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Biosafety and Health 5 (2023) 101–107

Contents lists available at ScienceDirect

Biosafety and Health

journal homepage: www.elsevier.com/locate/bsheal

Original Research

A multiplex method for detection of SARS-CoV-2 variants based on MALDI-TOF mass spectrometry

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ARTICLE INFO

Article history:
Received 11 January 2023
Revised 8 February 2023
Accepted 26 February 2023
Available online 1 March 2023

Keywords:
COVID-19
SARS-CoV-2
Multiplexing capability
MALDI-TOF mass spectrometry
Viral variation

ABSTRACT

The recent outbreak of the coronavirus disease 2019 (COVID-19) pandemic and the continuous evolution of the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) have highlighted the significance of new detection methods for global monitoring and prevention. Although quantitative reverse transcription PCR (RT-qPCR), the current gold standard for diagnosis, performs excellently in genetic testing, its multiplexing capability is limited because of the signal crosstalk of various fluorophores. Herein, we present a highly efficient platform which combines 17-plex assays with matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS), enabling the targeting of 14 different mutation sites of the spike gene. Diagnosis using a set of 324 nasopharyngeal swabs or sputum clinical samples with SARS-CoV-2 MS method was identical to that with the RT-qPCR. The detection consistency of mutation sites was 97.9% (47/48) compared to Sanger sequencing without cross-reaction with other respiratory-related pathogens. Therefore, the MS method is highly potent to track and assess SARS-CoV-2 changes in a timely manner, thereby aiding the continuous response to viral variation and prevention of further transmission.

1. Introduction

Coronavirus disease 2019 (COVID-19) is an infectious disease caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), with fever, cough, dyspnoea, myalgia, and fatigue as the most common nonspecific symptoms [1–3]. Humans are highly susceptible, with human-to-human transmission occurring primarily through inhalation of droplets, aerosols, and small airborne particles contaminated by viruses [4,5]. According to the World Health Organization (WHO; https://covid19.who.int/), there have been more than 660 million laboratory-confirmed cases of COVID-19 and over 6.69 million deaths worldwide by 10 January 2023 making it one of the deadliest pandemics in history and posing a serious threat to public health. Therefore, accurate and rapid identification of SARS-CoV-2 is of great significance, which has led to the emergence of a large number of SARS-CoV-2 detection techniques.

The main detection techniques for SARS-CoV-2 include viral cultures, immunological tests, and molecular tests. Viral culture involves inoculation of viral suspensions from clinical samples onto cultured cells, such as VeroE6, Huh7 and human airway epithelial cells, and amplification of the virus through cell passage and culture [6–8]. With its high accuracy, the isolation of viruses in cell culture is widely recognised as the gold standard for identifying viral pathogens. However, time-consuming and complicated protocols that require high levels of stringency and safety make it unsuitable for the large-scale, rapid diagnosis essential in severe pandemic situations such as COVID-19. Using the principle of specific antigen-antibody binding, a lateral flow immunoassay (LFIA) can detect and visualise the presence of a specific viral antigen within 15 min. LFIA can be used for point-of-care testing owing to its low cost, user-friendliness, and equipment-free nature. However, its sensitivity is greatly affected by the viral load, and false negative results can easily occur during the incubation period and in healthy carriers [9,10]. Therefore, it can only be used as an auxiliary method for rapid, primary screening of diseases. Nucleic acid amplification tests such as reverse transcription polymerase chain reaction (RT-PCR), quantitative reverse transcription PCR (RT-qPCR), nested RT-PCR, digital RT-PCR (dPCR), target RNA-dependent RNA polymerase (RdRp), open reading frames (ORFs), nucleocapsid (N), and envelope (E) genes to detect SARS-CoV-2 [7,11]. Among these molecular tests, RT-PCR is currently considered the gold standard for the detection of SARS-CoV-2 and diagnosis of COVID-19 by WHO and the Centers for Disease Control

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https://doi.org/10.1016/j.bsheal.2023.02.003
2590-0536/

HIGHLIGHTS

Scientific question

To design a reliable method for simultaneous detection of SARS-CoV-2 and identification of specific viral variants.

Evidence before this study

Accurate detection of SARS-CoV-2 is certainly of the essence during one of the deadliest pandemics, coronavirus disease 2019 (COVID-19). Previous studies have established various detection techniques, within which RT-PCR is typically regarded as the gold standard for disease diagnosis. However, its inability to target multiple genes limits clinical application in identifying SARS-CoV-2 variants.

New findings

Based on coupled methods combining multiplex PCR and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) designed by our research group, we had made some improvements on reagents and instruments. The new optimized method was further applied to the analysis of SARS-CoV-2, namely SARS-CoV-2 MS method.

