Simultaneous Detection of *SARS-CoV-2* and Six Other Human Coronaviruses by Multiplex PCR and MALDI-TOF MS

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**Abstract:** The outbreak of *severe acute respiratory syndrome coronavirus 2* (*SARS-CoV-2*) is challenging the health systems worldwide, and large population testing is a vital step to control this pandemic. Here, we developed a new method (named HCoV-MS), which combines multiplex PCR with matrix-assisted laser desorption/ionization-time of flight mass spectrometry to simultaneously detect and differentiate seven human coronaviruses (HCoVs). The HCoV-MS method had good specificity and sensitivity, with a detection limit of 1-5 copies/reaction. To validate the HCoV-MS method, we tested 151 clinical samples, and the results showed good concordance with real-time PCR. In addition, 41 D614G variants were identified, which were consistent with the sequencing results. This method was also used in EQAE-SARS-COV in 2020, and all the samples were accurately identified. Taken together, HCoV-MS could be used as an effective method for large-scale detection. It was also capable of detecting key single nucleotide polymorphism about variants.

**Keywords:** Coronavirus; MALDI-TOF MS; Detection; D614G Variant; Biotyping

**Introduction**

An outbreak of *severe acute respiratory syndrome coronavirus 2* (*SARS-CoV-2*) began in December 2019, and the associated coronavirus disease (COVID-19) rapidly distributed worldwide. CoVs are large, enveloped, positive-sense RNA viruses that could cause respiratory diseases in a range of animals, including humans(Xiu, Zhang et al. 2017). In addition, CoVs are divided into four genera, namely δ-CoVs, γ-CoVs, β-CoVs, and α-CoVs, among which β-CoVs and α-CoVs can infect mammals(Ezhilan, Suresh et al. 2021). Six human CoVs (HCoVs) have been identified so far, including *HCoV-NL63*, *HCoV-229E*, *HCoV-OC43*, *HCoV-HKU1*, *SARS-CoV*, and *MERS-CoV*(Xiu, Zhang et al. 2017). Approximately 15%–30% of respiratory tract infections worldwide each year are caused by *HCoV-229E*, *HCoV-OC43*, *HCoV-NL63*, and *HCoV-HKU1*. They are mild and self-healing diseases and will not pose a major threat to the human health of the public. However, the SARS that broke out in 2002, the Middle East Respiratory Syndrome (MERS) that started in Saudi Arabia in September 2012, and the novel coronavirus pneumonia caused by *SARS-CoV-2* that emerged in December 2019 were different. They are all thought to be zoonotic and have been associated with more severe and potentially fatal outcomes(Miller, McGrath et al. 2020). The World Health Organization named the 2019 novel coronavirus disease COVID-19 on February 11, 2020, and the International Committee on Taxonomy of Viruses named the 2019 novel coronavirus *SARS-CoV-2* on the same day. *SARS-CoV-2* is the 7th HCoV, which has been confirmed in history. The homology of the whole genome sequence with that of *SARS-CoV* is 76%(Wu, Peng et al. 2020).

The ability to quickly and accurately detect and diagnose infected persons or carriers is of vital importance and significance for epidemic prevention and control when an epidemic occurs. Currently, the most commonly used detection methods are real-time PCR(Gao and Quan 2020). Although real-time PCR is highly sensitive, it is difficult to detect multiple mutations in the variant using this method. *SARS-CoV-2* has spread rapidly in large areas around the world and has gradually appeared as a variant since December 2019, making it more contagious and causing serious harm. As of 6 May 2021, the U.S. Centers for Disease Control and Prevention and the European Centre for Disease Prevention and Control have identified multiple *SARS-CoV-2* lineages as variants of concern or variants of interest, including Alpha (B.1.1.7, Q.1-Q.8), Beta (B.1.351, B.1.351.2, B.1.351.3), Gamma (P.1, P.1.1, P.1.2), Epsilon (B.1.427 and B.1.429), Eta (B.1.525), Iota (B.1.526), Kappa (B.1.617.1), B.1.617.3, Mu (B.1.621, B.1.621.1), Zeta (P.2) and Delta (B.1.617.2 and AY.1 sublineages) (ECDC 2021, U.S.CDC 2021). Many share the same mutation site, such as D614G, E484K, N501Y, and/or L452R. The D614G variant refers to a strain, where aspartic acid (D) at amino acid position 614 of *SARS-CoV-2* is mutated to glycine (G). The variant only accounted for less than 10% of the global sequence of *SARS-CoV-2*, when it first appeared in Europe in early February 2020(Korber, Fischer et al. 2020). However, it had spread to the Americas, Oceania, South America, and Asia after just over four months. It accounted for more than 74% of the global sequence of *SARS-CoV-2* and became the main strain form during the pandemic by June 2020(Yurkovetskiy, Wang et al. 2020). Accurate identification of mutant strains is helpful for the surveillance, prevention, and control of COVID-19.

