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An adjusted ELISpot-based immunoassay for evaluation of SARS-CoV-2-specific T-cell responses

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Like antibody evaluation, using an effective antigen-specific T-cell immunity assessment method in coronavirus disease 2019 (COVID-19) patients, survivors and vaccines is crucial for understanding the immune persistence, prognosis assessment, and vaccine development for COVID-19. This study evaluated an empirically adjusted enzyme-linked immunospot assay for detecting severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2)-specific T-cell immunity in 175 peripheral blood samples from COVID-19 convalescents and healthy individuals. Results of viral nucleic acid were used as the gold standard of infection confirmation. The SARS-CoV-2M peptide pool had higher sensitivity of 85% and specificity of 71% for the single peptide pool. For combined peptide pools, the parallel evaluation (at least one of the peptide pools is positive) of total peptide pools (S1&S2&M&N) had higher sensitivity (up to 93%), and the serial evaluation (all peptide pools are positive) of total peptide pools had higher specificity (up to 100%). The result of the serial evaluation was better than that of the parallel evaluation as a whole. The detection efficiency of M and N peptide pool serial evaluation appeared the highest, with a sensitivity of 80% and specificity of 93%. This T-cell immunity detection assay introduced in this report can achieve high operability and applicability. Therefore, it can be an effective SARS-CoV-2-specific cellular immune function evaluation method.

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1. Introduction

Coronavirus disease 2019 (COVID-19), caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), spreads globally as a complex, highly variable syndrome [1,2]. As of late January 2022, more than 357.4 million cases and 5.6 million deaths have been reported worldwide. At the same time, 232.5 million people have been cured globally, including more than 125,000 in China [3]. Studies on the strength and duration of adaptive immune responses in COVID-19 patients, convalescents, and vaccinees may help understand how immune protection develops and continues after SARS-CoV-2 natural infection and provide helpful information for vaccine development [4]. A comprehensive evaluation of virus-specific immune function from humoral and cellular immunity should be carried out. Serological detection techniques are quite mature now, such as using neutralization tests to detect virus-specific neutralizing antibodies and thus assess antibody protection of COVID-19 convalescent patients. Studies have shown that the antibodies from SARS-CoV-2 convalescent patients persist over 14 months [5]. However, cellular immunity detecting methods require complex operations and specialized facilities and have not been strictly standardized yet. To measure the cytokines released by T-cell in responses to specific immune responses, the human interferon-γ (IFN-γ) enzyme-linked immunospot (ELISpot) assay is commonly used with the stimulation of pathogen-specific peptides, such as mycobacterium tuberculosis [6]. The detection of T-cell function for cellular immunity has high specificity and accessibility. Some studies have demonstrated that the functional response of patients.
HIGHLIGHTS

Scientific question
This study proposed and evaluated a modified SARS-CoV-2-specific immunoassay with high validity.

Evidence before this study
The number of confirmed COVID-19 cases is increasing globally. Compared with humoral immunity, the evaluation of SARS-CoV-2-specific cellular immunity has not been completely unified and standardized, and has the characteristics of complex operation and high requirements of equipment.

New findings
An assessment of the SARS-CoV-2-specific T-cell responses of COVID-19 convalescents and healthy individuals was conducted through an adjusted ELISpot-based immunoassay in this study. The data showed that the parallel combination test and serial combination test of SARS-CoV-2 peptide pools showed higher sensitivity and specificity, respectively. The combined detection of peptide pools could obtain higher detection efficiency, especially the serial test, whose Youden indices were generally better than that of the parallel test. Combination of M and N peptide pool serial evaluation is optimal.

Significance of the study
The SARS-CoV-2-specific T-cell immunoassay proposed in this study is practical and valid, which is conducive to promote the standardized assessment of immune responses to SARS-CoV-2 variants, vaccines and diagnosis.

SARS-CoV-2-specific T-cell can be maintained 6 months to 1 year after infection [7,8]. However, compared with antibody-related research, studies focusing on the SARS-CoV-2-specific cellular immunity using ELISpot assay are relatively lagging behind the standardization [9–11].