Significance of the study

The designed multiplex method is sensitive and high throughput to detect any known SARS-CoV-2 variants and to track its evolution. Furthermore, the improved aspects could benefit the flexibility and applicability of the SARS-CoV-2 MS method.

and Prevention (CDC) [12–16]. Molecular tests ensure rapid detection with high sensitivity and specificity. However, most molecular tests only target a single or few genes, leading to low detection accuracy, inability to identify SARS-CoV-2 variants, and ultimately, limitations in clinical application [17]. Therefore, it is necessary to develop a multiplexed method that can target multiple SARS-CoV-2 genes in a single reaction.

Our research group has developed a series of detection methods combining multiplex PCR and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS). These methods have been used for the analysis of various microorganisms with DNA or RNA genomes, including 30 oncogenic human papillomavirus genotypes, 11 different pathogens related to sexually transmitted infections, 21 common respiratory viruses, and six human coronaviruses (HCoVs) [18–21]. The ability to detect HCoVs is especially important as the high pathogenicity, infectivity, and susceptibility of HCoVs, especially severe acute respiratory syndrome coronavirus (SARS-CoV) and Middle East respiratory syndrome coronavirus (MERS-CoV), make them a great threat to global health. Xiu et al. [21] showed two 17-plex panels, in which panel A was designed to detect six known HCoVs, namely HCoV-229E, HCoV-OC43, HCoV-NL63, HCoV-HKU1, SARS-CoV, and MERS-CoV, and panel B was used to discover unknown HCoVs by detecting all alphacoronaviruses (α-CoV) and betacoronaviruses (β-CoV). In this study, we improved and optimised the high-throughput assay and applied it to 17-plex analysis of SARS-CoV-2, a strain of β-CoV subgroup B identified in 2019, for three coronavirus genes: N, RdRp, and spike glycoprotein (S) [7]. Performance characteristics of the MS method were compared with those of RT-qPCR, and PCR products from the clinical samples were sequenced to identify SARS-CoV-2 variants and subvariants.

Table 1
Primer and probe sequences for detecting SARS-CoV-2 gene.

Number	Gene target	Sequence
1	RNaseP	F[a]: ACGTTGGATGTGAGCGGCTGTCTCCACAAG R[b]: ACGTTGGATGTGGCGGTGTTTGCAGATTTG P[c]: CGAGCGGGTTCTGAC
2	N	F: ACGTTGGATGAATTGGAACGCCTTGTCCTC R: ACGTTGGATGTTGGTTCACCGCTCTCACTC P: TCTCACTCAACATGGC
3	RdRp	F: ACGTTGGATGCTACCTGGCGTGGTTTGTAT R: ACGTTGGATGCGAAATGCTGGTATTGTTGG P: GATTGTAACTGGTATGATTTCGG
4	D950N	F: ACGTTGGATGGACTCACTTTCTTCCACAGC R: ACGTTGGATGAGCGTGTTTAAAGCTTGTGC P: CATTTTGGTTGACCACAT
5	T19I	F: ACGTTGGATGTGCCACTAGTCTCTAGTCAG R: ACGTTGGATGTCAGGGTAATAAACACCACG P: GGGTAATTGAGTTCTGGTT
6	F486V	F: ACGTTGGATGAGTACTACTACTCTGTATG R: ACGTTGGATGAAATCTATCAGGCCGGTAGC P: GTGTAATGGTGTTGAAGGT
7	T95I	F: ACGTTGGATGAGTACCAAAAATCCAGCCTC R: ACGTTGGATGGAGGTTTGATAACCCTGTCC P: TGGTGTTTATTTTGCTTCCA
8	D614G	F: ACGTTGGATGTTCTAACCAGGTTGCTGTTC R: ACGTTGGATGACACGCCAAGTAGGAGTAAG P: GGGACTTCTGTGCAGTTAACA
9	A67V	F: ACGTTGGATGACTCAGGACTTGTTCTTACC R: ACGTTGGATGCCTCTTAGTACCATTGGTCC P: GTGGTCCCAGAGACATGTATA
10	L452Q	F: ACGTTGGATGCTTGATTCTAAGGTTGGTGG R: ACGTTGGATGCTACCGGCCTGATAGATTTC P: AGACTTCCTAAACAATCTATAC
11	Q493R	F: ACGTTGGATGAAATCTATCAGGCCGGTAGC R: ACGTTGGATGAGTACTACTACTCTGTATG P: AGTGGGTTGGAAACCATATGAT
12	V213G	F: ACGTTGGATGGGCAAATCTACCAATGGTTC R: ACGTTGGATGATATATTCTAAGCACACGCC P: GACGCACACGCCTATTAATTTAG
13	D405N	F: ACGTTGGATGTTGCCCTGGAGCGATTTGTC R: ACGTTGGATGTGTTATGGAGTGTCTCCTAC P: GAGATTCATTTGTAATTAGAGGT
14	P681R	F: ACGTTGGATGCCAAGTGACATAGTGTAGGC R: ACGTTGGATGGCGCTAGTTATCAGACTCAG P: TTTATCAGACTCAGACTAATTCTC
15	T376A	F: ACGTTGGATGGGAACAGGAAGAGAATCAGC R: ACGTTGGATGTAGGAGACACTCCATAACAC P: GGATACACTCCATAACACTTAAAAG
16	S704L	F: ACGTTGGATGCACTATGTCACTTGGTGCAG R: ACGTTGGATGCACTGGTAGAATTTCTGTGG P: AATAGAGTTATTAGAGTAAGCAACT
17	Y145D	F: ACGTTGGATGCACTTTCCATCCAACTTTTG R: ACGTTGGATGGACCCAGTCCCTACTTATTG P: GGGAGGATCCATTTTTGGGTGTTTA

