Establishing diagnostic algorithms for SARS-CoV-2 nucleic acid testing in clinical practice

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Abstract

Background: Coronavirus disease 2019 (COVID-19), which is caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), has become a global pandemic. Our laboratory initially used a two-step molecular assay, first reported by Corman et al, for SARS-CoV-2 identification (the Taiwan Center for Disease Control [T-CDC] method). As rapid and accurate diagnosis of COVID-19 is required to control the spread of this infectious disease, the current study evaluated three commercially available assays, including the TaqPath COVID-19 Combo kit, the cobas SARS-CoV-2 test, and the Rendu 2019-nCoV Assay kit, to establish diagnostic algorithms for clinical laboratories.

Methods: A total of 790 clinical specimens, including nasopharyngeal swabs, throat swabs, sputum, saliva, stool, endotracheal aspirate, and serum were obtained from patients who were suspected or already confirmed to have COVID-19 at the Taipei Veterans General Hospital from February to May 2020. These specimens were tested for SARS-CoV-2 using the different assays and the performance variance between the assays was analyzed.

Results: Of the assays we evaluated, the T-CDC method and the TaqPath COVID-19 Combo kit require lots of hands-on practical laboratory work, while the cobas SARS-CoV-2 test and the Rendu 2019-nCoV Assay kit are fully automated detection systems. The T-CDC method and the TaqPath COVID-19 Combo kit showed similar detection sensitivity; however, the T-CDC method frequently delivered false-positive signals for envelope (*E*) and/or RNA-dependent RNA polymerase (*RdRP*) gene detection, thus increasing the risk of reporting false-positive results. A manual test-based testing strategy combining the T-CDC method and the TaqPath COVID-19 Combo kit was developed, which demonstrated excellent concordance rates (>99%) with the cobas and Rendu automatic systems. There were a few cases showing discrepant results, which may be due to the varied detection sensitivities as well as targets among the different platforms. Moreover, the concordance rate between the cobas and Rendu assays was 100%.

Conclusion: Based on our evaluation, two SARS-CoV-2 diagnostic algorithms, one focusing on the manual assays and the other on the automatic platforms, were proposed. Our results provide valuable information that allows clinical laboratories to implement optimal diagnostic strategies for SARS-CoV-2 testing based on their clinical needs, such as test volume, turn-around time, and staff/resource limitations.

Keywords: Assay comparison; Coronavirus disease 2019; Nucleic acid amplification test; Severe acute respiratory syndrome coronavirus 2

1. INTRODUCTION

Coronavirus disease 2019 (COVID-19) is caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), a novel enveloped RNA beta-coronavirus that first emerged in Wuhan, China in December 2019.[1–3] This disease rapidly spread worldwide and has become a global pandemic with approximately 16 million confirmed infections and >600 thousand deaths as of July 27, 2020. COVID-19 is now a global public health disaster and many countries are still fighting against a surge in cases. The clinical presentation of patients with COVID-19 includes acute onset fever, myalgia, cough, dyspnea, and radiological evidence of ground-glass lung opacities compatible with atypical pneumonia, although cases of asymptomatic or mildly symptomatic COVID-19 have also been reported.[4,5] Due to the nonspecific presentation of COVID-19, molecular diagnostic tests for the detection of SARS-CoV-2 in clinical specimens are currently required for confirmation of COVID-19 infection. Accurate and rapid diagnosis of SARS-CoV-2 in suspected cases is crucial for efficient control of the outbreak such as patient isolation, contact tracking, and infection control.

Of the methods available for virus detection, nucleic acid amplification tests (NAATs) are considered the gold standard because of their superior sensitivity and specificity.[6] As the

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complete genome of SARS-CoV-2 was rapidly decoded early in the epidemic, the first protocol that targeted the RNA-dependent RNA polymerase (*RdRP*), envelope (*E*), and nucleocapsid (*N*) genes of SARS-CoV-2 using real-time reverse-transcriptase-polymerase chain reaction (rRT-PCR) was published on January 23, 2020.[7,8] For a routine workflow, the authors recommended the *E* gene assay as the first-line screening tool, followed by confirmatory testing with *RdRP* gene assays.

