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Original Article

Multicenter study evaluating novel multi-specimen pooling assay for the detection of SARS-CoV-2: High sensitivity and high throughput testing

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KEYWORDS
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Abstract *Background/purpose:* Mass screening for severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is important to prevent the spread of coronavirus disease 2019 (COVID-19). Pooling samples can increase the number of tests processed. LabTurbo AIO 48 is an automated platform that allows ribonucleic acid extraction and sample analysis on the same

Abbreviations: COVID-19, 2019 novel coronavirus; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2; LoD, limit of detection.

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coronavirus 2;
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Pooled specimen

instrument. We created a novel pooling assay on this platform for SARS-CoV-2 detection and demonstrated that the pooling strategy increases testing capacity without affecting accuracy and sensitivity.

Methods: Comparative limit of detection (LoD) assessment was performed on the LabTurbo AIO 48 platform and the current standard detection system based on real-time reverse transcription polymerase chain reaction (rRT-PCR) using 55 clinically positive samples. An additional 330 primary clinical samples were assessed.

Results: Six samples pooled into one reaction tube were detected in approximately 2.5 h using the World Health Organization rRT-PCR protocol. LabTurbo AIO 48 also demonstrated a higher throughput than our reference rRT-PCR assay, with an LoD of 1000 copies/mL. The overall percentage agreement between the methods for the 330 samples was 100%.

Conclusion: We created a novel multi-specimen pooling assay using LabTurbo AIO 48 for the robust detection of SARS-CoV-2, allowing high-throughput results; this assay will aid in better control and prevention of COVID-19. The diagnostic assay was cost-effective and time-efficient; thus, the pooling strategy is a practical and effective method for diagnosing large quantities of specimens without compromising precision.

Introduction

There are more than 124 million confirmed cases of coronavirus disease 2019 (COVID-19) caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), with over 2 million deaths globally, as of March 30, 2021.[1] COVID-19 has become a global problem as it causes social disruption due to high morbidity and mortality. Moreover, its rapid spread significantly increases the demand on healthcare systems.[2] Therefore, the identification of SARS-CoV-2 in the initial stage is crucial for COVID-19 diagnosis.[3,4]

Several diagnostic methods for SARS-CoV-2 have been developed; however, they have different infrastructure requirements, which affect the capacity of batch production. In addition, these methods have variable throughput, turnaround times (ranging from a few minutes to several hours), and analytical performances.[5–7] Real-time reverse-transcription PCR (rRT-PCR) for the detection of SARS-CoV-2 is one of the confirmatory diagnostic methods for COVID-19. However, implementation of SARS-CoV-2 testing has been difficult in some areas due to the shortage of reagents.[8] Therefore, it is important to consider how rRT-PCR testing can be efficiently conducted with high accuracy, when the availability of reagents and well-trained staff is limited during this global disaster.[6,7,9]

Pooling samples as a cost- and time-saving approach may help overcome these limitations.[10–12] Studies have suggested two protocols for pooling: (1) extracting RNA from a mixture of the original samples and (2) mixing the extracted RNA from individual samples.[13,14] However, this pooling approach directly affects the analytical sensitivity of the rRT-PCR assay, potentially leading to reduced diagnostic sensitivity.[13]

Here, we evaluated a novel sample-pooling strategy using an rRT-PCR platform without any volume loss. We investigated the extent to which this was possible, while detecting weakly positive samples in the largest pool of samples thus far.

Methods

Specimen collection

Upper respiratory tract specimens from 55 SARS-CoV-2-positive- and 275 SARS-CoV-2-negative individuals from different medical centers in Taiwan were pooled. All positive samples were further confirmed by the Taiwan Centers for Disease Control and Prevention (CDC) central laboratory. Each specimen was either a nasopharyngeal or an oropharyngeal swab. The Institutional Review Board of the clinical and genomic research committee at the Tri-Service General Hospital approved this retrospective study (Approval No.: C202020504-1) and the use of an opt-out consent method. All methods were performed in accordance with the 1964 Declaration of Helsinki and other relevant guidelines and regulations.