Nucleic acid mass spectrometry analysis, combining multiplex PCR (mPCR) with matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF MS), designs sequence targets based on single nucleotide polymorphism (SNP) sites to achieve multiple detection and mutation identification(Settanni and Corsetti 2007, Tsuchida, Umemura et al. 2020). At present, nucleic acid mass spectrometry has been widely used in multiple detection and typing of bacteria or viruses, such as the simultaneous detection of 10 duck viruses(Liu, Wang et al. 2019), multi-site typing of Mycoplasma pneumoniae, simultaneous detection of drug resistance(Zhao, Zhang et al. 2021), and simultaneous detection of 21 common respiratory viruses(Zhang, Xiao et al. 2015).

In this study, we developed a new method named HCoV-MS to simultaneously detect and identify seven HCoVs and further type *SARS-CoV-2* based on the D614G mutation site(Korber, Fischer et al. 2020, Plante, Liu et al. 2020). We take the D614G mutation site as an example and incorporate it into our detection system to prove the ability to detect the mutation site. We also validated the system using plasmids and simulated samples and applied them to identify 151 clinical samples.

**Materials and Methods**

**Target gene selection**

The genome sequences of the seven HCoV strains used in this study were obtained from GenBank (http://www.ncbi.nlm.nih.gov/genbank/) (Table S1). Two genes, *N* and *RdRp*, were chosen as the target genes for the detection and differentiation of *HCoV-229E*, *HCoV-NL63*, *HCoV-OC43*, and *HCoV-HKU1*. Three target genes, *N*, *E*, and *ORF1b*, were chosen for *SARS-CoV*; four genes (*N*, *RdRp*, *E*, and *ORF1b*) were chosen for *MERS-CoV*, and four genes (*N1*, *N2*, *ORF1b*, *S*) were chosen for *SARS-CoV-2*. The SNP site S(D614G) was chosen to detect the D614G mutation of the spike protein. The sequences of the target genes were compared and analyzed using Clustalx2 and DNAMAN. Moreover, the conserved sequences within each target gene species and species-specific fragments were selected as target gene sequences.

**Primer design**

The mPCR primers were designed using Primer Premier 6 software, requiring 19-21 bp primer length, 55-65℃ Tm value, and 40%–60% GC content. The universal sequence ACGTTGGATG was added to all mPCR primers. The gene locus analysis and design system (Rongzhi Biotechnology (Qingdao) Co., Ltd.) was used to design mass probes according to the base quality and the position of the mPCR primers (Table 1). All primers were synthesized by Shenggong Bioengineering (Shanghai) Co., Ltd. (Shanghai, China).