Abbreviation: SARS-CoV-2, severe acute respiratory syndrome coronavirus 2.
[a] F, forward primer.
[b] R, reverse primer.
[c] P, extension probe.

2. Materials and methods

2.1. Design of SARS-CoV-2 MS method

Based on published studies, we selected 17 highly conserved genetic regions as targets, containing 14 distinct regions of the S gene

for detection, N and RdRp genes for virus identification, and ribonuclease P gene (RNaseP gene) as an internal control to verify the presence of nucleic acids.

All available target sequences were downloaded from GenBank (https://www.ncbi.nlm.nih.gov/genbank/) and GISAID database (https://www.gisaid.org/, 5 records, Accession ID: EPI_ISL_10757906, EPI_ISL_11360236, EPI_ISL_11360241, EPI_ISL_7398392, and EPI_ISL_14560351) [22]. Primer pairs and extension probes were designed using Assay Design 4.1 software (Agena Bioscience, Inc., San Diego, CA, USA) and their specificities were confirmed using Primer-BLAST (https://www.ncbi.nlm.nih.gov/tools/primer-blast/). Both the multiplex PCR primer pairs and extension probes for single base extension (SBE) were synthesised by Tsingke Biotechnology (Beijing, China). The 17 genetic target regions and their respective primers and probes are listed in Table 1.

2.2. Sample collection and nucleic acid extraction

Nasopharyngeal swabs or sputum samples were collected from 324 patients confirmed or suspected to be positive for SARS-CoV-2 infection. Three SARS-CoV-2 isolates from clinical specimens (Delta, Omicron BA.1, and Omicron BA.5) were obtained from Christophe Mérieux Laboratory, Institute of Pathogen Biology, Chinese Academy of Medical Sciences & Peking Union Medical College. And we also used 49 clinical samples and a viral strain isolated from bats that were positive for 34 common respiratory pathogens to assess the potential cross-reactivity of this assay.

Nasopharyngeal swab samples were collected from the nose and throat with nylon swabs, which were rotated three times for 15 s. All collected samples were transferred to 200 μL viral transport medium (VTM) and transported at 2 ℃ ~ 8 ℃. Nucleic acids were extracted from VTM using the QIAamp Viral RNA Mini Kit and the QIAamp DNA Mini Kit (QIAGEN, Hilden, Germany) following the manufacturer's instructions. And all the extracted DNA and RNA were stored at −80 ℃.

2.3. Experimental procedures of SARS-CoV-2 MS method

All three reaction systems, including PCR, shrimp alkaline phosphatase (SAP), and SBE, were performed using a ProFlex PCR system (Applied Biosciences, Foster City, CA, USA). The 10 μL multiplex PCR reaction consisted of 2 μL SARS-CoV-2 sample (extracted viral RNA), 2.6 μL primer mix, 5 μL 2 × Reaction Mix, and 0.4 μL SuperScript III RT/Platinum Taq Mix (Invitrogen, Carlsbad, CA, USA). The reaction mix was loaded in a 384-well PCR plate and the thermal cycling parameters were as follows: RNA reverse transcription PCR at 55 ℃ for 30 min; a pre-denaturation step at 94 ℃ for 2 min; 45 PCR cycles, each of which included denaturation at 94 ℃ for 15 s, annealing at 56.5 ℃ for 30 s, and extension at 68 ℃ for 1 min; and final elongation at 68 ℃ for 5 min. At this stage, different targets of SARS-CoV-2 were amplified by multiplex PCR.

After 45 PCR cycles, SAP was used to dephosphorylate unincorporated dNTPs, making them unavailable for further stages [23]. According to the manufacturer's instructions, 0.3 μL SAP (Agena Bioscience, Inc., San Diego, CA, USA) and the reaction buffer were mixed with the PCR amplification products and incubated at 37 ℃ for 40 min and then 85 ℃ for 5 min.