Furthermore, data on long-term SARS-CoV-2-specific T-cell responses are limited. This study aimed to evaluate the results of an improved simple ELISpot assay for detecting SARS-CoV-2-specific T-cell immune responses. Different combinations of SARS-CoV-2 peptide pools were assessed to select the desired evaluation system. In the long run, it may facilitate the standardized assessment of immune responses to SARS-CoV-2 variants, vaccines and also promote the diagnosis.

2. Materials and methods

2.1. Experimental design

271 SARS-CoV-2 overlapping peptides (length 15–18 amino acids) were synthesized, which spanned the entire spike (S), membrane (M), and nucleocapsid (N) proteins. Under the natural conditions of SARS-CoV-2 attack on cells, S protein is cleaved into the S1 (residues, 1–685) domain at the N-terminal and S2 (res. 686–1273) domain at C-terminal under the action of host cell proteases, such as the furin protease [12]. According to the natural cleavage sites of SARS-CoV-2 S protein, the peptides of S protein are divided into the S1 region and S2 region, containing 92 and 93 peptides, respectively. The M and N protein pools have 29 and 57 peptides, respectively. After being prepared by mixing the corresponding peptides in each region, these four peptide pools were used as an antigenic stimulus for the stimulation of peripheral blood mononuclear cells (PBMCs) after resuscitation. The peptides were dissolved in dimethyl sulfoxide (DMSO) at the target concentration of 20 μg/μL for storage and 1 μg of each peptide at use. The concentration of each peptide in the peptide pool used for PBMCs culture and subsequent stimulation for IFN-γ expression was 2 μg/mL. Biological factors such as recombinant interleukin-7 (IL-7) (15–25 ng/mL) and interleukin-2 (IL-2) (175–225 U/mL) were added during the culture period, and human IFN-γ ELISPOT assay (BD Corp, USA) was performed after nine days of culture as described previously [13,14] (Fig. 1).

2.2. Result determination

The antigen-specific T-cell responses can be quantified by subtracting the number of spots counted by the ELISpot Reader System (CTL-Immunospot S5 Versa, USA) in the negative control from the corresponding experimental well. The evaluation criteria were as follows: If the negative control spot-forming cells (SFCs) < 5/10^5 PBMCs, the positive reaction was defined as SFCs > 10/10^5; Otherwise, a positive reaction was described as a result at least twice that of the negative control well. To avoid the influence of subjective factors, researchers conducted experiments and judged results with blinding. Parallel and serial tests were used to consider and evaluate the method to improve the detection efficiency of the four SARS-CoV-2 peptide pools. The parallel test refers to the simultaneous conduction of peptide pools detection within the combination, which is conducive to improving detection sensitivity. If any peptide pool detection result is positive, it can be judged as positive. At the same time, the serial test means that only the positive results of all peptide pools in the specified combination are considered positive, which is an effective method to improve the specificity of detection. Accuracy, sensitivity, specificity, Youden index, positive likelihood ratio, and negative likelihood ratio were calculated to evaluate and judge the detection results. As an index to assess the total ability in identifying genuinely infected and non-infected participants, the Youden index was an essential parameter for evaluating different detection efficiency of peptide pools.

2.3. Data source

The above method was used to evaluate the T-cell immunity of a COVID-19 convalescent cohort recruited from Macheng, Hubei Province, China, approximately 6 months to 1 year after diagnosis of COVID-19. In addition, 28 participants who were not infected with SARS-CoV-2 and had not been vaccinated against COVID-19 were tested as healthy controls. All participants (or legal guardians of minors) signed the written informed consent.

2.4. Analyses

This study selected the SARS-CoV-2 specific nucleic acid test results at the admission of COVID-19 patients as the gold standard for the T-cell method evaluation [15]. Descriptive statistical analyses were conducted to summarize the characteristics of participants. The rates comparison was examined by the chi-square test or Fisher’s exact test. The McNemar test was used for the chi-square test of paired data. All analyses of method evaluation were conducted in SAS (University Edition; SAS Institute) and Prism (Version8.0.2, GraphPad). All tests were two-tailed. We performed peptide sequence alignment in Clustal 2.1.