**Table 1.** The Primers of mPCR and MPE

|  |  |  |  |
| --- | --- | --- | --- |
| **Target gene** | **mPCR primer (F)****1** | **mPCR primer (R) 1** | **MPE primer** |
| Human *RNase P* | AGATTTGGACCTGCGAGCG | GAGCGGCTGTCTCCACAAGT | CAGTAGCTGTTTCTGAACT |
| HCoV-229E-*N* | ACGGTGTTAGGCGCAAGAAT | AGGAGCACGGGAGTCAGGTT | CCAACCAGAGATACCACACTTCAA |
| HCoV-229E-*RdRp* | GGACCACGAGCAGTCCATGT | GTTCTGCCCTCATGCCAAGT | CCCCATGTATAACTTACTTAAAGG |
| HCoV-NL63-*N* | GTTGCTGCTGTTACTTTGGCT | CCCTGGGTTGAGAAAGAGGCT | TACCAGTCGAAGTCACCT |
| HCoV-NL63-*RdRp* | CACTTGTTACAACTGCTGGTT | TGTCAACCTAACTGARTGTGT | GAAAGCAATTAGGTTTGGT |
| HCoV-OC43-*N* | AGCAACCAGGCTGATGTCAA | GGCGGAAACCTAGTCGGAAT | ATTCGCTACTTGGGTCCCGAT |
| HCoV-OC43-*RdRp* | CCCAGGATGTGGTGTTGCTAT | CGCAATCCAATGCATGACACA | CGCATGACACATGGTCAG |
| HCoV-HKU1-*N* | ACTCCCGGTCATYATGCTGG | TTCGYTCAGATTGGTCARCC | GGAGAAGTTTTCTTGAGGATT |
| HCoV-HKU1-*RdRp* | ACACACCGYTATCGTTTGTCT | CAAGCAGAGCACTAGCAGATG | GGGTGCATAGCAGGATCTGCTGCATA |
| MERS-CoV-*N* | TTGGCGGAGACAGGACAGAA | GGAATGGGAGTGCTGCTTCG | CCAAAATTAATACCGGGAATGGA |
| MERS-CoV-*E* | ACACTCTTGGTGTGTATGGCT | GCGGGCTGAACTAACAGGGTA | CCGGCTACTAGATTATGTGTGCAAT |
| MERS-CoV-*RdRp* | GGAGAACGTGTACGCCAAGC | AGCACACCGACTAAACCAGC | AGCCAAGCTATCTTAAACA |
| MERS-CoV-*ORF1b* | GCTGCTCTTCTTGCCGGTTC | TGGTCAAGGGCTGTGCATCA | CCCCACAGGGTCATCAACAAT |
| SARS-CoV-*N* | TGATGAAGCTCAGCCTTTGCC | AATCATCCATGTCAGCCGCAG | GGGCAGAGACAAAAGAAGCAGCCC |
| SARS-CoV-*E* | GCCATCCTTACTGCGCTTCG | ACGCGAGTAGACGTAAACCG | TCTTGTTAACGTGAGTTTA |
| SARS-CoV-*ORF1b* | AGAAACGCCCGTAATGGTGT | CTAGCTTGTGCTGGTCCCTT | GGGTTTTAATAACAGAAGGTTCAGT |
| SARS-CoV2-*ORF1b* | TGCTGTAGATGCTGCTAAAGC | GCCTGACCAGTACCAGTGTG | CCCATCTTAACACAATTAGTGATTGG |
| SARS-CoV2-*N1* | AGAATGGAGAACGCAGTGGG | CGGTGAACCAAGACGCAGTA | CGACGTTGTTTTGATCG |
| SARS-CoV2-*N2* | CAACTCCAGGCAGCAGTAGG | TGTCAAGCAGCAGCAAAGCA | TCTGGCTGGCAATGGCGGTGAT |
| SARS-CoV2-*S* | ACAGGCACAGGTGTTCTTACT | TGGATCACGGACAGCATCAGT | CTCAATTTGGCAGAGACATT |
| SARS-CoV2-*S*（D614G） | ACTTCTAACCAGGTTGCTGTTCTT | CACGCCAAGTAGGAGTAAGTTGAT | CCAGGGACTTCTGTGCAGTTAACA |

1 The specific primers used in this study contained a 5’ 10-base extension (ACGTTGGATG)

**Method establishment and parameter optimization**

Plasmids were used to establish the method. Constructed plasmids of human *RNase P*, *HCoV-229E*, *HCoV-OC43*, *HCoV-NL63*, *HCoV-HKU1*, *SARS-CoV*, *MERS-CoV*, and *SARS-CoV-2*, among which *HCoV-HKU1* showed relatively more conservative sequence mutation sites within the species, so the two plasmids *HCoV-HKU1-1* and *HCoV-HKU1-2* were constructed according to mutation frequency selection and synthesis. Each plasmid contained all target genes. All plasmids were carried on the pUC57 vector (Shenggong Bioengineering Co., Ltd., Shanghai, China).

For detection of RNA viruses, viral RNA was extracted using the QIAamp Viral RNA kit (52904), and then reverse-transcribed to DNA using the InRcute IncRNA First-Strand cDNA Kit (KR202) according to the manufacturer’s instructions. For the mPCR section, the mixes (5 µl) contained 2 μL PCR master mix, 1 μL deionized water, 1 μL PCR mix primer, and 1 μL DNA template. The reaction mixtures were pre-denatured for 15 min at 95 °C, denatured for 15 s at 95 °C, annealed for 30 s at 59 °C, then extended for 30 s at 72 °C for 45 cycles, and extended for 10 min at 60 °C. Shrimp alkaline phosphatase was directly added to the reaction system to dephosphorylate the mPCR mixture as follows: 37 °C for 40 min and then 85 °C for 5 min. After shrimp alkaline phosphatase treatment, the mass probe extension (MPE) reaction mix, which included 1 μL E-ddNTPmix, 1.4 μL MPE buffer, 0.6 μL MPE enzyme, and 1 μL mixed mass probe was added. The reaction conditions were 95 °C for 30 s; 95 °C for 5 s, then 52 °C for 5 s and 80 °C for 5 s for 5 cycles, and then from 95 °C for 5 s to 80 °C for 5 s for 40 large cycles; 72 °C for 3 min. The final product from the last step was desalinated using a resin column. The SNP typing kit of Rongzhi Biotechnology (Qingdao) Co., Ltd. was used in the previous steps. The last step was MS detection using a QuanTOF I mass spectrometer (Rongzhi Biotechnology Co., Ltd., Qingdao, China). One microliter of α-Cyano-4-hydroxycinnamic acid (10 mg/ml in 50% acetonitrile and 0.1% trifluoroacetic acid) was spotted on the target plate before 0.5μL of sample was spotted on each well, 3 wells for each sample. The acquisition mode of the instrument was linear positive ion mode, and the parameters were as follows: Accelerate Voltage: 20 kV; Mass Range: 3000–1100 Da; Laser Frequency: 3000 Hz; Shots/Spectrum: 800; Laser energy: 30 uJ, signal-to-noise ratio>4. The instrument was calibrated using calibrators (4k-10 kDa) with molecular weights of 4550.0 Da, 5478.6 Da, 6332.2 Da, 7717.0 Da, and 9507.2 Da (Fig 1). The theoretical values of MPE mass and product mass are shown in Table S2. The allowable deviation of the actual value is ±1Da.