Following SAP incubation, site-specific oligonucleotide primer mixtures were bonded directly to their respective amplicons, and single base extension (SBE) was carried out, using ddNTPs as the substrate. The SBE reaction containing 0.94 μL extension probe cocktail, 0.2 μL 10 × iPLEX Buffer Plus, 0.2 μL terminator mix, and 0.041 μL ThermoSequenase enzyme (Agena Bioscience, Inc., San Diego, CA, USA) was added to the reactions and incubated in accordance with standard conditions (95 ℃ for 30 s; 45 cycles, each of which included 94 ℃ for 5 s, followed by 5 cycles at 52 ℃ for 5 s and 80 ℃ for 5 s; 72 ℃ for 3 min).

Finally, the reaction products were mixed with 11 μL nuclease-free water and fully contacted with a cationic ion exchange resin (Beijing JOHN LUNDA Technology Development Co., Ltd., Beijing, China) by rotation at 80 rpm for 40 min to absorb the cations. 1 μL matrix composed of 10:1 3-hydroxypicolinic acid: ammonium citrate dibasic solution was spotted on an AXIMA 384 well sample plate (SHIMADZU, Kyoto, Japan) and dried for 1 min at room temperature of 21°C – 25° C. The 1 μL purified reaction products were then spotted onto the target plate coated with the dried matrix. After allowing to stand at room temperature for about 2 min, the target plate was performed at a power of 120 V and profiles of 50 using an AXIMA performance™ mass spectrometer (SHIMADZU, Kyoto, Japan) and analysed by MALDI-MS software. The AXIMA performance™ ionises the reaction products by irradiation. The DNA ions were then separated at different speeds based on their mass-to-charge ratios (m/z) and stratified by a highly sensitive ion detector. Finally, the mass spectra of these DNA ions were obtained by the signal recording system of the mass spectrometer for data analysis and further identification and genotyping of the novel coronavirus [24]. A brief flowchart of the SARS-CoV-2 MS method is shown (Fig. 1).

2.4. Evaluation of SARS-CoV-2 MS method

Viral RNA of the three SARS-CoV-2 clinical isolates (Delta, Omicron BA.1, and Omicron BA.5) was quantified using a Qubit 4 fluorometer (Thermo Fisher Scientific Inc.). Next, the three isolates were mixed with equal proportions of the extracted human genome and then serially diluted to concentrations of 50,000, 5,000, 500, 50, and 5 copies/μL (100,000, 10,000, 1,000, 100, and 10 copies per reaction, respectively). This dilution series was used to determine the limit of detection (LOD) of the SARS-CoV-2 MS method. A common group of respiratory pathogens, including viral and bacterial pathogens, were also analysed using the MS method to determine the specificity of this assay. Each sample was performed in duplicate and nuclease-free water was used as a negative control.

2.5. Confirmation of SARS-CoV-2 MS method

To confirm the results of the SARS-CoV-2 MS method, RNA extracted from 324 clinical samples and 3 clinical isolates was simultaneously analysed by the MS and RT-qPCR methods. A total of 327 samples were performed in QuanStudio™ 6 Flex Real-time PCR system (Thermo Fisher Scientific Inc., Waltham, USA) using a Novel Coronavirus Nucleic Acid Detection Kit (Biogerm, Shanghai, China), and then viral RNA was analysed using the quantitative standard curve.

To further verify the positive results of the MS method, viral RNA of 327 samples was reverse transcribed into cDNA using SuperScript III Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA) and then amplified using Takara Taq DNA Polymerase (Takara, Kyoto, Japan). The amplification products were analysed by Sanger sequencing (Tsingke Biotechnology Co., Ltd., Beijing, China). Primer pairs used for Sanger sequencing are shown in Supplementary Table S1.

3. Results

3.1. Performance of the SARS-CoV-2 MS method

In this study, we developed a 17-plex method targeting the N, RdRp, and S genes and tested its efficiency in detecting various variants of SARS-CoV-2. The N and RdRp genes were used for virus detection, the S gene was used to analyse the important mutation sites of SARS-CoV-2 and the RNaseP gene was used as an internal control.

