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Accuracy management survey of nucleic acid amplification tests using inactivated SARS-CoV-2 in Hiroshima Prefecture

Toshihito NOMURA[1,2,5)], Tanuza NAZMUL[1)], Akifumi HIGASHIURA[1)], Naoki SHIGEMOTO[3,5)], Kunie ARIYOSHI[3,5)], Hiroki KITAGAWA[2)], Keitaro OMORI[2)], Norihumi SHIGEMOTO[2)], Masao KUWABARA[4,5)], Ko KO[6)], Junko TANAKA[6)], Hidetoshi TAHARA[7)], Hideki OHDAN[8)], Tatsuhiko KUBO[9)], Hiroki OHGE[2)], and Takemasa SAKAGUCHI[1,*)]

1) Department of Virology, Graduate School of Biomedical and Health Sciences, Hiroshima University, 1-2-3 Kasumi, Minami-ku, Hiroshima 734-8551, Japan
2) Department of Infectious Diseases, Hiroshima University Hospital, 1-2-3 Kasumi, Minami-ku, Hiroshima 734-8551, Japan
3) Public Health and Environment Center, Hiroshima Prefectural Technology Research Institute, 1-6-29 Minamimachi, Minami-ku, Hiroshima 734-0007, Japan
4) Hiroshima Center for Disease Control and Prevention, 1-6-29 Minamimachi, Minami-ku, Hiroshima 734-0007, Japan
5) Meeting for the standardization of PCR tests for COVID-19 in Hiroshima Prefecture, 1-6-29 Minamimachi, Minami-ku, Hiroshima 734-0007, Japan
6) Department of Epidemiology and Disease Control, Graduate School of Biomedical and Health Sciences, Hiroshima University, 1-2-3 Kasumi, Minami-ku, Hiroshima 734-8551, Japan
7) Department of Cellular and Molecular Biology, Graduate School of Biomedical and Health Sciences, Hiroshima University, 1-2-3 Kasumi, Minami-ku, Hiroshima 734-8551, Japan
8) Department of Gastroenterological and Transplant Surgery, Graduate School of Biomedical and Health Sciences, Hiroshima University, 1-2-3 Kasumi, Minami-ku, Hiroshima 734-8551, Japan
9) Department of Public Health and Health Policy, Graduate School of Biomedical and Health Sciences, Hiroshima University, 1-2-3 Kasumi, Minami-ku, Hiroshima 734-8551, Japan

ABSTRACT

At the beginning of 2020, the number of laboratories performing SARS-CoV-2 testing increased with the rapid expansion of COVID-19 in Hiroshima Prefecture. Thus, it is necessary to compare and verify the validity of the test results among local laboratories. In this study, we distributed the same standard samples to laboratories that performed COVID-19 testing using the nucleic acid amplification method and confirmed the accuracy of the tests. The SARS-CoV-2 strain distributed by the National Institute of Infectious Diseases (NIID), Japan, was used for testing. As measured by RT-qPCR, a specific amount of the virus was inactivated by ethanol and dried as specimens for distribution. This study included 27 tests performed at 15 laboratories conducting or planning to conduct nucleic acid amplification tests (RT-qPCR and LAMP methods) for SARS-CoV-2. The detection limit of each test method was set at the value provided by the NIID. The accuracy of the tests was examined to determine whether they met the required accuracy criteria. SARS-CoV-2 genomic RNA was reliably detected in all 27 tests. The inactivated specimens used in this study were safe to distribute and could be used as positive controls for all methods.

Key words: *COVID-19*, *Laboratory Test*, *RT-qPCR*, *LAMP*

INTRODUCTION

Novel coronavirus disease (COVID-19) continues to prevail[6)]. The rapid spread of SARS-CoV-2 worldwide has increased the need for testing. A wide range of testing methods are available due to the development of various reagents and instruments[5,10)]. Each laboratory adopted a method considering its budget and the demand for testing. As testing expands, discrepancies in results obtained by different laboratories may become an issue. Owing to the different testing and specimen collection methods, it is often difficult to determine the cause and solve the problem when discrepancies are observed in results.

In this study, at the request of the laboratories in

* Corresponding author: Takemasa Sakaguchi, M.D., Ph.D.
Department of Virology, Graduate School of Biomedical and Health Sciences, Hiroshima University, 1-2-3 Kasumi, Minami-ku, Hiroshima 734-8551, Japan
Tel: +81-82-257-5157, E-mail: tsaka@hiroshima-u.ac.jp

Hiroshima Prefecture, we checked the accuracy of the tests conducted at each laboratory. We distributed the same standard samples to laboratories in Hiroshima Prefecture. The laboratories performed COVID-19 tests using nucleic acid amplification. Each laboratory was responsible for the tests and was asked to provide the results as if they were actual tests. Comparing the results verified that the test was performed with a sensitivity that met the criteria of the National Institute of Infectious Diseases (NIID), Japan.