As the pandemic has spread rapidly around the world, the U.S. Food and Drug Administration has approved several commercial assays for use in clinical laboratories by granting them 'emergency use approval'. Most of these commercial tests are rRT-PCR assays, on either manual or automatic platforms.[9] In the current study, we compared the performance of several commercial SARS-CoV-2 molecular tests, including one manual assay—the TaqPath COVID-19 Combo kit (Thermo Fisher Scientific, Inc.) and two automatic and robust platforms—the cobas SARS-CoV-2 test (Roche Diagnostics) and the 2019-nCoV assay kit (Rendu Biotechnology). Based on our evaluation, two molecular diagnostic algorithms for SARS-CoV-2 were proposed, which can be used to provide accurate molecular diagnostic results to help the fight against the COVID-19 pandemic.

2. METHODS

2.1. Clinical specimens

A total of 790 clinical specimens were included in this study. These were obtained from patients who were suspected or confirmed to have COVID-19 at the Taipei Veterans General Hospital from February to May 2020. These specimens include 650 nasopharyngeal swabs, 18 throat swabs, 87 sputum specimens, 16 saliva specimens, nine stool specimens, nine aspiration specimens from endotracheal tubes, and one serum specimen. The study was approved by the Taipei Veterans General Hospital Institutional Review Board (approval no. 2020-06-011B).

2.2. NAATs

The NAATs used in the current study included the Taiwan Center for Disease Control (T-CDC) method, the TaqPath COVID-19 Combo kit (Thermo Fisher Scientific, Inc.), the cobas SARS-CoV-2 test (Roche Diagnostics), and the 2019-nCoV assay kit (Rendu Biotechnology). They are all summarized in Table 1. Among these assays, the T-CDC method, the TaqPath COVID-19 Combo kit, and the cobas SARS- CoV-2 test use rRT-PCR-based methodology, while the Rendu 2019-nCoV assay kit uses transcription-mediated isothermal amplification methodology.

Both the T-CDC method and the TaqPath COVID-19 Combo kit are manual assays. The T-CDC method is a laboratory developed test designed to target the *E* and *RdRP* genes of SARS-CoV-2. The primers and probes used in this assay were as described previously.[7] The TaqPath COVID-19 Combo kit is a multiplex commercial assay that targets the *Orf1ab*, *N*, and *S* genes of SARS-COV-2. Briefly, total nucleic acid was extracted from the specimens using a LabTurbo virus mini kit on a LabTurbo 48 Compact System (LabTurbo, Taiwan). For the T-CDC method, the extracted nucleic acid was mixed with specific primers and probes and the LightCycler Multiplex RNA Virus Master reaction master mix (Roche Life Science), and the PCR reactions were run on a Cobas z480 real-time PCR analyzer (Roche Diagnostics). For the TaqPath COVID-19 Combo kit, the extracted nucleic acids were mixed with the TaqMan 2019-nCoV Assay kit v2 reaction mixture according to manufacturer's instructions and the PCR reactions were run on a QuantStudio 5 real-time PCR system (Thermo Fisher Scientific, Inc.). For result interpretation, any signal crossing the detection threshold before 40 cycles was considered positive.

The cobas SARS-CoV-2 test was performed on a Cobas 6800 system (Roche Diagnostics). After loading the clinical specimens and reagents into the machine as per the manufacturer's protocol, the system automatically performs nucleic acid extraction and rRT-PCR, which targets the *Orf1ab* and *E* genes. The system directly reports a positive or negative result for the amplification of each gene; a positive result is accompanied by a Ct value < 40. If both genes are negative, SARS-CoV-2 RNA is not present in the specimen. If the *Orf1ab* gene is positive, regardless of the *E* gene test result, the test concludes that SARS-CoV-2 RNA is present. If only the *E* gene is positive, the test result is equivocal.