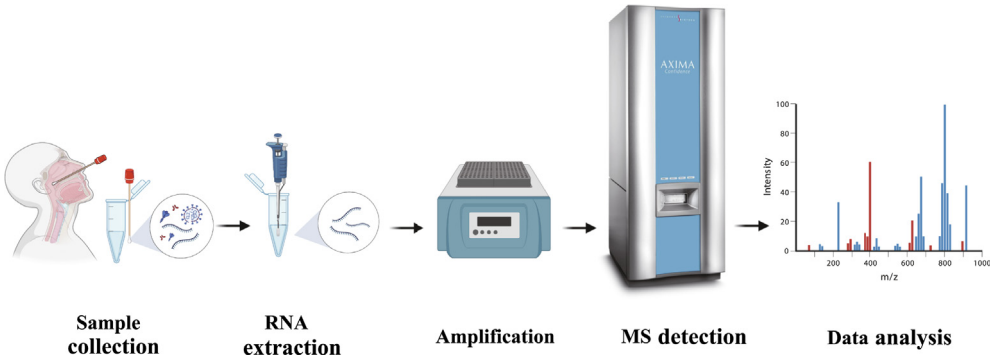

Fig. 1. Schematic workflow of the SARS-CoV-2 MS method. The workflow mainly contained four steps. First, Nasopharyngeal swabs or sputum samples were collected. Second, viral RNA was extracted from all collected samples. Thirdly, multiplex PCR was used to amplify different genetic target regions of SARS-CoV-2. Finally, the reaction mix was analysed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) to determine the types of viral variants. Abbreviations: SARS-CoV-2, severe acute respiratory syndrome coronavirus 2; MS, mass spectrometry.

Table 2
The identification criteria for target mutation sites.

Number	Gene target	The m/z[a] of negative results	The m/z[a] of positive results
1	RNaseP	4,609	4,796
2	SARS-CoV-2_N	4,801	5,028
3	SARS-CoV-2_RdRp	7,225	7,386
4	D950N	5,465	5,736
5	T19I	5,605	5,876
6	F486V	5,954	6,225
7	T95I	6,095	6,282
8	D614G	6,161	6,332
9	A67V	6,570	6,757
10	L452Q	6,846	6,934
11	Q493R	6,839	7,010
12	V213G	7,208	7,555
13	D405N	7,833	7,984
14	P681R	8,002	8,289
15	T376A	8,227	8,354
16	S704L	8,337	8,464
17	Y145D	8,374	8,461

[a] *m/z*, mass to charge ratio.

All primer pairs and extension probes were confirmed as robust by nucleotide BLAST. According to the mass spectra and the identification criteria for target mutation sites (Table 2), we determined whether there were genetic mutations in each sample target. The same MS result of *m/z* as the negative value indicated that the target was not mutated, whereas one matching the positive value allowed the identification of the specific mutation sites. According to the interpretation criteria formulated from GISAID (https://gisaid.org/resources/statements-clarifications/) and Nextstrain database (https://nextstrain.org/) for the SARS-CoV-2 MS method (Table 3), the viral variants of Delta, Omicron BA.1, Omicron BA.2, and Omicron BA.5 were distinguished. Mutation sites were detected for the Delta variant (D950N, D614G, L452Q, and P681R), the Omicron BA.1 subvariant (T95I, D614G, A67V, Q493R, and Y145D), the Omicron BA.2 subvariant (T19I, D614G, Q493R, V213G, D405N, and T376A), and the Omicron BA.5 subvariant (F486V, D614G, L452Q, V213G, and D405N). The internal reference genes RNaseP, N, and RdRp, were all positive in the results of these four viral variants. By further analysing the mass spectra of the three clinical isolates (Delta, Omicron BA.1, and Omicron BA.5), the identification criteria in Table 2 were calibrated to ascertain the mutation of each target. The mass spectrum of Omicron BA.1 is shown (Fig. 2). Using the ultimate judgement criteria (including identification criteria from Table 2 and interpretation criteria from Table 3), the MS method was shown to be highly sensitive for species identification of viral variants in clinical applications.

3.2. Analysis of sensitivity and specificity

The analytical sensitivity of the SARS-CoV-2 MS method was evaluated using 10-fold serial dilutions of the three clinical isolates (Delta, Omicron BA.1, and Omicron BA.5) with concentrations ranging from 5 to 50,000 copies/μL. The mass spectra peaks showed that the MS method was successful in detecting all targets. Moreover, the LOD of each target calculated using the MS method was as low as 5 copies/μL (10 copies per reaction). The nuclease-free water, used as the negative control, produced negative results.

To determine the specificity of the MS method, 34 nonspecific respiratory pathogens in 50 samples were investigated using the same experimental steps. This panel of common respiratory pathogens, including adenovirus, human enterovirus, HCoV-OC43, HCoV-229E, HCoV-NL63, HCoV-HKU1, MERS-CoV, human bocavirus 1, human metapneumoviruses (hMPV A and B), human rhinovirus, influenza A H1N1, influenza A H3N2, influenza B viruses, parainfluenza virus (PIV 1 to 4), respiratory syncytial viruses (RSV A and B), SARS-Like coronavirus, Legionella pneumophila, Bordetella pertussis, Mycoplasma pneumoniae, *Chlamydophila pneumoniae, Haemophilus influenzae, Staphylococcus aureus, Moraxella catarrhalis, Klebsiella pneumoniae, Pseudomonas aeruginosa, Acinetobacter baumannii, Streptococcus pneumoniae, Escherichia coli, Neisseria meningitidis*, were used for the method validation (Supplementary Table S2). The mass spectra showed negative results for all 34 common viral and bacterial pathogens, indicating no cross-reactivity which would interfere with the identification of the target analytes.