Viral nucleic acid extraction

Total viral nucleic acid was automatically extracted from each nasopharyngeal/oropharyngeal swab in a universal viral transport medium (300 μL) to a final eluate volume of 60 μL using the LabTurbo Virus Mini Kit (Cat. No. LVN48-300; LabTurbo, Taipei City, Taiwan) and an automated LabTurbo 48 Compact extraction system.

One-step RT-PCR

SARS-CoV-2 screening and confirmatory assays were performed by targeting the envelope (E) and RNA-dependent RNA polymerase (RdRp) viral genes according to the Taiwan CDC recommendations and World Health Organization (WHO) guidelines. In addition, we also targeted the nucleocapsid (N1) viral gene according to the United States CDC guidelines. A 25-μL reaction mixture, containing 6 μL of RNA and 19 μL of either of the three in-house master mix

solutions, was prepared for each sample. The RNA testing kit (TaiGen Bioscience Corp., Taipei, Taiwan) composed of reverse transcriptase, the primer/probe mixture, and 2 × PCR master mix solutions was utilized according to the manufacturer's instructions.[13] We used three different reagents to detect the target genes (*E*, *RdRp*, and *N1*) in the specimens from Tri-Service General Hospital (TSGH) and Cathay General Hospital (CGH).

Assessment of limit of detection using serial dilutions

We purchased purified RNA controls (Vircell, Granada, Spain) of the above viral genes for absolute quantification. These controls were used to prepare a serial dilution panel with approximately 5—20 replicates. We serially diluted the RNA controls (2000, 1000, and 500 copies/mL) using nuclease-free water to assess the limit of detection (LoD). We mixed 300 μL of each serial dilution of the SARS-CoV-2 RNA controls with different specimens using nuclease-free water. Samples were prepared as 1-, 3-, 5-, 6-, and 7-sample pools, of total volumes 300, 900, 1500, 1800, and 2100 μL, respectively. For example, the 3p-sample pool of 1000 copies/mL was prepared by mixing 300 μL of 1000 copies/mL SARS-CoV-2 RNA control with 600 μL of nuclease-free water. These samples were then designated as 1P, 3P, 5P, 6P, and 7P and used in the RNA extraction and rRT-PCR steps. LoD was defined as a 95% probability of 20 replicates testing positive.

Sample pooling strategy to detect COVID-19

Representative SARS-CoV-2-positive specimens were used in our 6P pooling strategy. All positive samples were divided into high, intermediate, and low viral loads. For the 6p-sample pool, 300 μL of each sample, one positive specimen was mixed with five negative specimens. We used all pooled sample (total of 1800uL) for RNA extraction. We tested the effectiveness of our strategy in detecting these positive samples in the pooled samples (Fig. 1).

Table 1 Limit-of-detection results of pooling strategy with SARS-CoV-2 RNA control in *N1* and *E* gene assay on the LabTurbo AIO 48 platform.

RNA control Sample pool	2000 copies/mL		1000 copies/mL		500 copies/mL	
Strategy (total input volume)	*N1* gene	*E* gene	*N1* gene	*E* gene	*N1* gene	*E* gene
1P (300 μL)	5/5	5/5	20/20	20/20	20/20	19/20
3P (900 μL)	5/5	5/5	20/20	20/20	20/20	18/20
5P (1500 μL)	10/10	10/10	20/20	20/20	17/20	14/20
6P (1800 μL)	10/10	10/10	20/20	19/20	14/20	13/20
7P (2100 μL)	10/10	10/10	19/20	15/20	13/20	9/20

Statistical analysis

Data are expressed as mean ± standard deviation. Treatment groups were compared using the one-way analysis of variance and Scheffe post-hoc test with statistical significance indicated at $p > 0.5$. The software used for statistical analyses was SPSS version 19.0 (SPSS Inc., Chicago, IL, USA) for Windows. The detail results are shown in Fig. 3.

Results

Analytical sensitivity of the 1800 μL/sample SARS-CoV-2 pool

The LoD was determined for solutions with 2000, 1000, and 500 copies/mL SARS-CoV-2 RNA-positive controls by testing 5—20 replicates. The LoD of 20 replicate tests at 1000 copies/mL was 100% and 95% for the *N1* and *E* genes, respectively, on the LabTurbo AIO 48 platform (Table 1). The LOD did not appear to change in different pooled strategies. In our method, we pooled 300 μL of each specimen, and subjected the pooled mixture to the extraction procedure.

