, saliva, sputum, and other physiological fluids.

MATERIALS AND METHODS

Sample Collection

All procedures for the collection, transport, and preparation of the samples were carried out according to the restrictions and protocols of SR 1.3.3118-13 "Safety procedures for work with microorganisms of the I−II groups of pathogenicity (hazard)". Swabs of the mucosa of the lower part of the nasopharynx and posterior wall of the oropharynx were used for the study. The sample was collected via a sterile velor swab with a plastic applicator. The swab was introduced along the outer wall of the nose to a depth of 2 to 3 cm to the lower shell, and, after performing a rotational movement, it was removed along the outer wall of the nose. After obtaining the material, the swab (up to the place of breakage) was placed into a sterile disposable tube with transport medium, and the end of the probe was broken off. Oropharyngeal swab samples were taken with a dry sterile viscose swab by rotational movements along the surface of the tonsils, palatine arches, and the posterior wall of the oropharynx. To increase the viral concentration, both the nasopharyngeal and oropharyngeal swabs were placed into a single tube, which was then sealed and marked.

The samples were collected from five patients with COVID-19 infection confirmed by RT-qPCR (Table 1). The negative control samples were collected from three healthy individuals.

Virus Inactivation

Before the virus has been inactivated and the outside of the tubes has been disinfected, all work must be carried out in accordance with the rules of biological safety level 3.[7]

Table 1. Viral Load in All Collected Samples According to RT-qPCR

sample	number of PCR cycles
1	24
2	19
3	21
4	31
5	36
6	control
7	control
8	control

1. Transfer 0.25 mL of the specimen to a clean polypropylene 1.5 to 2.0 mL Eppendorf tube.
2. Inactivate by heating to 65 °C for 30 min using a water bath or a thermostated block heater.[8] (It is important to note that during heat treatment, all parts of the tube are heated, so that all of the virus, even that on "inaccessible" surfaces, will be inactivated.)
3. After incubation, add 0.75 mL of 100% isopropanol to obtain a 75% solution and allow the sample to sit for 10 min at room temperature.[9]
4. Treat the outer surfaces of the tubes with 70% isopropanol or 1% sodium hypochlorite: Spill from a jet wash and let stand for 15 min without getting wet.

After this procedure, the virus can be considered deactivated, and the surface of the tube is safe to handle.

Sample Preparation

Protocol 1: Standard Proteomics Preparation Procedure. Inactivated samples were lyophilized and resuspended in 50 mM ammonium bicarbonate buffer containing 0.1% Rapigest SF Surfactant (Waters). Reduction was carried out by incubating for 30 min at 50 °C in 20 mM dithiothreitol (DTT), followed by alkylation with 50 mM iodacetamide for 45 min at room temperature in the dark and tryptic digestion for 4 h at 37 °C. The reaction was terminated by adding formic acid to a final concentration of 0.5%.

Protocol 2: Express Preparation Procedure. Inactivated samples were cooled to −20 °C for 2 h and centrifuged at 20 000*g* for 20 min. The pellet was resuspended in 50 mM ammonium bicarbonate buffer containing 0.1% Rapigest SF Surfactant (Waters) and subjected to tryptic digestion for 4 h at 37 °C. The reaction was terminated by adding formic acid to a final concentration of 0.5%.

LC−MS/MS Method. The tryptic peptides were analyzed in duplicate on a nano-HPLC (high-performance liquid chromatography) Dionex Ultimate3000 system (Thermo Fisher Scientific, USA) coupled to a timsTOF Pro (Bruker Daltonics, USA) mass spectrometer. The sample volume loaded was 2 μL per injection. HPLC separation was carried out using a packed emitter column (C18, 25 cm × 75 μm 1.6 μm) (Ion Optics, Parkville, Australia)[10] by gradient elution. Mobile phase A was 0.1% formic acid in water; mobile phase B was 0.1% formic acid in acetonitrile. LC separation was achieved at a flow of 400 nL/min using a 40 min gradient from 4 to 90% of phase B.