3.3. Application of the SARS-CoV-2 MS method in clinical samples

We used 327 samples (including 324 clinical samples and 3 identified clinical isolates) to demonstrate the applicability of the SARS-CoV-2 MS method. The RNaseP, N, and RdRp genes for virus identification were detected in parallel by the MS method and RT-qPCR diagnosis. Positive results for SARS-CoV-2 were detected for 48 samples, while 279 samples were negative using our method. These detection results matched the RT-qPCR diagnosis. Sanger sequencing was used to detect the specific mutation sites of the S gene for all 48 positive samples selected by RT-qPCR, so as to determine viral variants accurately. The MS method and Sanger sequencing were congruent in the identification of 47 positive results (16 Delta variants, 16 Omicron BA.1, 7 Omicron BA.2, and 8 Omicron BA.5 lineages). In the single sample where results differed, only the N gene and a few mutation sites of the S gene (L452Q) were successfully detected using our method. A high cycle threshold (CT) value of 39, implying low viral load, was detected for this sample using RT-qPCR. Further, its agarose gel electrophoresis band was too weak to be visualised, resulting in the

Table 3
The interpretation criteria of the SARS-CoV-2 MS method results.

Viral variant strain	1 RNaseP	2 N	3 RdRp	4 D950N	5 T19I	6 F486V	7 T95I	8 D614G	9 A67V	10 L452Q	11 Q493R	12 V213G	13 D405N	14 P681R	15 T376A	16 S704L[a]	17 Y145D
Delta	+	+	+	+	–	–	–	+	–	+	–	–	–	+	–	–	–
Omicron-BA.1	+	+	+	–	–	–	+	+	+	–	+	–	–	–	–	–	+
Omicron-BA.2	+	+	+	–	+	–	–	+	–	–	+	+	+	–	+	–	–
Omicron-BA.5	+	+	+	–	–	+	–	+	–	+	–	+	+	–	–	–	–

+, positive detection results of RNaseP, N, and RdRp genes or specific mutation sites.
−, negative detection results of specific mutation sites, representing wild viral strains.
Abbreviations: SARS-CoV-2, severe acute respiratory syndrome coronavirus 2; MS, mass spectrometry.
[a] S704L mutation site positive for SARS-CoV-2 Omicron BA.2.12.1 variant strain.

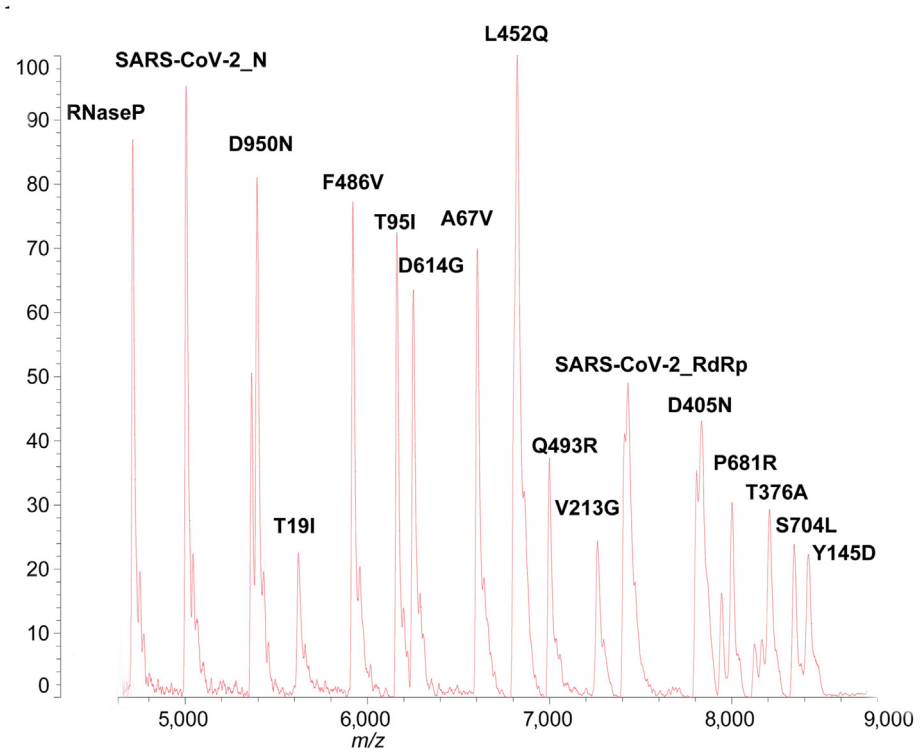

Fig. 2. The mass spectrum of SARS-CoV-2 Omicron BA.1. Three SARS-CoV-2 clinical isolates (Delta, Omicron BA.1, and Omicron BA.5) were detected using the MS method, among which the detection results of Omicron BA.1 variants were shown. The mutation sites of each target were confirmed using the identification criteria in Table 2. Abbreviation: SARS-CoV-2, severe acute respiratory syndrome coronavirus 2; MS, mass spectrometry.

inability to determine the S gene mutation sites by Sanger sequencing, leading to only some mutation sites being successfully detected using the MS method. The positive detection rate of specific mutation sites in clinical samples was 97.9% (47/48). The mass spectra of the Delta variant, Omicron BA.1, Omicron BA.2, and Omicron BA.5 variant are shown in Fig. 3 and Supplementary Figure S1.