3. Results

3.1. Characteristics of participants

A total of 101 COVID-19 convalescents and 28 healthy individuals were involved. Our laboratory has quantified the immune function of...
this group of participants in a previous publication [8]. 6 COVID-19 convalescents did not participate in the evaluation due to blood sample limitations. The remaining 95 convalescents ranged in age from 13 to 77, 50 (52.6%) were male and 45 (47.4%) were female, including 8 (8.4%) asymptomatic, 47 (49.5%) mild, 30 (31.6%) moderate and 10 (10.5%) severe or critical clinical types. 149 SARS-CoV-2-specific IFN-γ ELISpot tests were involved in these COVID-19 convalescents (including different time points). Of the 28 healthy controls included, 15 (53.6%) were male, and 13 (46.4%) were female, aged 22–71 years. Thus a total of 177 person-times tests were conducted (Supplementary Table 1).

### 3.2. Evaluation of SARS-CoV-2 IFN-γ ELISpot assay with single peptide pools

Using M peptide pool as stimulus, the proportion of detection results consistent with gold standard (laboratory-confirmed cases) was 83.1% (147/177), the sensitivity 85.2% (95% CI: 78.5–90.5%).

| ELISpot assay/Peptide pool | Nucleic acid test | Sum | Accuracy | Sensitivity (95% CI) | Specificity (95% CI) | Youden index | +LR | -LR | Chi square† | P value† |
|---------------------------|-----------------|-----|----------|----------------------|----------------------|--------------|------|-----|------------|----------|
| S1                        | Positive        | 110 | 7        | 74.0%                | 73.8% (66.0–80.7%)   | 75.0% (55.1–89.3%) | 0.49 | 2.95 | 0.35      | 22.2609  | <0.0001 |
|                           | Negative        | 39  | 21       |                      |                      |              |      |     |            |          |
|                           | Sum             | 149 | 60       |                      |                      |              |      |     |            |          |
| S2                        | Positive        | 99  | 10       | 66.1%                | 66.4% (58.3–74.0%)   | 64.3% (44.1–81.4%) | 0.31 | 1.86 | 0.52      | 26.6667  | <0.0001 |
|                           | Negative        | 50  | 18       |                      |                      |              |      |     |            |          |
|                           | Sum             | 149 | 68       |                      |                      |              |      |     |            |          |
| M                         | Positive        | 127 | 8        | 83.1%                | 85.2% (78.5–90.5%)   | 71.4% (51.3–86.8%) | 0.57 | 2.98 | 0.21      | 6.5333   | 0.0106  |
|                           | Negative        | 22  | 20       |                      |                      |              |      |     |            |          |
|                           | Sum             | 149 | 42       |                      |                      |              |      |     |            |          |
| N                         | Positive        | 126 | 10       | 81.4%                | 84.6% (77.7–90.0%)   | 64.3% (44.1–81.4%) | 0.49 | 2.37 | 0.24      | 5.1212   | 0.0236  |
|                           | Negative        | 23  | 18       |                      |                      |              |      |     |            |          |
|                           | Sum             | 149 | 41       |                      |                      |              |      |     |            |          |

Abbreviations: ELISpot = Enzyme-linked immunospot; S = Spike protein; M = Membrane protein; N = Nucleocapsid protein; +LR = Positive likelihood ratio; -LR = Negative likelihood ratio; CI = Confidence interval.

*S1&S2: Spike protein (S) were divided into S1 (res. 1–685) and S2 (res. 686–1273) pools according to the natural cleavage site.

† McNemar test for the paired data of different peptide pool detecting assay compared to the gold standard.
the specificity 71.4% (95% CI: 51.3%–86.8%), and the accuracy index (Youden index) was 0.57. When S1, S2 and N peptide pools were used as stimulus, the proportion of detection results met the gold standard was 74.0% (131/177), 66.1% (117/177) and 81.4% (144/177), the sensitivity 73.8% (95% CI: 66.0%–80.7%), 66.4% (95% CI: 58.3%–74.0%), 84.6% (95% CI: 77.7%–90.0%), specificity of 75.0% (95% CI: 55.1%–89.3%), 64.3% (95% CI: 44.1%–81.4%), with the Youden indexes was 0.49, 0.31 and 0.49, respectively (Table 1).