The Rendu 2019-nCoV assay kit integrates nucleic acid extraction and transcription-mediated isothermal nucleic acid amplification in an automatic AutoSAT system. This assay only targets the viral *Orf1ab* gene. Briefly, after automatic nucleic acid extraction, the system utilizes magnetic beads to capture viral RNA, which is then subjected to reverse transcription using primers with a T7 promoter sequence at the 5′ end, followed by RNA polymerization to transcribe multiple copies of the RNA amplicons. TaqMan fluorescent probes targeting the *Orf1ab* gene were used to detect the presence of SARS-CoV-2 in a real-time manner. Samples with a dt value (time when the signal intensity crosses threshold) ≤35 were considered as positive for SARS- CoV-2.

2.3. Statistical analysis

We compared the final results from each method (presence or absence of SARS-CoV-2 RNA). The concordance rates between each pair of methods were calculated. Cohen's kappa coefficient for the qualitative results (positive/negative) between each pair of assays was also calculated. Cohen's kappa values > 0.8 were interpreted as an excellent agreement.

Table 1

Nuclei acid amplification test methods used in the present study

Method	Assay type	Test principle	Target gene(s)	Internal control
T-CDC	Manual	rRT-PCR	*E, RdRP*	RNase P
TaqPath COVID-19 Combo kit	Manual	rRT-PCR	*N, Orf1ab, S*	MS2
Roche	Automatic	rRT-PCR	*Orf1ab, E*	MS2
Rendu	Automatic	iAMP	*Orf1ab*	Synthetic RNA

iAMP = isothermal amplification; MS2 = RNA from bacteriophage MS2; *RdRP*, RNA-dependent RNA polymerase; rRT-PCR = real-time reverse-transcriptase-polymerase chain reaction; T-CDC = Taiwan Center for Disease Control.

3. RESULTS

In January 2020, to help limit the COVID-19 outbreak in in Taiwan, under the auspices of T-CDC, our laboratory set up a SARS-CoV-2 rRT-PCR assay based on the Corman et al method for the clinical diagnosis of patients with suspected infections.[7] Before virus isolates were available, this was the earliest molecular assay protocol for the identification of SARS-CoV-2. In this diagnostic workflow, the initial screening test was conducted using the *E* gene assay for pan-sarbecovirus detection, and the *E* gene positive samples were then further confirmed by an *RdRP* gene assay. Due to the lack of commercial assays at that time, the T-CDC adopted this assay as the standard identification method in Taiwan (T-CDC method). After using this method in clinical practice for a period of time, we noticed a significant number of cases with positive signals for *E* or *RdRP* genes; however, all of these suspicious cases were eventually shown negative for SARS-CoV-2 through additional laboratory and clinical

evidence. Similar findings were also reported by other groups in Taiwan.[10] These events could increase the risk of reporting false-positive results, thus leading to unnecessary contact tracking and quarantines for individuals who are not actually infected with SARS-CoV-2.

At the beginning of March 2020, the first commercial kit for SARS- CoV-2 detection was made available in Taiwan. It was the TaqPath COVID-19 Combo kit (Thermo Fisher Scientific, Inc.), which is a manual rRT-PCR-based multiplex assay targeting the *Orf1ab*, *S*, and *N* genes of SARS-CoV-2. Unlike the T-CDC method, which requires three separate PCR reactions, this assay incorporates primers and probes for all three target genes in one reaction, which reduces the hands-on laboratory time required to complete the test.

Since the virus culture of SARS-CoV-2 was not performed at our hospital, we initially compared the detection sensitivity between the T-CDC method and the TaqPath COVID-19 Combo kit using a positive RNA sample extracted from the nasopharyngeal swab of a confirmed case. The positive RNA was serially diluted from 10^{-1} to 10^{-4}, and then subjected to the T-CDC method and the TaqPath COVID-19 Combo kit. As shown in Fig. 1, both of the assays had a detection sensitivity of up to 10^{-4} among the different target genes. However, in the T-CDC method, weakly positive signals for the *E* and *RdRP* genes with a Ct ranging from 36 to 40 were discovered in the negative control. We further compared these two methods using nine clinical specimens in which three were confirmed positive and six were negative. As shown in Table 2, all of the positive cases had Ct values detected in all target genes among the two assays, but in the T-CDC method, five out of six negative cases were shown to have weakly positive signals on either the *E* or *RdRP* genes, which may be misdiagnosed as suspicious positive cases requiring repetition of the experiments. As the T-CDC method was the gold standard diagnostic method used in Taiwan at the beginning of the epidemic, in order to minimize the risk of reporting false-positive results, we established a molecular diagnostic algorithm for SARS-CoV-2 based on manual assays of the TaqPath COVID-19 Combo kit and the T-CDC method (Fig. 2).