4. Discussion

Assay coupled multiplex PCR with MALDI-TOF MS was researched previously for rapid detection of coronaviruses to prevent viral spread [21]. Nevertheless, the approach had some limitations. First, DNA was necessary as the initial template for multiplex PCR, which means that RNA viruses must be reverse-transcribed into DNA. This extra step after sample extraction increased the complexity and duration of the assay. Second, the MassARRAY® System (Agena Bioscience, Inc., San Diego, CA, USA) was used to scan and measure the masses of DNA molecules. Although the genetic testing and data analysis for such an approach is now simplified, the practical application is limited by the closed nature of this commercial platform.

Based on these studies, we modified several aspects of the previous method. The SARS-CoV-2 MS method uses a universal MALDI-TOF mass spectrometer and can be performed with other MALDI-TOF mass spectrometers that meet these requirements. This improved method possesses the advantages of flexibility and applicability, mainly reflected in the flexible use of reagents and the convenient operation of instruments.

For the flexibility of reagents, we introduced a one-step RT-PCR system, SuperScript III RT/Platinum Taq Mix, containing both reverse transcriptase and DNA polymerase. The extracted viral RNA can be directly used without additional reverse transcription from RNA to cDNA, thus efficiently saving time and labour. Furthermore, we tested 15 different types of resins and selected a domestic cationic ion exchange resin from Beijing JOHN LUNDA Technology Development, expanding the range of commercial reagents and reducing their costs.

For convenience and intended general application of the instruments, we first replaced the MassARRAY® System with AXIMA performance™. This new coupled method using a universal mass spectrometer can be integrated with other mass spectrometers of any brand, reducing the limitations of the original work using a closed

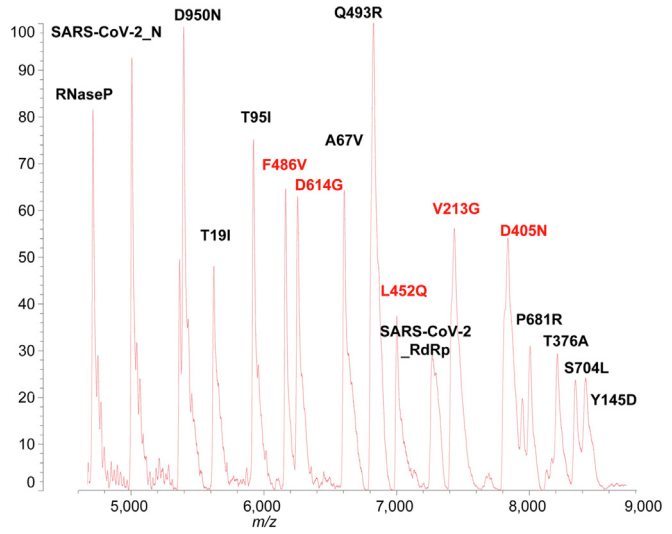

Fig. 3. The detection results of SARS-CoV-2 Omicron BA.5 variants using the MS method. The types of viral variants in 327 samples were identified by the judgement criteria in Tables 2 and 3.

platform. Using AXIMA performance™ also extends the types of samples able to be analysed, such as carbohydrates and metabolites, and the possibility of their subsequent application in experiments, such as the process of pharmacokinetics and characterisation of proteins. This highlights the versatility of the universal mass spectrometer, rather than the MassARRAY® System specialising in molecular diagnostics. Second, instead of transferring analytes of extremely small size using the MassARRAY® Nanodispenser RS1000 automatic spotter, the manual sample-spotting technique used was more flexible in terms of the number of samples to be tested. The MS method was successfully employed on various sample plates depending on the number of samples required for testing, including 48-well, 96-well, and 182-well plates, avoiding wastage of substrates and target plates. Finally, for the mass spectrometry data analysis, we also made some improvements based on SHIMADZU's original software, to replace Typer software (Agena Bioscience, Inc., San Diego, CA, USA) in the previous method, making it more suitable for multiplex detection of pathogens. The improved software is capable of superimposing and comparing the mass spectra of different samples, making the presentation of detection results more apparent and intuitive. Meanwhile, the recorded experimental data can be represented by either bar charts or broken-line graphs as required, which also highlights the flexibility of this new method and the ease of reading and analysis.