### 3.3. Evaluation of SARS-CoV-2 IFN-γ ELISpot assay with combined peptide pools

For the parallel evaluation of four SARS-CoV-2 peptide pools, the positive result was defined as at least one of the peptide pools under test being positive. The results of the combined analysis of the four peptide pools were as follows: accuracy 82.5% (146/177), sensitivity 92.6% (95% CI: 87.2%–96.3%), specificity 28.6% (95% CI: 13.2%–48.7%), and Youden index was 0.21. The combination of S1&M, S1&N, and S2&M performed a better profile in the parallel evaluation of pairwise peptide pool combinations. The accuracy of S1&M and S1&N was 82.5% (146/177) and 81.9% (145/177), sensitivity of 88.6% (95% CI: 82.4%–93.2%) and 87.2% (95% CI: 80.8%–92.1%), specificity of 50.0% (95% CI: 30.7%–69.4%) and 53.6% (95% CI: 33.9%–71.5%), and the Youden index was 0.39 and 0.41, respectively (Table 2).

| ELISpot assay/Peptide pool | Nucleic acid test | Sum | Accuracy | Sensitivity (95% CI) | Specificity (95% CI) | Youden index | +LR | 1-LR | Chi square | P value |
|---------------------------|------------------|-----|----------|----------------------|---------------------|--------------|-----|------|-----------|---------|
| SARS-CoV-2                |                  |     |          |                      |                     |              |     |      |           |         |
| Positive                  | Positive         | 138 | 20       | 158                  | 82.5%               | 92.6% (87.2%–96.3%) | 28.6% (13.2%–48.7%) | 0.21 | 1.30 | 0.26     | 2.6129  | 0.1060 |
|                           | Negative         | 11  | 8        | 19                   |                      |               |     |      |           |         |
|                           | Sum              | 149 | 28       | 177                  |                      |               |     |      |           |         |
| S1&S2                     | Positive         | 120 | 13       | 133                  | 76.3%               | 80.5% (73.3%–86.6%) | 53.6% (33.9%–72.5%) | 0.34 | 1.73 | 0.36     | 6.0952  | 0.0136 |
|                           | Negative         | 29  | 15       | 44                   |                      |               |     |      |           |         |
|                           | Sum              | 149 | 28       | 177                  |                      |               |     |      |           |         |
| S1&M                      | Positive         | 132 | 14       | 146                  | 82.5%               | 88.6% (82.4%–93.2%) | 50.0% (30.7%–69.4%) | 0.39 | 1.77 | 0.23     | 0.2903  | 0.5900 |
|                           | Negative         | 17  | 14       | 31                   |                      |               |     |      |           |         |
|                           | Sum              | 149 | 28       | 177                  |                      |               |     |      |           |         |
| S1&N                      | Positive         | 130 | 13       | 143                  | 81.9%               | 87.2% (80.8%–92.1%) | 53.6% (33.9%–71.5%) | 0.41 | 1.88 | 0.24     | 1.1250  | 0.2888 |
|                           | Negative         | 19  | 15       | 34                   |                      |               |     |      |           |         |
|                           | Sum              | 149 | 28       | 177                  |                      |               |     |      |           |         |
| S2&M                      | Positive         | 132 | 14       | 146                  | 82.5%               | 88.6% (82.4%–93.2%) | 50.0% (30.7%–69.4%) | 0.39 | 1.77 | 0.23     | 0.2903  | 0.5900 |
|                           | Negative         | 17  | 14       | 31                   |                      |               |     |      |           |         |
|                           | Sum              | 149 | 28       | 177                  |                      |               |     |      |           |         |
| M&N                       | Positive         | 134 | 16       | 150                  | 82.5%               | 89.9% (83.9%–94.3%) | 42.9% (24.5%–62.8%) | 0.33 | 1.57 | 0.23     | 0.0323  | 0.8575 |
|                           | Negative         | 15  | 12       | 27                   |                      |               |     |      |           |         |
|                           | Sum              | 149 | 28       | 177                  |                      |               |     |      |           |         |

Abbreviations: ELISpot = Enzyme-linked immunospot; S = Spike protein; M = Membrane protein; N = Nucleocapsid protein; +LR = Positive likelihood ratio; -LR = Negative likelihood ratio; CI = Confidence interval.