Analytical specificity of the 1800 μL/sample SARS-CoV-2 pool

We tested retrospective samples of viruses commonly detected in the upper respiratory tract, including influenza A, influenza B, rhinovirus, respiratory syncytial virus, parainfluenza virus, rhinovirus/enterovirus, and adenovirus, using the pooling strategy to determine the analytical specificity of the assays in detecting SARS-CoV-2 *N1*, *E*, and *RdRp* genes. There was no cross-reactivity with the abovementioned viruses using this strategy.

Clinical performance of the 1800 μL/sample SARS-CoV-2 pool

Of the 330 retrospective specimens, 275 and 55 were identified as negative and positive for SARS-CoV-2, which was confirmed by the WHO guideline, respectively. We mixed one SARS-CoV-2 positive and several negative specimens in our pooling strategy (for example: 3P is a combination of one positive and two negative specimens, 6P is one positive with five negative specimens) in one tube. After automated extraction of the pooled specimen, we used the same primer sets recommended by the US Food and Drug Association and WHO to target the SARS-CoV-2 *N1* and *E* genes. The 6p pooling strategy showed 100% positive agreement in the *N1* and *E* gene-positive specimens ($n = 55$) for low (C_t value 30—35), medium (C_t value 20—29), and high (C_t value < 20) SARS-CoV-2 viral loads (Table 2). Further analysis of the C_t values of the SARS-CoV-2 E gene-positive specimens ($n = 55$) confirmed that the data obtained from the LabTurbo AIO 48 system are highly correlated with those obtained from the WHO (followed by

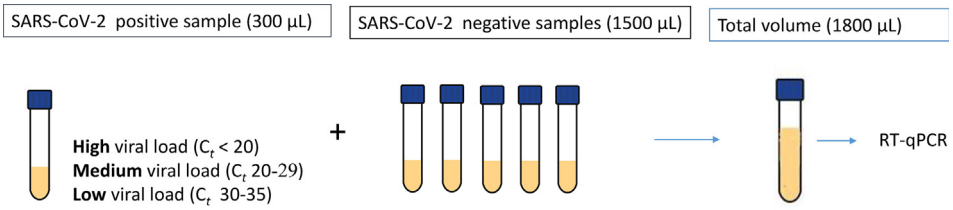

Figure 1. Schematic diagram of sample pooling strategy for detecting SARS-CoV-2 in clinical samples.

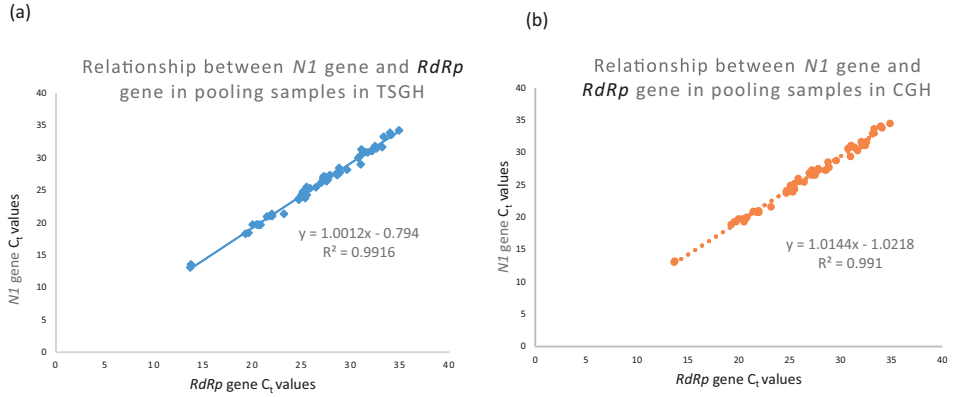

Figure 2. Correlation of Ct values for clinically positive pooled samples from the WHO (followed by Taiwan CDC)-recommended rRT-PCR assay for *RdRp* gene and the LabTurbo AIO 48 targeting *N1* gene for severe acute respiratory coronavirus 2 detection in (a) the Tri-Service General Hospital (TSGH) and (b) Cathay General Hospital (CGH).