The COVID-19 pandemic remains a serious issue for global healthcare systems. Timely and accurate COVID-19 testing is essential for minimising the infection prevalence and initiating adequate therapy. Among the different kinds of viral tests, RT-PCR is typically the most reliable and is regarded as the gold standard for disease diagnosis. However, previous studies have shown that false-negative rates ranged from 2% [25] to 58% [26], and the total false-negative rate was 12% (95% CI 0.10–0.14; $P < 0.01$) among 18,565 COVID-19 confirmed participants [27]. Error results come from three phases: the pre-analytical phase (sampling, storage, and transfer), the analytical phase (RNA extraction, cDNA synthesis, and PCR amplification), and the post-analytical phase (interpretation and analysis) [28]. Various laboratory tests involve the same steps, such as sample collection and SARS-CoV-2 RNA extraction, resulting in similar inevitable errors [29]. Samples with insufficient viral load, the occurrence of genome mutations, and the inexact determination of baseline and threshold are also important sources of false-negative results.

Compared with RT-PCR, the SARS-CoV-2 MS method has proven to be more accurate for detecting the RNaseP, N, and RdRp genes, and further identifying SARS-CoV-2. Our method is sensitive enough to detect samples with lower viral loads (LOD of 10 copies per reaction). DNA molecules can be identified by calculating and analysing their exact molecular weight, a direct detection of their inherent physical properties without the need for fluorescence and labelling [30]. In addition, it is more economical for large-scale epidemiological surveillance because of its scalable throughput and lower price per sample.

A surge in the number of infected persons during the pandemic has contributed to increased opportunities for viral genome mutations. Multiple variants of SARS-CoV-2 have emerged and circulated around the world over time, such as the Alpha variant (B.1.1.7 and Q lineages) in December 2020, Delta (B.1.617.2 and AY lineages) in May 2021, and Omicron (B.1.1.529, BA.1, BA.2, BA.3, BA.4, and BA.5 lineages) in November 2021. The major global viral variants of concern have been characterised differently in terms of transmissibility, virulence, reinfection risk, and immune escape [31], highlighting the importance of the accurate identification of different viral variants. Multiplex RT-PCR tests have been developed in many countries [32–36] involving the simultaneous targeting of ORF1ab and N genes in China or different genetic regions of the N gene in the USA. However, each fluorophore has different excitation and emission spectra. The various fluorophores used for multiplex RT-PCR produce potential crosstalk and increase the risk of false-positive amplification. To minimise the crosstalk between fluorescent signals, fluorophores should have minimal spectral overlap and the most separate emission spectra [37], which limits the multiplexing capacity of RT-PCR. By contrast, the MS method can simultaneously target 14 genetic regions of the S gene without the limitation of fluorophores. Because of the extensive coverage of targets, our method has a strong ability to detect any known variants and to track the evolution of SARS-CoV-2.

However, despite optimisation, the SARS-CoV-2 MS method still has some limitations. First, the experimental results depended on the quality of the sample. If the viral load of a clinical sample is too low, the MS method may not be able to detect the presence of SARS-CoV-2. As only a small initial volume of the sample is used (2 μL), increasing sample volume and rounds of RT-qPCR could compensate for the low viral load of the sample. Second, the workflow turnaround time is nearly 8 h after nucleic acid extraction, making it impractical for a small number of samples [30]. Third, the coupled method involves multiple additions and transfers of the reagents. The risk of cross-contamination between samples increases with the multiple tube lid openings during the experiment. The need for highly trained technicians to operate complicated manual tasks hinders its clinical application. Despite these shortcomings, our method does have great potential to accurately detect different viral variants compared to current methods.

Ethics statement

All experiments were performed according to the ethical standards of the national research committee and approved by the Institutional Review Boards of the Institute of Pathogen Biology. All samples were obtained under approved ethical protocols and with informed consent from each patient.

Acknowledgements

The research project was funded by grants from the CAMS Innovation Fund for Medical Sciences (CIFMS, 2021-I2M-1-038), Non-profit Central Research Institute Fund of Chinese Academy of Medical Sciences (2019PT310029), and the Fundamental Research Funds for the Central Universities (3332021092). We are grateful to Lili Ren for kindly providing us with clinical isolates of SARS-CoV-2. And we also would like to thank Editage (www.editage.cn) for English language editing.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

Author contributions

Ziyuan Zhao: Conceptualization, Methodology, Data curation, Formal analysis, Writing – original draft, Writing – review & editing. **Liying Sun:** Conceptualization, Methodology, Validation, Data curation, Formal analysis, Writing – review & editing. **Liqin Wang:** Methodology, Validation, Data curation. **Xiaodong Li:** Data curation. **Junping Peng:** Conceptualization, Methodology, Formal analysis, Writing – review & editing, Project administration, Funding acquisition, Resources.

Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bsheal.2023.02.003.

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