According to our algorithm, for specimens with positive (two genes showing positive) or equivocal (only one gene showing

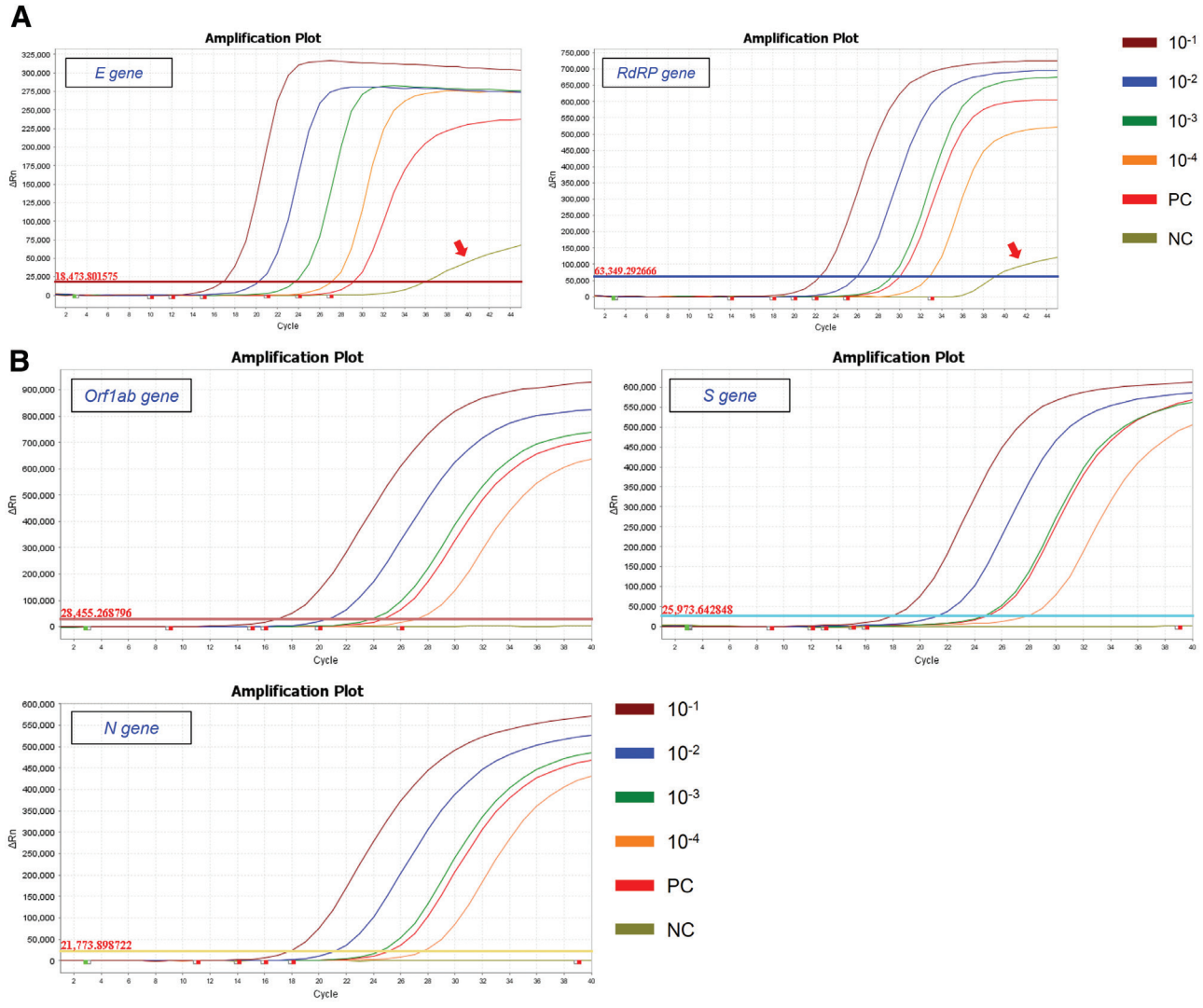

Fig. 1 Detection sensitivity for the T-CDC method and the TaqPath COVID-19 Combo kit. Total nucleic acid was extracted from a COVID-19 confirmed patient and serially diluted from 10^{-1} to 10^{-4}, then evaluated using the (A) T-CDC method and the (B) TaqPath COVID-19 Combo kit, along with positive and negative controls. Both of these assays showed a detection sensitivity up to 10^{-4}, but the T-CDC method showed false-positive signals for the *E* and *RdRP* genes in the negative control, which are indicated by arrows. COVID-19 = coronavirus disease 2019; T-CDC = Taiwan Center for Disease Control.

Table 2

Assay comparison between the T-CDC method and the TaqPath COVID-19 Combo kit

		T-CDC		TaqPath COVID-19 Combo Kit		
No.	SARS-CoV-2	*E*	*RdRP*	*Orf1ab*	*N*	*S*
1	Positive	32.58	39.3	32.32	31.98	32.56
2	Positive	24.39	29.66	26.14	26.77	26.47
3	Positive	27.53	30.98	26.51	26.44	27.79
4	Negative	39.39	ND	ND	ND	ND
5	Negative	ND	ND	ND	ND	ND
6	Negative	ND	39.99	ND	ND	ND
7	Negative	36.92	ND	ND	ND	ND
8	Negative	33.56	ND	ND	ND	ND
9	Negative	38.77	ND	ND	ND	ND

SARS-CoV-2 = severe acute respiratory syndrome coronavirus 2; T-CDC = Taiwan Center for Disease Control.

positive) results as determined by the T-CDC method, the tests should be repeated together with the TaqPath COVID-19 Combo kit. In the repeating assay, if the T-CDC method was equivocal and the TaqPath COVID-19 Combo kit was negative, the specimen would be reported as negative for SARS-CoV-2; if both the T-CDC and the TaqPath COVID-19 Combo kit were positive, the specimen would be