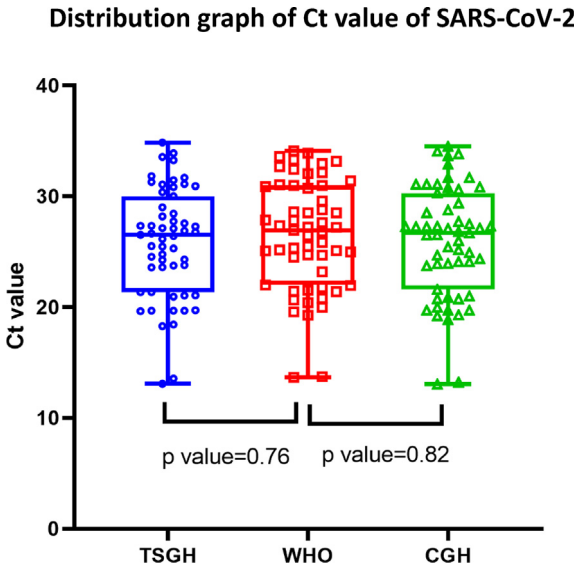

Figure 3. Comparative results of the Ct values detected in the pooled sample containing severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) positive samples from two medical centers. p-values were evaluated using Scheffe's method.

the Taiwan CDC)-recommended rRT-PCR assay (Fig. 2, Table 3). Analysis of variance tests indicated that there were no significant differences between the groups regarding the WHO reference genes for SARS-CoV-2 diagnosis and several SARS-CoV-2 rRT-PCR kits used by the two medical centers ($p > 0.5$) (Figure 3).

Discussion

In this COVID-19 pandemic situation, pooling samples is a good strategy to meet the huge demand for resources, as it reduces reagent use, hands-on time, and expenses required for the SARS-CoV-2 rRT-PCR test. Notably, testing pooled samples is an efficient method for mass screening and increasing testing capacity when resources are finite. Robert et al. successfully identified syphilis from pooled samples in 1943. This pooled strategy was also implemented in blood-donor screening for transfusion-transmitted viral infections, such as human immunodeficiency virus 1 or hepatitis C virus infection.[15] However, this strategy might negatively affect sensitivity because pooling of samples dilutes the virus.

Pooling strategies have been previously used for SARS-CoV-2 screening in many countries. There are three types of pooling methods and each has advantages and disadvantages. The first is swab pooling, wherein all individuals' swabs are put together in one medium. This method can avoid a decrease in sensitivity, and the amount of human RNA can be increased. However, when a positive result is obtained from the pool, it might be necessary to resample the specimens to determine individuals who are positive.[11,19,20]

The second method is transport medium pooling, which involves pooling of individual transport media after collecting the swabs separately in each tube. The mixed transport media are added to the inspection machine for analysis according to the maximum allowable amount for each run. Equal amounts of medium are taken from each tube and mixed well for RNA extraction; the total volume should not

exceed the maximum allowable limit for the instrument. According to comprehensive results, the C_t values detected from the pooled media may be higher than that of single samples, and false-negative results may occur if the number of media pooled is too large. Additionally, the test should be repeated to identify the individual positive sample.[16,21]

Table 2

(a). C_t value of the high viral load of severe acute respiratory syndrome coronavirus 2-positive specimens (*N1* gene C_t value < 20). The C_t value of severe acute respiratory syndrome coronavirus 2 positive specimens in TSGH and CGH with different viral load.