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**Fig 1. Operation steps of HCoV-MS method.** Samples could be nasopharyngeal swabs, sputum, and alveolar lavage fluid.

**Specificity test of the HCoV-MS method**

Nine high-concentration CoV plasmids (105 copies/μL) were used to verify the specificity of the HCoV-MS method. The number of mPCR cycles was set to 30, 35, 40, or 45 cycles. The respiratory viruses *ADV7*, *InfB*, *H1N1*, and *H3N2* (>105 CFU/μL) were also used to carry out relevant specific verifications to test whether other influenza viruses would cross-react.

**Sensitivity test of the HCoV-MS method by plasmids**

To verify the sensitivity of the system, the HCoV-MS method was used to test the plasmids that had undergone concentration gradient dilution. Plasmids with different concentration gradients were tested when the mPCR was set to 30 cycles, 35 cycles, 40 cycles, or 45 cycles. At the same time, the FastFire qPCR PreMix (Probe) (FP208-02) produced by Tiangen Biochemical Technology (Beijing, China) was used for real-time PCR experiments to verify the quality of plasmid dilution.

**Sensitivity test of HCoV-MS method by simulated samples**

The HCoV-MS method and real-time PCR were used simultaneously to test the simulated samples with a 2-fold dilution of 10 gradients, which was leveled from to 1-10, to compare the detection sensitivity. The real-time PCR was performed using the 2019-nCoV nucleic acid detection kit (Shengxiang Biotechnology, Hunan, China) and according to the manufacturer’s instructions.

**Sensitivity test of the HCoV-MS method by clinical samples**

Twenty-six confirmed *SARS-CoV-2* clinical samples with a total of 1-5 gradients of 4-fold dilutions were used for simultaneous experiments to compare the sensitivity of the detection. The real-time PCR experiment with positive *SARS-CoV-2* clinical samples used the 2019-nCoV nucleic acid detection kit (Shengxiang Biotechnology, Hunan, China).

**Performance evaluation and clinical sample test**

The HCoV-MS method was used to detect CoVs from 12 samples in an external quality assessment exercise (UN-CoV-2020) organized by the Robert Koch Institute in the framework of the UNSGM project RefBio. The 12 samples provided were divided into four groups, and one of the three samples in each group was a known negative sample, and the rest were positive or negative (Table 2). A total of 151 clinical samples (oropharyngeal swabs) were collected in virus transport medium and stored at −80 °C. All of the samples were collected from patients who have travel experience abroad (collected from February to April 2021). The HCoV-MS method and real-time PCR were used to test all of the samples. The real-time PCR was performed using the 2019-nCoV nucleic acid detection kit (Shengxiang Biotechnology, Hunan, China) and according to the manufacturer’s instructions.