reported positive for SARS-CoV-2; if the T-CDC method was positive and the TaqPath COVID-19 Combo kit was negative, or the T-CDC method was equivocal and the TaqPath COVID-19 Combo kit was positive, re-extraction of nucleic acids and further repetition of both tests is required. In addition, to assist frontline healthcare workers with the proper management of patients and to prevent nosocomial infections, we also integrated infection control reporting systems into our diagnostic algorithm and classified the reporting score as D0 (negative), D1 (suspicious), D2 (highly suspicious), and D3 (confirmed).

Although this algorithm achieved our goal of providing accurate SARS-CoV-2 diagnosis, it requires a lot of handling in the laboratory, which may increase the risk of personnel infection and specimen contamination. As the number of COVID-19 cases is growing worldwide, there is an urgent need for automated and robust testing approaches. In the middle of April 2020, two automatic assays, the cobas SARS-CoV-2 test running on the cobas 6800 system and the 2019-nCoV assay kit running on the AutoSAT system were made available in our laboratory. These two assays are capable of completing nucleic acid extraction, amplification, and detection as a fully automated system. The cobas SARS-CoV-2 test detects the *E* and *Orf1ab* genes using rRT-PCR methodology and has a batch capacity providing 384 reports in 8 hours. The Rendu 2019-nCoV assay kit only detects the *Orf1ab* gene using isothermal transcription-based amplification coupled with TaqMan probe detection methods, and has a shorter turn-around time for individual tests: the time-to-first report is 90 minutes, and a further 10 minutes for each subsequent report.