Sample no.	Tri-Service General Hospital		World Health Organization		Cathay General Hospital		Result Interpretation
	N1 gene	*E* gene	*RdRp* gene	*E* gene	*N1* gene	*E* gene	
1	19.65	20.76	20.64	19.72	19.72	20.45	Positive
2	19.68	20.60	20.80	19.95	19.98	20.60	Positive
3	19.72	20.91	20.41	19.40	19.72	20.45	Positive
4	19.68	20.51	21.52	19.64	19.20	20.68.	Positive
5	18.42	19.02	19.57	18.71	19.28	19.60	Positive
6	13.07	13.64	13.65	13.19	13.04	13.29	Positive
7	18.28	18.68	19.26	18.99	18.85	19.19	Positive
8	19.69	20.28	19.98	20.47	19.72	20.45	Positive
9	13.54	13.49	13.72	13.28	13.19	13.47	Positive
(b) C_t value of the medium viral load of severe acute respiratory syndrome coronavirus 2 specimens by (*N1* gene C_t value: 20—29)							
10	29.00	29.76	30.98	29.43	29.38	30.02	Positive
11	28.17	30.00	29.58	29.05	28.75	29.52	Positive
12	27.75	29.33	30.87	29.07	27.70	28.02	Positive
13	26.40	27.21	28.51	26.50	26.51	26.49	Positive
14	26.52	26.54	26.8	26.20	26.49	26.51	Positive
15	25.30	26.64	25.84	25.46	25.97	26.13	Positive
16	23.57	24.48	24.68	23.87	23.72	23.79	Positive
17	24.28	25.11	24.49	21.69	24.35	24.96	Positive
18	21.35	22.39	23.17	22.44	21.59	21.81	Positive
19	23.59	24.30	21.38	20.94	23.93	24.09	Positive
20	21.34	21.63	21.95	21.03	20.99	21.58	Positive
21	26.64	27.71	27.63	27.10	27.13	27.28	Positive
22	27.27	27.53	27.81	27.45	27.49	27.31	Positive
23	25.50	25.75	25.48	25.63	25.17	25.39	Positive
24	23.78	24.90	25.32	23.53	23.90	24.36	Positive
25	23.74	24.61	24.70	22.62	24.08	24.59	Positive
26	28.43	28.37	28.78	28.02	28.51	28.68	Positive
27	27.34	27.84	28.59	27.01	27.30	27.58	Positive
28	27.12	27.20	27.19	27.10	27.25	27.22	Positive
29	27.33	27.96	27.86	27.10	27.30	27.58	Positive
30	27.57	27.31	28.50	27.55	27.26	27.28	Positive
31	26.89	27.92	27.22	26.63	27.25	27.22	Positive
32	25.46	26.25	26.42	26.21	25.43	25.72	Positive
33	27.08	27.65	27.35	26.14	27.09	27.10	Positive
34	26.15	27.24	26.93	26.56	26.81	27.07	Positive
35	26.76	27.56	25.16	23.74	26.68	26.76	Positive
36	24.77	25.57	25.10	24.97	24.69	25.06	Positive
37	24.54	25.63	25.08	24.72	24.90	24.80	Positive
38	24.24	24.97	24.96	24.01	24.08	24.21	Positive
39	20.94	21.52	21.44	21.15	20.88	21.28	Positive
40	21.05	21.62	21.76	20.98	20.76	21.39	Positive
41	21.03	21.51	21.98	21.64	20.74	21.23	Positive
(c) C_t value of the low viral load of severe acute respiratory syndrome coronavirus 2 specimens by (*N1* gene C_t value: 30—35).							
42	31.11	32.1	32.04	31.37	31.67	31.83	Positive
43	30.39	31.28	30.97	30.49	30.84	30.88	Positive

(*continued on next page*)

Table 2 (*continued*)

(c) C_t value of the low viral load of severe acute respiratory syndrome coronavirus 2 specimens by (*N1* gene C_t value: 30—35).							
44	31.67	32.10	33.16	32.07	32.89	32.35	Positive
45	30.92	31.00	31.38	30.64	30.79	30.62	Positive
46	30.84	31.43	32.68	30.81	30.30	30.89	Positive
47	31.45	31.12	33.56	31.13	31.67	31.83	Positive
48	30.01	30.02	30.73	30.06	30.60	30.73	Positive
49	31.82	31.90	32.44	31.42	31.09	31.10	Positive
50	33.26	34.93	33.31	34.31	33.66	34.92	Positive
51	31.05	31.99	32.13	30.68	31.08	31.21	Positive
52	31.29	32.85	31.04	29.55	31.07	31.25	Positive
53	34.85	34.22	33.87	34.55	34.49	33.73	Positive
54	33.88	34.32	32.95	33.11	34.07	34.25	Positive
55	33.55	32.81	34.08	33.00	33.81	34.09	Positive