*Positive: At least one peptide pool is positive. The others are the same as Table 1.

90.5%), the specificity 71.4% (95% CI: 51.3%–86.8%), and the accuracy index (Youden index) was 0.57. When S1, S2 and N peptide pools were used as stimulus, the proportion of detection results met the gold standard was 74.0% (131/177), 66.1% (117/177) and 81.4% (144/177), the sensitivity 73.8% (95% CI: 66.0%–80.7%), 66.4% (95% CI: 58.3%–74.0%), 84.6% (95% CI: 77.7%–90.0%), specificity of 75.0% (95% CI: 55.1%–89.3%), 64.3% (95% CI: 44.1%–81.4%), with the Youden indexes was 0.49, 0.31 and 0.49, respectively (Table 1).

### 4. Discussion and conclusion

The measurement of T-cell responses based on the IFN-γ releasing assay has been widely used to diagnose and evaluate tuberculosis vaccines candidates [16–18]. This study proposed and evaluated a modified IFN-γ releasing ELISpot assay for SARS-CoV-2-specific T-cell detection. The viral nucleic acid test results were compared as the gold standard in the parallel evaluation

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standard, and representative asymptomatic, mild, moderate, and severe cases were included. In addition, the blind method was carried out during the experiment.

In general, this T-cell immunoassay method has the advantages of simple operation, strong practicability, and ease of acceptance. González et al. evaluated a commercially available SARS-CoV-2 IFN-γ secreting kit with 81.1% and 90.9% sensitivity and specificity in 3- and 12-month COVID-19 convalescents, respectively [19], which is concordant with our study. However, our evaluation system has higher flexibility and could choose the combinations of peptide pools with higher detection efficiency according to actual needs. The maximum sensitivity was 93%, and the maximum specificity was 100%. For example, if it is necessary to improve the ability to detect COVID-19 patients in clinical practice, a high sensitive choice of total SARS-CoV-2 (S1&S2&M&N combined) peptide pools parallel test can be selected. On the other hand, if the ability to exclude the non-COVID-19 patient

| ELISPOT assay/Peptide pool | Nucleic acid test | Sum | Accuracy | Sensitivity (95% CI) | Specificity (95% CI) | Youden index | +LR | -LR | Chi square | P value |
|----------------------------|------------------|-----|----------|---------------------|----------------------|--------------|-----|-----|------------|---------|
| SARS-CoV-2 Parallel 6m     | Positive         | 71  | 91       | 76.0%               | 93.4% (85.3%–97.8%)  | 28.6% (13.2%–48.7%) | 0.22          | 0.7019 | 0.7619   |
|                           | Negative         | 5   | 8        | 67.0%               | 53.9% (42.1%–65.4%)  | 100.0% (87.7%–100.0%) | 0.54          | 0.3418 | 0.4075   |
|                           | Sum              | 76  | 28       | 91.0%               | 80.9% (66.6%–92.8%)  | 28.6% (13.2%–48.7%) | 0.20          |        |          |
| SARS-CoV-2 Parallel 12m    | Positive         | 67  | 63       | 74.3%               | 91.8% (83.0%–96.9%)  | 28.6% (13.2%–48.7%) | 0.20          |        |          |
|                           | Negative         | 6   | 8        | 67.0%               | 53.9% (42.1%–65.4%)  | 100.0% (87.7%–100.0%) | 0.54          | 0.3418 | 0.4075   |
|                           | Sum              | 73  | 28       | 91.0%               | 80.9% (66.6%–92.8%)  | 28.6% (13.2%–48.7%) | 0.20          |        |          |
| SARS-CoV-2 Serial 6m       | Positive         | 41  | 45       | 72.3%               | 61.6% (49.5%–72.8%)  | 100.0% (87.7%–100.0%) | 0.20          |        |          |
|                           | Negative         | 45  | 56       | 72.3%               | 61.6% (49.5%–72.8%)  | 100.0% (87.7%–100.0%) | 0.20          |        |          |
|                           | Sum              | 73  | 28       | 91.0%               | 80.9% (66.6%–92.8%)  | 28.6% (13.2%–48.7%) | 0.20          |        |          |

Abbreviations: ELISPOT = Enzyme-linked immunospot; CI = Confidence interval; 6m = 6 months of recovery; 12m = 12 months of recovery.