**Table 2.** Sample Information and Identification Results of UN-CoV-2020

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Sample**  **set ID** | **Sample ID** | **Sample Type** | **Sample description** | **Result** |
| A | RKI-SW-1.1 | Human throat swab | possibly contaminated  (CoV pos1 or neg2) | CoV pos: *SARS-CoV-2* |
| RKI-SW-1.2 | Human throat swab | possibly contaminated  (CoV pos or neg) | CoV pos: *HCoV-NL63* |
| RKI-SW-1.3 | Human throat swab | Uncontaminated  (neg control) | CoV neg |
| B | RKI-SW-2.1 | Human throat swab | possibly contaminated  (CoV pos or neg) | CoV pos: *SARS-CoV-2* |
| RKI-SW-2.2 | Human throat swab | possibly contaminated  (CoV pos or neg) | CoV neg |
| RKI-SW-2.2 | Human throat swab | Uncontaminated  (neg control) | CoV neg |
| C | RKI-SW-3.1 | Livestock throat swab | possibly contaminated  (CoV pos or neg) | CoV pos: *SARS-CoV-2* |
| RKI-SW-3.2 | Livestock throat swab | possibly contaminated  (CoV pos or neg) | CoV pos: *SARS-CoV* |
| RKI-SW-3.3 | Livestock throat swab | Uncontaminated  (neg control) | CoV neg |
| D | RKI-SW-4.1 | Cell culture supernatant | possibly contaminated  (CoV pos or neg) | CoV pos: *SARS-CoV-2* |
| RKI-SW-4.2 | Cell culture supernatant | possibly contaminated  (CoV pos or neg) | CoV pos: *SARS-CoV* |
| RKI-SW-4.3 | Cell culture supernatant | Uncontaminated  (neg control) | CoV neg |

1. pos: positive.

2. neg: negative.

**Results**

**Performance of the HCoV-MS method**

The results obtained by detecting the amplification efficiency of mixed mPCR primers and mixed mass probes showed that none of the primers and probes participated in an extension reaction when deionized water was used as the template for detection. Except for the low amplification efficiency of NL63-*RdRp*, the target genes of other plasmids could be subjected to MPE and form specific product peaks when the plasmid was used as a template for detection. We increased the concentration of the NL63-*RdRp* mPCR primers in the mixed primer to optimize the system. After optimization, each primer was specifically amplified at 45 cycles.

**Specificity verification of the HCoV-MS method**

Nine high-concentration (105 copies/μL) plasmids containing target genes were used to verify the specificity of the system when the cycles of mPCR were 30, 35, 40, or 45 cycles. The results showed that high-concentration plasmids amplified well in the detection system, and all the target genes of each plasmid could be specifically amplified in 45 cycles (Fig S1).

Respiratory samples containing high concentrations of *ADV7*, *InfB*, *H1N1*, and *H3N2* viruses were used to evaluate the specificity of the HCoV-MS method, and no cross-reactivity was observed. These results showed that the specificity of the HCoV-MS method was excellent.

**Sensitivity of the HCoV-MS method**

Serial dilutions of plasmids were used to evaluate the sensitivity of the HCoV-MS method. The detection limits of part of the plasmid target genes are shown in Fig 2. The limit of detection of each target gene is listed in Table 3. The limit of detection of the HCoV-MS method was 1-5 copies/reaction.

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**Fig 2. The limit of detection of the part of target genes.** (a) 1 copy/reaction. (b) 2.5 copies/reaction. (c) 5 copies/reaction. (d) 10 copies/reaction. The red arrow in the figure refers to extended or unextended primer.

**Table 3** Detection limit of HCoV-MS method

|  |  |  |
| --- | --- | --- |
| **Name** | **Target** | **Detection limit (copies/reaction)** |
| Human RNase P | Human *Rnase P* | 1 |
| HCoV-NL63 | *N* | 1 |
| *RdRp* | 1 |
| HCoV-229E | *N* | 2.5 |
| *RdRp* | 2.5 |
| HCoV-OC43 | *N* | 2.5 |
| *RdRp* | 2.5 |
| HCoV-HKU1 | *N* | 1 |
| *RdRp* | 2.5 |
| MERS-CoV | *N* | 1 |
| *RdRp* | 2.5 |
| *E* | 2.5 |
| *ORF1b* | 2.5 |
| SARS-CoV | *E* | 5 |
| *N* | 5 |
| *ORF1b* | 5 |
| SARS-CoV-2 | *N1* | 2.5 |
| *N2* | 2.5 |
| *S* | 2.5 |
| *S（D614G）* | 2.5 |
| *ORF1b* | 2.5 |

**Comparison of HCoV-MS method and real-time PCR in sensitivity**

A *SARS-CoV-2*-spiked sample with serial dilutions was used to compare the detection sensitivity of HCoV-MS and real-time PCR for *SARS-CoV-2*. The detection limits of the HCoV-MS method and real-time PCR were the same (Table 4). Twenty-six *SARS-CoV-2* clinical samples with serial dilutions were used to evaluate the detection sensitivity of HCoV-MS. The gradient of the detection limit of the 26 *SARS-CoV-2* clinical samples is shown in Table 5. Most results for clinical samples obtained by the HCoV-MS method and real-time PCR were the same or differed by only 1-2 gradients, and only a few samples showed a significantly different detection gradient in the experiment. Evidently, the detection sensitivity of the HCoV-MS method we constructed was almost the same as real-time PCR sensitivity in actual clinical sample detection.