Mass-spectrometric measurements were carried out using the parallel accumulation serial fragmentation (PASEF)[11] acquisition method. The electrospray ionization (ESI) source settings were as follows: 4500 V capillary voltage, 500 V end plate offset, and 3.0 L/min of dry gas at temperature of 180 °C. The measurements were carried out over the m/z range from 100 to 1700 Th. The range of ion mobilities included values from 0.60 to 1.60 V s/cm^2 ($1/k_0$). The total cycle time was set to 1.16 s, and the number of PASEF MS/MS scans was set to 10. For low sample amounts, the total cycle time was set to 1.88 s.

Data Analysis

The obtained data were analyzed using PEAKS Studio 8.5 and MaxQuant version 1.6.7.0 using the following parameters: parent mass error tolerance −20 ppm; fragment mass error tolerance −0.03 Da. Because of the light denaturation conditions, the absence of reduction and alkylation steps in one of the sample preparation approaches, and the short hydrolysis time, up to three missed cleavages were allowed, but only peptides with both trypsin-specific ends were considered.

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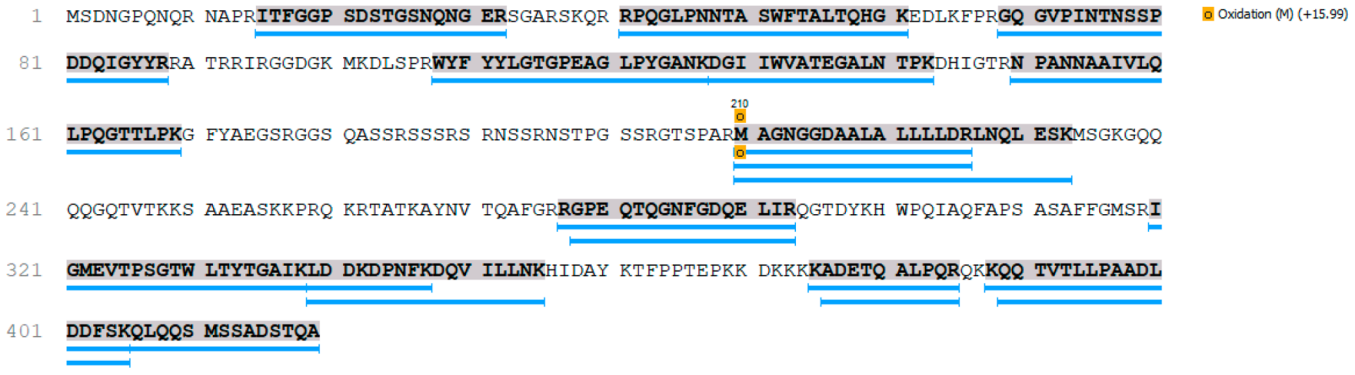

Figure 1. Sequence coverage of the P0DTC9|NCAP_SARS2 nucleoprotein from SARS CoV-2.

Table 2. Peptides from the P0DTC9|NCAP_SARS2 Nucleoprotein Identified via PEAKS Studio in Different Samples[a]