*Chi square test between 6m and 12m.
needs improvement, the best choice is to combine the total peptide immunoassay evaluation, which can be analogous to the series circuit in physics, even though there may be pre-existing T-cell immunity in healthy people because of the possibility of previous exposure to common cold coronaviruses (CCCs) [20]. This modified evaluation system does not affect its ability to exclude the non-COVID-19 patient, as the test specificity can be 100%. We performed peptide sequence alignments between SARS-CoV-2 and CCCs (HCoV-229E, HCoV-HKU1, HCoV-OC43, and HCoV-NL63). The identity between SARS-CoV-2 and the CCC structural proteins is only 26.1%–35.3%, confirming that the evaluation system can effectively exclude the non-COVID-19 patient (Table 5).

The study was limited by the number of blood samples and the number of subjects, which could potentially affect the significance of the McNemar test. Significantly, the relatively small number of non-patients may affect the effectiveness of the method assessment. In our study, 149 case-group and 28 non-case-group samples were evaluated. It needs to be acknowledged that the number of healthy controls is insufficient. This gap may result in a relatively small proportion of false-positive and valid negative results in the test, making it difficult to determine the sensitivity and specificity and thus over- or under-estimating validity. Another limitation of this study is that no repeated measurements were made, which may affect the extrapolation of the conclusions. Further improvement will be made in subsequent studies. More experiments are needed to assess the evaluation system of the T-cell immunoassay ELISPOT assay.

This study proposed and evaluated a modified IFN-γ releasing ELISPOT assay for SARS-COV-2-specific T-cell detection, which can serve as an effective SARS-CoV-2-specific cellular immune function evaluation method. The M&N peptide pool serial test was the most accurate combination, with 80% sensitivity and 93% specificity. In addition, maximum sensitivity (93%) and specificity (100%) were achieved using parallel and serial assessments of SARS-CoV-2 total peptide libraries. Considering the conservation of the T-cell epitopes among SARS-CoV-2 and its variants, this detection assay would still maintain equivalent sensitivity and specificity for the SARS-CoV-2 variants, including the Omicron variant, the fifth reported variant of concern by WHO, the main mutation sites of which are concentrated in the RBD region of S1 protein [21]. The T-cell responses-based assay will play an essential role in the requirements of immunity assessment for T-cell-related COVID-19 diagnosis, prognosis or vaccine evaluation, as well as the retester of SARS-CoV-2 omicron [22].

Table 5
Sequence identity matrix between SARS-CoV-2 and CCCs*. % Identity
Matrix-created by Clustal 2.1

| SARS-CoV-2 | HCoV-229E | HCoV-HKU1 | HCoV-OC43 | HCoV-NL63 |
|------------|-----------|-----------|-----------|-----------|
| S protein  | 27.21     | 31.46     | 32.44     | 32.57     |
| M protein  | 29.22     | 35.29     | 39.19     | 27.76     |
| N protein  | 27.27     | 35.03     | 35.77     | 27.59     |

*SARS-CoV-2: severe acute respiratory syndrome coronavirus 2; CCCs: common cold coronaviruses.

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Conflict of interest statement

The authors declare that there are no conflicts of interest.

Author contributions

**Hao Lin:** Data Curation, Formal Analysis, Methodology, Visualization, Writing – Original Draft. **Jie Zhang:** Data Curation, Formal Analysis, Investigation, Validation, Writing – Review & editing. **Shaobo Dong:** Data Curation, Investigation, Resources. **Yaning Liu:** Project Administration, Supervision, Visualization, Writing – Original Draft. **Peipei Liu:** Project Administration, Resources. **George F. Gao:** Conceptualization, Supervision. **William J. Liu:** Conceptualization, Funding Acquisition, Project Administration, Validation, Writing – Review & Editing. **Guizhen Wu:** Conceptualization, Funding Acquisition, Supervision, Resources.

Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bsheal.2022.04.005.

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Ethics statement

The Ethics Committee of the National Institute for Viral Disease Control and Prevention, China CDC (Ethical approval No. IVDC2021-007), approved the study. Written informed consent was obtained from all participants (or legal guardians of minors).
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