To evaluate the performance of these two automatic platforms, a total of 790 specimens were enrolled in which 28 were

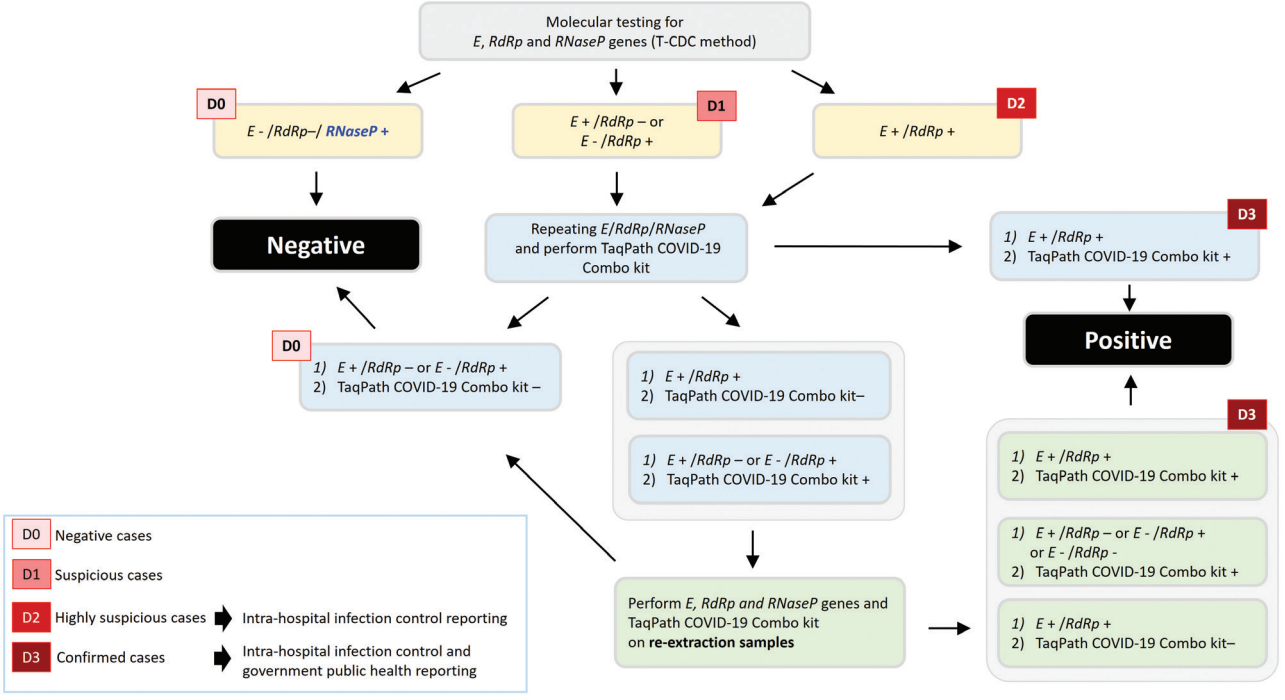

Fig. 2 The molecular diagnostic algorithm for SARS-CoV-2 based on manual rRT-PCR assays. This figure demonstrates the diagnosis workflow for suspected specimens of SARS-CoV-2. After nucleic acid is extracted from the clinical specimens, we first perform the T-CDC method targeting the viral *E* and *RdRP* genes, using the human *RNaseP* gene as an internal control. If both the *E* and *RdRP* genes are undetected, we report the case as SARS-CoV-2 negative. If either viral gene is detected, we repeat the T-CDC method and also perform the TaqPath COVID-19 Combo kit to evaluate viral *N*, *S*, and *Orf1ab* genes. If only one gene is detected by the T-CDC method (equivocal for T-CDC) and the results of the TaqPath COVID-19 Combo kit are negative, we report the case as SARS-CoV-2 negative. If both the T-CDC and TaqPath COVID-19 Combo kit are positive, we report the case as SARS-CoV-2 positive. If the T-CDC and TaqPath COVID-19 Combo kit show discrepant results with the T-CDC negative and the TaqPath COVID-19 Combo kit positive, or T-CDC positive and the TaqPath COVID-19 Combo kit negative, re-extraction of the samples and subsequent performance of the two assays are required. When testing re-extraction samples, if either the TaqPath COVID-19 Combo kit or the T-CDC is positive, we report the case as SARS-CoV-2 positive, otherwise as negative. rRT-PCR = real-time reverse-transcriptase-polymerase chain reaction; SARS-CoV-2 = severe acute respiratory syndrome coronavirus 2; T-CDC = Taiwan Center for Disease Control.