Start	End	Sequence	Missed cleavages	Score	Intensity															
					COVID positive										COVID negative					
					Standard method						Express method				Express method					
					1-1	1-2	2-1	2-2	3-1	3-2	4-1	4-2	5-1	5-2	6-1	6-2	7-1	7-2	8-1	8-2
15	32	R.ITFGGPSDSTGSNQNGER.S	0	114.82	0	0	1	1	2	2	0	0	1	1	0	0	0	0	0	
41	61	R.RPQGLPNNTASWFTALTQHGK.E	1	111.41			1		2	2		1		1						
69	88	R.GQGVPINTNSSPDDQIGYYR.R	0	92.5	0	0	1	2	2	2	2	0	2	2	0	0	0	0	0	0
108	127	R.WYFYYLGTGPEAGLPYGANK.D	0	66.93	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0
128	143	K.DGIIWVATEGALNTPK.D	0	97.47	0	0	1	1	2	4	0	0	1	1	0	0	0	0	0	0
150	169	R.NPANNAAIVLQLPQGTTLPK.G	0	112.96	0	0	1	0	1	2	1	0	3	1	0	0	0	0	0	0
210	226	R.MAGNGGDAALALLLLDR.L	0	128.02	0	0	2	0	3	3	0	0	0	3	0	0	0	0	0	0
210	226	R.M(+15.99)AGNGGDAALALLLLDR.L	0	113.48	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0
210	233	R.MAGNGGDAALALLLLDRLNQLESK.M	1	92.12							1	1	1	3						
277	293	R.RGPEQTQGNFGDQELIR.Q	1	91.31	1	1		1	3	1	1		2	1						
278	293	R.GPEQTQGNFGDQELIR.Q	0	92.81	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0
320	338	R.IGMEVTPSGTWLTYTGAIK.L	0	105.48	1	1	1	1	5	2	0	1	2	2	0	0	0	0	0	0
339	347	K.LDDKDPNFK.D	1	55.92					2	3										
339	355	K.LDDKDPNFKDQVILLNK.H	2	109.74			1	1	1	1	1	1	1	1						
375	385	K.KADETQALPQR.Q	1	65.84						1		1	1							
376	385	K.ADETQALPQR.Q	0	72.36	1	0	0	0	0	1	1	1	0	0	0	0	0	0	0	0
388	405	K.KQQTVTLLPAADLDDFSK.Q	1	87.26							3		1							
389	405	K.QQTVTLLPAADLDDFSK.Q	0	102.7	0	0	1	0	1	1	1	0	1	1	0	0	0	0	0	0
406	419	K.QLQQSMSSADSTQA	0	63.54	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0

[a]Sample preparation protocol: Samples 1−5 were collected from five patients with COVID-19 (1−3 were prepared by Protocol 1; 4 and 5 were prepared by Protocol 2). Samples 6−8 are negative controls (healthy individuals). Two LC−MS/MS runs were performed for each sample. The numbers in the table correspond to spectral counts for each peptide.

Oxidation of methionine and carbamidomethylation of cysteine residues were set as possible variable modifications, and up to three variable modifications per peptide were allowed. The search was carried out using the SwissProt SARS-COV-2 database with the human one set as the contamination database. False discovery rate (FDR) thresholds for all stages were set to 0.01 (1%) or lower. The mass spectrometric proteomic data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD019648.[12]

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Table 3. Peptides from the P0DTC9|NCAP_SARS2 Nucleoprotein Identified via MaxQuant in Different Samples Depending on the Sample Preparation Protocol[a]