**Table 4** The detection results of *SARS-CoV-2* simulated samples by two detection methods

|  |  |  |
| --- | --- | --- |
| **Gradient** | **Result of QuanSNP** | **Result of RT-PCR (mean±SD)** |
| 1 | neg1 | neg (43.61±0.33) |
| 2 | neg | neg (40.86±0.15) |
| 3 | pos2 | pos (39.45±0.25) |
| 4 | pos | pos (37.50±0.40) |
| 5 | pos | pos (36.31±0.35) |
| 6 | pos | pos (35.06±0.11) |
| 7 | pos | pos (32.79±0.32) |
| 8 | pos | pos (31.06±0.08) |
| 9 | pos | pos (29.37±0.09) |
| 10 | pos | pos (28.91±0.55) |

The concentration of gradient 1-10 is gradually increasing. 1. neg: negative. 2.pos: positive.

**Table 5** The gradient of detection limit of 26 *SARS-CoV-2* clinical samples

|  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| **Sample** | **Gradient** | | | | | | | | | |
| **1** | | **2** | | **3** | | **4** | | **5** | |
| **real-time PCR** | **HCoV-MS** | **real-time PCR** | **HCoV-MS** | **real-time PCR** | **HCoV-MS** | **real-time PCR** | **HCoV-MS** | **real-time PCR** | **HCoV-MS** |
| 1 | No Ct/No Ct | -1 | No Ct/No Ct | - | 40.74/No Ct | +2 | 36.87/No Ct | + | 34.17/No Ct | + |
| 2 | No Ct/No Ct | - | No Ct/No Ct | - | 40.65/No Ct | - | 36.30/No Ct | + | 34.36/No Ct | + |
| 3 | 35.94/No Ct | - | 32.84/No Ct | - | 33.35/No Ct | - | 32.56/No Ct | + | 30.16/No Ct | + |
| 4 | 36.88/34.01 | + | 35.96/32.03 | + | 32.92/29.77 | + | 31.24/27.63 | + | 28.52/25.92 | + |
| 5 | 39.64/33.68 | + | 37.01/32.01 | + | 34.79/30.05 | + | 33.03/27.92 | + | 30.6/25.83 | + |
| 6 | 37.33/35.04 | + | 36.67/32.81 | + | 35.45/31.17 | + | 34.28/29.21 | + | 32.9/27.14 | + |
| 7 | 40.63/34.79 | + | 40.11/32.60 | + | 38.66/30.97 | + | 35.83/28.46 | + | 33.83/26.86 | + |
| 8 | No Ct/No Ct | - | No Ct/39.02 | - | 39.35/36.45 | - | 36.3/34.71 | + | 35.15/32.30 | + |
| 9 | No Ct/No Ct | - | No Ct/No Ct | - | No Ct/No Ct | - | 39.33/36.50 | + | 36.32/34.60 | + |
| 10 | No Ct/No Ct | - | 37.23/37.43 | - | 37.06/34.76 | + | 35.16/32.90 | + | 33.24/30.99 | + |
| 11 | 39.02/36.78 | - | 36.35/33.84 | + | 34.35/31.44 | + | 32.36/30.16 | + | 30.66/28.16 | + |
| 12 | No Ct/No Ct | + | No Ct/No Ct | + | No Ct/38.39 | + | 35.95/35.15 | + | 34.61/32.06 | + |
| 13 | No Ct/No Ct | + | No Ct/No Ct | + | No Ct/No Ct | + | 34.99/No Ct | + | 33.94/38.01 | + |
| 14 | No Ct/No Ct | - | No Ct/No Ct | - | No Ct/38.16 | + | 37.12/35.66 | + | 34.52/33.22 | + |
| 15 | No Ct/No Ct | + | 37.15/36.10 | + | 37.04/33.67 | + | 35.26/31.76 | + | 33.01/30.57 | + |
| 16 | No Ct/No Ct | + | 40.81/No Ct | + | No Ct/38.45 | + | 39.89/36.62 | + | 37.84/35.04 | + |
| 17 | No Ct/No Ct | - | No Ct/39.73 | - | 39.31/37.82 | + | 37.87/36.36 | + | 36.43/34.64 | + |
| 18 | No Ct/No Ct | - | No Ct/40.08 | - | No Ct/39.48 | - | 42.18/38.98 | + | 40.41/36.18 | + |
| 19 | No Ct/No Ct | + | 37.22/36.00 | + | 36.90/34.46 | + | 34.81/33.69 | + | 32.92/31.26 | + |
| 20 | 37.80/32.63 | + | 36.22/30.56 | + | 33.91/28.28 | + | 32.24/26.46 | + | 29.53/23.98 | + |
| 21 | 39.93/34.69 | + | 36.10/32.38 | + | 32.28/30.01 | + | 30.51/27.60 | + | 27.69/25.37 | + |
| 22 | No Ct/No Ct | - | No Ct/38.56 | + | 38.56/39.32 | + | 37.10/34.75 | + | 36.13/33.54 | + |
| 23 | No Ct/No Ct | - | 35.15/No Ct | - | 34.93/No Ct | - | 34.15/38.72 | - | 30.79/35.09 | + |
| 24 | No Ct/No Ct | - | 39.17/36.95 | + | 36.61/34.35 | + | 35.95/31.70 | + | 33.32/29.73 | + |
| 25 | No Ct/No Ct | - | No Ct/No Ct | + | No Ct/38.88 | + | 36.66/37.20 | + | 33.94/35.70 | + |
| 26 | No Ct/No Ct | - | 36.46/No Ct | + | 34.97/38.94 | + | 35.39/35.67 | + | 33.65/34.16 | + |