The last method is RNA pooling. RNA is extracted from each sample, and the extracted RNA from different samples are mixed and used for rRT-PCR. After obtaining a positive response in the pool, it is easy to identify the sample responsible for the positive signal. Usually, resampling is not required for confirmation. However, this method is time-consuming, particularly, the loading process during extraction. It also exhibits decreased sensitivity when too many specimens are pooled. These three methods demonstrate that multi-sample pools are a good method to increase testing throughput, while using fewer reagents and offering faster results.[20,22] However, the implementation of these methods might be negatively affected by the resulting low sensitivity. The above methods were summarized in Supplementary Table 1.[11,16—22]

Here, we propose a solution for sample pooling by expanding the volume limit. Though this could cause double dilution, we propose an increase in the tolerance of sample volume without directly reducing the number of samples and subsequently mixing them for simultaneous analyses. During the analysis of high-throughput samples, we can observe whether there is a decline in sensitivity, because the pooled specimens may be used as screening tools, and the clinical sensitivities differ by pool size. In Table 1, for the *N1* and *E* genes, the number of samples that can be mixed is 1, 3, 5, and 6, and the obtained sensitivity is 100%. The performance of *N1* (100%) better than *E* (95%) in the 6P strategy. In our study, we selected the 6P strategy as the clinical pooling strategy. When the 6p-sample is screened positive, even if just one gene is positive, each specimen should be re-tested following the WHO protocol using the *RdRp* and *E* genes.

Our protocol will be helpful for quickly screening individuals in groups at a high risk for COVID-19, enabling quarantining of confirmed positive people, even in situations with limited time and resources.

In conclusion, we propose a pooling strategy to detect SARS-CoV-2. The LoD was estimated according to the US Food and Drug Association (*N*1 gene) and WHO (*E* gene) guidelines, respectively. We propose that a six-in-one mixture might be a feasible strategy (Table 1). For clinical efficiency analysis, we spiked one positive SARS-CoV-2 sample with five negative SARS-CoV-2 samples in the pool. We poured the mixture into the LabTurbo AIO system

Table 3 Primer and probe sequences used in this study Primers and probe sequences used for real-time RT-PCR detecting severe acute respiratory syndrome coronavirus 2.

Target gene	Primer name	Sequence (5′→3′)	Reference
RdRP gene	RdRP_SARSr-F2	GTGARATGGTCATGTGTGGCGG	[5]
	RdRP_SARSr-R1	CARATGTTAAASACACTATTAGCATA	
	RdRP_SARSr-P2	FAM-CAGGTGGAACCTCATCAGGAGATGC-BBQ	
E gene	E_Sarbeco_F1	ACAGGTACGTTAATAGTTAATAGCGT	[5]
	E_Sarbeco_R2	ATATTGCAGCAGTACGCACACA	
	E_Sarbeco_P1	FAM-ACACTAGCCATCCTTACTGCGCTTCG-BBQ	
*N*1 gene	2019-nCoV_N1-F	GACCCCAAAATCAGCGAAAT	[22]
	2019-nCoV_N1-R	TCTGGTTACTGCCAGTTGAATCTG	
	2019-nCoV_N1-P	/56FAM/ACCCCGCAT/ZEN/TACGTTTGGTGGACC/3IABkFQ/ FAM/ACCCCGCAT/ZEN/TACGTTTGGTGGACC/3IABkFQ/	
RNase P	RP-F	AGATTTGGACCTGCGAGCG	[22]
	RP-R	GAGCGGCTGTCTCCACAAGT	
	RP-P	/5Cy5/TTCTGACCT/TAO/GAAGGCTCTGCGCG/3IAbRQSp/	

without volume loss for SARS-CoV-2 detection by rRT-PCR. Sample pooling strategy was also tested in SARS-CoV-2 positive specimens with high, medium, and low viral loads. We detected SARS-CoV-2 in six samples pooled in one reaction tube. This strategy increases the efficiency of LabTurbo AIO to 288 samples in 2.5-h turnaround time. With this strategy, the overall percentage agreement between both methods for the 330 samples was 100%.

Declaration of competing interest

None.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jmii.2021.08.003.