Start	End	Sequence	Missed cleavages	Total Intensity	Intensity															
					COVID positive										COVID negative					
					Standard method					Express method					Express method					
					1-1	1-2	2-1	2-2	3-1	3-2	4-1	4-2	5-1	5-2	6-1	6-2	7-1	7-2	8-1	8-2
15	32	ITFGGPSDSTGSNQNGER	0	5E+5	0	0	3E+4	1E+5	9E+4	1E+5	0	0	7E+4	6E+4	0	0	0	0	0	0
41	61	RPQGLPNNTASWFTALTQHGK	1	1E+5					1E+5											
69	88	GQGVPINTNSSPDDQIGYYR	0	1E+6	0	4E+4	2E+4	9E+4	2E+5	3E+5	0	0	1E+5	2E+5	0	0	0	0	0	0
108	127	WYFYYLGTGPEAGLPYGANK	0	1E+5	0	1E+4	0	2E+4	0	0	0	0	3E+4	5E+4	0	0	0	0	0	0
128	143	DGIIWVATEGALNTPK	0	7E+5	0	8E+3	5E+4	1E+4	1E+5	3E+5	2E+4	1E+4	7E+4	1E+5	0	0	0	0	0	0
150	169	NPANNAAIVLQLPQGTTLPK	0	7E+5	0	0	0	9E+4	3E+4	2E+5	0	0	9E+4	2E+5	0	0	0	0	0	0
210	226	MAGNGGDAALALLLLDR	0	5E+5	0	0	0	7E+4	7E+4	2E+5	4E+3	1E+5	2E+4	2E+4	0	0	0	0	0	0
210	226	M(+15.99)AGNGGDAALALLLLDR	0	2E+5	0	0	0	0	0	8e+4	4e+3	1e+5	0	1E+4	0	0	0	0	0	0
210	233	MAGNGGDAALALLLLDRLNQLESK	1	4E+5							2E+4	4E+4	1E+5	2E+5						
277	293	RGPEQTQGNFGDQELIR	1	1E+5						9E+4			8E+3	4E+3						
320	338	IGMEVTPSGTWLTYTGAIK	0	8E+5	4E+4	0	1E+4	1E+5	1E+5	2E+5	8E+4	1E+4	0	2E+5	0	0	0	0	0	0
339	355	LDDKDPNFKDQVILLNK	2	6E+5			2E+4	5E+4		8E+4	4E+4	8E+4	2E+5	2E+5						
388	405	KQQTVTLLPAADLDDFSK	1	9E+4						9E+3		7E+3		7E+4						
389	405	QQTVTLLPAADLDDFSK	0	5E+5	0	0	0	1E+5	1E+5	1E+5	1E+4	0	0	1E+5	0	0	0	0	0	0

[a]Sample preparation protocol: Samples 1−5 were collected from five patients with COVID-19 (1−3 were prepared by Protocol 1; 4 and 5 were prepared by Protocol 2). Samples 6-8 are negative controls (healthy individuals). Two LC−MS/MS runs were performed for each sample. The numbers in the table correspond to the intensities of each peptide.

RESULTS

Approximately 1000−1500 proteins were identified in each sample, among which the P0DTC9|NCAP_SARS2 nucleoprotein of severe acute respiratory syndrome coronavirus 2 was registered (Figure 1).

Depending on the viral content in the samples, the preparation protocol, and the processing software used, 1−17 peptides of the N protein were reliably detected and identified in the COVID-19 patient samples (Tables 2 and 3; MS/MS spectra of these peptides can be found in the Supporting Information).

The N protein, being the most abundant protein in the virion, is the best candidate for mass-spectrometric detection of the infection, and so its detection is expected. Also, the MS-based detection of several peptides from the SARS-COoV-2 nucleoprotein has been previously reported by Ihling et al.[6] in the gargle solution samples of COVID-19 patients. We have performed a pilot study on nasopharynx epithelial swabs already collected from patients with COVID-19 for RT-qPCR and showed confident identification of the N protein with the use of a very basic sample preparation procedure.

More than that, the express procedure allowed better detection of the N protein than the more thorough one standardly used for proteomic analysis. This is probably due to the significantly lower amounts of peptides from the much more abundant host proteins, which require reduction, alkylation, deglycosylation, and other preparative steps for good sequence coverage.

Also, because an untargeted LC−MS/MS with data-dependent acquisition approach was used to identify as many proteins as possible, it is expected that the use of targeted approaches aimed at monitoring the presence of this exact protein will result in significantly lower detection limits. For example the use of PRM approaches on triple−quadrupole mass spectrometers based on the peptides identified in this study may allow us to go below the sensitivity of RT-qPCR, whereas the application of immunoprecipitation methods with subsequent MALDI analysis or even MALDI detection of viral proteins directly on the immobilized antibodies (iMALDI) will allow us to shorten the processing times to as low as 1 h.

The observation of over 1000 host proteins also suggests that this approach could be used for detecting and studying the changes caused by the viral infection in the proteome of host cells as well as the response of the organism to these conditions.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.jproteome.0c00412.

Annotated MS/MS spectra with sequence alignment, error maps, and ion tables for all detected peptides of P0DTC9|NCAP_SARS2 nucleoprotein of severe acute respiratory syndrome coronavirus 2 (PDF)

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Notes

The authors declare no competing financial interest.

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