The first value of real-time PCR results represents real-time PCR-ROX-*N* and the second value represents real-time PCR-FAM-*ORF1ab*. The determination of real-time PCR results was based on the 2019-nCoV nucleic acid detection kit (Shengxiang Biotechnology, Hunan, China.). The concentration of gradient 1-5 is gradually increasing.

1. “-” represents the result of HCoV-MS method is negative.

2. “+” represents the result of HCoV-MS method is positive.

**EQAE-SARS-COV 2020**

We participated in the UN-CoV-2020, in which sample categories included human/animal throat swabs or cell cultures detected using the HCoV-MS method. Seven samples were positive, including four *SARS-CoV-2* samples, two *SARS-CoV* samples, and one *HCoV-NL63* sample. The results were consistent with the announced answers (Table 2).

**Validated by clinical samples**

A total of 151 nasal and throat swabs were analyzed using the HCoV-MS method, and the results were compared with those of real-time PCR (Table 6). There were 92 positive samples and 59 negative samples. Both the HCoV-MS method and real-time PCR could detect one case of *HCoV-229E*. The detection sensitivity of the HCoV-MS method for *SARS-CoV-2* was 100% (92/92), and the detection specificity was 100% (61/61). *HCoV-NL63*, *HCoV-OC43*, *HCoV-HKU1*, *SARS-CoV*, and *MERS-CoV* were not detected in any of the samples.

**Table 6** Comparison of the results of HCoV-MS method and real-time PCR

|  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- |
| Name | | HCoV-MS | | |  |  | real-time PCR | |
|  | pos1 | F-pos2 | neg3 | F-neg4 |  | pos | | neg |
| *HCoV-229E* | 1 | 0 | 0 | 0 |  | 1 | | 0 |
| *SARS-CoV-2* | 91 | 0 | 59 | 0 |  | 91 | | 59 |

1. pos: positive. 2. F-pos: False positive. 3. neg: negative. 4. F-neg: False negative.

**Mutation identification of *SARS-CoV-2***

A total of 41 samples were identified as *SARS-CoV-2* D614G variants in the clinical samples. The detection result of the D614G variant in the HCoV-MS method showed that the MPE extension product at the target gene S (including the D614G mutation site) was base G (Fig 3). All strains were verified by high-throughput sequencing, and the results were the same as those in our study (unpublished data).\



a



b

**Fig 3. MS peak of the MPE probes.** (a) MS peaks of the D614G probes. (b) The zoomed MS peaks of the D614G probes (extended with G).

**Discussion**

The outbreak of *SARS-CoV-2* was first identified at the end of 2019 and is still challenging health systems worldwide. This virus is the seventh known CoV that infects humans (Cheng, Papenburg et al. 2020). *SARS-CoV* and *MERS-CoV* are zoonotic and highly pathogenic HCoVs that have resulted in regional and global outbreaks(Fung and Liu 2019). These HCoVs may make a comeback at any time and may even mutate to produce more dangerous variants(Hu, Zeng et al. 2017) , except *HCoV-229E*, *HCoV-OC43*, *HCoV-NL63*, and *HCoV-HKU1*(Miller, McGrath et al. 2020). The initial symptoms of HCoV infections are similar, but the treatment methods are different. It is very important to accurately identify the type of HCoV infection to prescribe treatment. In addition, the ability to type and identify HCoV mutant strains is helpful for monitoring epidemic strains(Younes, Al-Sadeq et al. 2020).