Table 3

Summary of interassay comparison results

Assay		Manual diagnostic algorithm			Concordance	κ
		Positive	Negative	Total		
Cobas	Positive	16	1	17	99.74%	0.94
	Negative	1	750	751		
	Total	17	751	768		
Rendu	Positive	16	1	17	99.23%	0.93
	Negative	1	241	242		
	Total	17	242	259		
Assay		**Cobas**			**Concordance**	**κ**
		Positive	**Negative**	**Total**		
Rendu	Positive	6	0	6	100%	1
	Negative	0	231	231		
	Total	6	231	237		

positive and 762 were negative for SARS-CoV-2, according to the results obtained from our manual diagnostic algorithm as shown in Figure 2. The positive specimens included 19 nasopharyngeal swabs, four throat swabs, two sputum specimens, two saliva specimens, and one stool specimen. The cobas SARS-CoV-2 test was performed on 768 specimens. The concordance rate between the cobas assay and our manual diagnostic algorithm was 99.74% (Table 3). The Rendu 2019-nCoV assay kit was performed on 259 specimens, and the concordance rate between the Rendu assay and our manual diagnostic algorithm was 99.23% (Table 3). One of the positive cases was reported as negative by both the cobas and Rendu assays. This case was a follow-up nasopharyngeal swab from a confirmed COVID-19 patient. The T-CDC method showed that the Ct value of the *E* gene was 37.84 and that of the *RdRP* gene was undetected, while the TaqPath COVID-19 Combo kit showed the Ct values of the *N* and *S* genes were 37 to 40, and that of *Orf1ab* was undetected. According to our diagnostic algorithm, this is considered as a positive case.

Conversely, one of the negative cases was found to be positive by both the cobas and Rendu assays. In this case, the cobas assay showed that the Ct values of both the *Orf1ab* and *E* genes were around 25, while the Rendu assay obtained positive results with an *Orf1ab* dt value of 9.5. After testing the recollected specimen, this patient was confirmed as SARS-CoV-2 positive. This discrepancy may come from the varied detection sensitivities among these methods, ie, in a COVID-19 patient whose viral copy number was falling near the limit of detection, discrepancies between methods may occur, and clinical follow up and re-testing are necessary to clarify the situation. Assays that target different viral genes may also contribute to the controversial results. For a comparison of the cobas and Rendu assays, a total of 237 specimens were tested, all of them showed identical results, with a concordance rate of 100% (Table 3).

4. DISCUSSION

Overall, our data showed that three commercially available SARS-CoV-2 assays have excellent agreement in performance, and that optimal testing strategies can be selected based on clinical and laboratory needs, such as throughput volume, turnaround time, and staff/resource limitations. However, considering their reduced turnaround time and the minimized infection risk to laboratory staff, a closed automatic testing platform, such as the Roche and Rendu systems, should be recommended in clinical laboratories. The diagnostic algorithm for automatic testing of SARS-CoV-2 currently performed in our laboratory is illustrated in Fig. 3. For cases with negative results for the *E* and *Orf1ab* genes, the case is directly reported as SARS-CoV-2 negative, while for cases with *Orf1ab* gene detection regardless of the *E* gene status, the case is directly reported as SARS-CoV-2