The HCoV-MS method we have established could simultaneously detect seven HCoVs and quickly identify which type of HCoV is at an early stage of infection. The HCoV-MS method showed strong specificity by increasing the number of detection target. For example, a total of five targets were designed for *SARS-CoV-2*, which greatly reduced the occurrence of false negatives. The viral sequence could still be amplified specifically under conditions of high plasmid concentration and high cycle number (45 cycles). The sensitivity of the method was high, compared to the mCoV-MS detection system constructed by Xiu et al. (Xiu, Zhang et al. 2017). The throughput was high. It only took 30 minutes to detect 384 targets simultaneously every time on the machine, and the results could also be automatically determined by the software.

Real-time PCR is an important method for diagnosing *SARS-CoV-2* infection that have made great contributions to the efforts against the epidemic(Chau, Strope et al. 2020). These methods have strong specificity and high sensitivity and have become the gold standard for *SARS-COV-2* detection(Vogels, Brito et al. 2020). In this study, we used simulated and clinical samples to compare the detection sensitivity of the HCoV-MS method and real-time PCR. Our results showed that the detection limit of the HCoV-MS method was almost the same as that of real-time PCR. Thus, the HCoV-MS method could become a potential alternative tool and a supplement to molecular technology to realize its application in HCoV detection.

HCoV is an RNA virus that is prone to mutations. The average frequency of base changes at each nucleic acid site is 10-4 per year(Pyrc, Dijkman et al. 2006). Currently, *SARS-CoV-2* is still widely spread worldwide and continues to produce variants. These variants may be more contagious and may cause serious harm. The ability to identify mutant strains is beneficial for monitoring local epidemic strains in large-scale screening and contributes to the control of the epidemic. High-throughput sequencing could identify mutation sites, but at a high cost(Zhao, Wang et al. 2019). Commercial real-time PCR kits to detect mutations have been developed, but their sensitivity is low, and no clinical application data have been published (thermofisher.com/mutation panel). MALDI-TOF MS has the potential to develop into a ‘Gold Standard’ for high-throughput SNP genotyping. Recently, Agena Bioscience® has developed a research use only panel for the detection and differentiation of *SARS-CoV-2* variants using the MassARRAY® System (https://china.agenabio.com/products/panel/coronavirus-sars-cov-2-variant-detection-research-panel/). However, the current system also lacks verification of actual samples. The HCoV-MS method we established could detect the mutation site D614G on the S protein of *SARS-CoV-2*. More importantly, the HCoV-MS method has been validated using clinical samples and has shown high accuracy and sensitivity. The 41 mutation samples detected were all correct. The successful detection of the D614G mutation provides an example for the identification and typing of other mutation sites.

In summary, the HCoV-MS method was developed to accurately detect *SARS-CoV-2* and 6 other HCoVs simultaneously, further typing *SARS-CoV-2* as well. The proposed nucleic acid mass spectrometry method has shown its merits of good specificity, high sensitivity, and high throughput. The detection sensitivity of the HCoV-MS method was comparable to that of real-time PCR, which could contribute in the efforts against the epidemic. It should be pointed out that our method is based on the known HCoV sequence, covering it as far as possible, based on what is currently known. It is difficult to detect a new HCoV using only the HCoV-MS method. As SNP sites can amplify any kind of base, they could provide clues for improved HCoV detection, compared to current methods.

**Author Contributions**

Tingting Liu, Lin Kang, Yanwei Li and Zhixiang Zhai performed experiments. Yi Hu, Xiaoping Kang, Tao Jiang, Hao Li, Jing Wang, Shan Gao, Jiaxin Li performed sample processing. Jing Huang, Hexing Song, Xiaoguang Zhou and Yuan Yuan analysed data. Baohua Zhao, Jinglin Wang and Wenwen Xin designed the study. Tingting Liu, Lin Kang and Zishuo Guo wrote the paper. All authors have read and agreed to the published version of the manuscript.

**Institutional Review Board Statement**

This study was carried out in accordance with the recommendations of national ethics regulations and approved by the ethics committee of Academy of Military Medical Sciences with written informed consent from all subjects. All subjects gave written informed consent in accordance with the Declaration of Helsinki. The protocol was approved by the ethics committee of Academy of Military Medical Sciences.

**Data Availability Statement**

The data that support the findings of this study are available within the article and its Supplementary Information files, or are available from the corresponding author upon reasonable request.

**Acknowledgments**

The authors thank Yujun Cui from Beijing Institute of Microbiology and Epidemiology, Academy of Military Medical Sciences (AMMS), for providing the result of sequencing analysis. This work was supported by the project to strengthen the reference laboratories in the UNSGM.

**Conflicts of Interest**

Author Zhixiang Zhai, Hexing Song and Xiaoguang Zhou are employed by Rongzhi Biological Technology (Qingdao) Co., Ltd. The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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