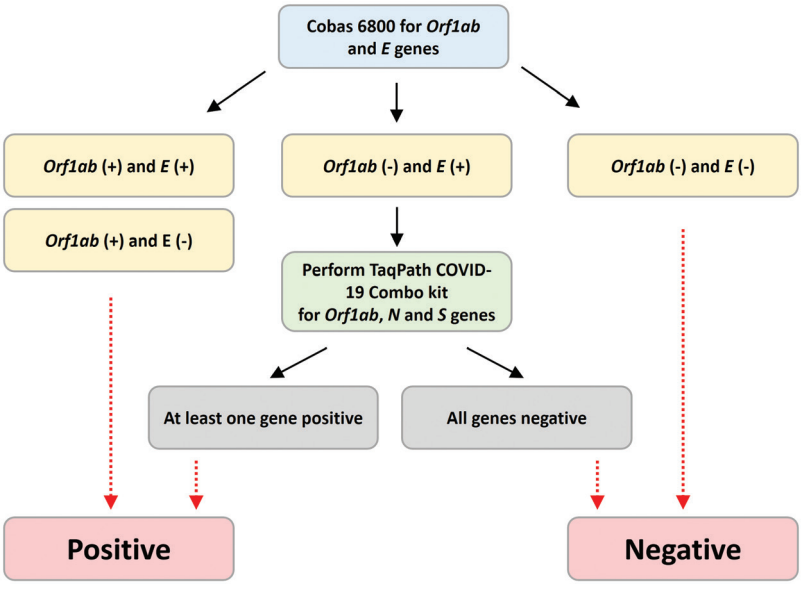

Fig. 3 The molecular diagnostic algorithm for SARS-CoV-2 based on the Automatic cobas 6800 System. In the optimized workflow, all specimens are first processed with the Roche cobas 6800 System, which incorporates automatic nucleic acid extraction and rRT-PCR reactions targeting the viral *Orf1ab* and *E* genes. If both genes are undetected, the specimen is reported as negative for SARS-CoV-2. If the *Orf1ab* gene is detected, the specimen is reported as positive for SARS-CoV-2. If only the *E* gene is detected, the TaqPath COVID-19 Combo kit is performed. An all-gene-negative result indicates the specimen is negative for SARS-CoV-2. If any one gene is positive in the TaqPath COVID-19 Combo kit, the specimen is considered positive for SARS-CoV-2. rRT-PCR = real-time reverse-transcriptase-polymerase chain reaction; SARS-CoV-2 = severe acute respiratory syndrome coronavirus 2.

positive according to manufacturer specifications. For cases with equivocal results, that is, only the *E* gene is detected, a TaqPath COVID-19 Combo kit is used as the confirmation test, since this assay targets the *N* and *S* genes. The Rendu system is also considered comparable in terms of test performance and may be deployed to community hospitals or designated clinics if point-of-care detection of SARS-CoV-2 is required in the future.

The current study had some limitations. First, the small number of positive specimens included. This was due to the low number of COVID-19 cases in Taiwan. Because of this, the specificity of each evaluated method may be more stringently examined, while their sensitivity may not be fully evaluated. The majority of the tested specimens were nasopharyngeal swabs. The other specimen types constituted less than 1/5 of the total number of specimens. The performance of the three methods regarding different specimen types still needs to be elucidated. The sensitivity and specificity of a diagnostic test is usually evaluated by comparing it with the gold standard. The lack of a definite gold standard diagnostic test for this novel SARS-CoV-2 makes any evaluation challenging.

Recently, a few people who were provided a negative COVID-19 test result certificate upon leaving Taiwan were reported as being positive for SARS-CoV-2 when arriving in foreign countries. One of the possible explanations for this discrepancy could be the different platforms or assays used among different countries, which have variable detection sensitivities. Moreover, since the standard materials for SARS-CoV-2 were difficult to obtain at the beginning of the pandemic, it was not possible to evaluate the detection limit of these methods using viral load analysis.

The World Health Organization (WHO) has published a list of protocols for the identification of SARS-CoV-2, and all of the listed assays use rRT-PCR-based methodology to detect more than one target, including the *Orf1ab*, *E*, *N*, and *RdRP* genes.[9] A study comparing all of the primer-probe sets published by the WHO found that the most sensitive primer-probe sets were the E-Sarbeco (Charité), HKU-ORF1 (HKU), HKU-N (HKU), and 2019-nCoV_N1 (US CDC); the primer-probe set with the lowest sensitivity was the RdRP-SARSr confirmatory primer-probe set, suggesting that assay performance, such as sensitivity and specificity, may be influenced by target selection.[11] Although the mutation rate of SARS-CoV-2 is still unclear, the most recent genotyping analysis showed that the genes encoding the S proteins and the RNA polymerase, RNA primase and nucleoprotein, undergo frequent mutations.[12] Therefore, when the sequences for primer-probe sets are placed on these target genes, they should avoid targeting the highly variable regions. In the present study, the Rendu assay showed a 100% concordance rate with the cobas assay when only detecting the *Orf1ab* gene of SARS-CoV-2, suggesting that *Orf1ab* might be a reliable target for single viral gene assays, such as one-step point-of-care test systems.

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