MATERIALS AND METHODS

Viruses and cells

SARS-CoV-2 (2019-nCoV/Japan/AI/I-004/2020 strain) (Pango lineage: B, GISAID accession ID: EPI_ISL_407084) was provided by the National Institute of Infectious Diseases, Japan. The virus was grown in TMPRSS2-expressing Vero cells (VeroE6/TMPRSS2 cells, JCRB1819; purchased from JCRB Cell Bank) as described previously[3,7]. The culture supernatant was collected and used as a virus solution.

Standard specimen preparation for distribution

The quantity of RNA genome was determined by RNA purification using NucleoSpin RNA Virus (TAKARA BIO, Otsu, Japan), reverse transcription-quantitative polymerase chain reaction (RT-qPCR) using One-Step PrimeScript III RT-qPCR Mix (TAKARA BIO), and Roche LightCycler II (Roche Diagnostics, Basel, Switzerland). A New Coronavirus Positive Control RNA (Nippon Gene Research Institute, Sendai, Japan) was used as the standard.

The viral solution was mixed with 99.5% ethanol at a ratio of 1:9 and aliquoted into 2,600 copies/tube. The samples were dried in a Savant SpeedVac SC11A (Thermo Scientific, Waltham, U.S.A.) and used as standards. Before use, the dried viruses were dissolved in 1 ml of RNase-free water or Dulbecco's phosphate-buffered saline (PBS).

Test methods used by the laboratories

The QIAamp viral RNA mini kit (QIAGEN, Hilden, Germany) and NucleoSpin RNA Virus (TAKARA BIO) were used for RNA extraction. One-Step PrimeScript III RT-qPCR Mix (TAKARA BIO), QuantiTect Probe RT-PCR kit (QIAGEN), TaqMan Fast Virus 1-Step Master Mix (Thermo Fischer Scientific, Waltham, MA, U.S.A.), or LightMix Modular SARS-CoV (COVID19) (Roche Diagnostics) were used for RT-PCR reactions. The viral RNA extraction and RT-qPCR kits conformed to the standard methods indicated by the NIID.

In addition, the 2019 Novel Coronavirus Detection Reagent kit (Shimadzu, Kyoto, Japan) or the SARS-CoV-2 Direct Detection RT-qPCR kit (TAKARA BIO) was used for RNA extraction and RT-qPCR.

In the loop-mediated isothermal amplification (LAMP) method, the QIAamp viral RNA mini kit (QIAGEN) or influenza virus extraction reagent (Eiken Chemical Co., Ltd., Tokyo) was used as the nucleic acid extraction reagent. After RNA extraction, a loopamp novel coronavirus 2019 (SARS-CoV-2) detection kit (Eiken Chemical) was used.

For primers used in one step PrimeScript III RT-qPCR mix (TAKARA BIO), QuantiTect Probe RT-PCR kit (QIAGEN), and TaqMan Fast Virus 1-Step Master Mix (Thermo Fischer Scientific), the following two primer sets for the N gene region were used: 5'-CACATTGGCACCCGCAATC-3', 5'-GAGGAACGAGAAGAGGCTTG-3', and 5'-FAM-ACTTCCTCAAGGAACAACATTGCCA-BHQ-3'. N2 set: 5'-AAATTTTGGGGACCAGGAAC-3', 5'-TGGCAGCTGTGTAGGGTCAAC-3', and 5'-FAM ATGTCGCGCATTGGCATGGA-BHQ-3 LightMix Modular SARS-CoV (COVID-19) (Roche Diagnostics) included primer sets for the orf1a and E regions. Direct kits that combine RT and PCR in a single tube, the 2019 Novel Coronavirus Detection Reagent Kit (Shimadzu), and the SARS-CoV-2 Direct Detection RT-qPCR Kit (TAKARA BIO), each containing primer sets for the N gene region. A positive result was determined if either primer set was positive in the tests using the two primer sets.

RESULTS

Virus inactivation for standard specimens

Inactivation of enveloped viruses, including Zika virus, Ebola virus, and SARS and MERS coronaviruses by ethanol has already been reported[8]. Inactivation of SARS-CoV-2 has also been reported[2,7]. In this study, 99.5% ethanol was mixed with the SARS-CoV-2 solution at a ratio of 9:1. We confirmed that the virus was inactivated by inoculating cultured cells with the treated virus. When observed by electron microscopy, no virus was detected in the ethanol-treated specimens (data not shown).

Investigation of inspection accuracy

We invited laboratories in Hiroshima Prefecture to participate in nucleic acid amplification for SARS-CoV-2. Testing was conducted in 15 laboratories from July 9 to August 17, 2020. A total of 27 tests, 20 using the RT-qPCR and seven using the LAMP method, were conducted at each laboratory. The laboratories in which the tests were conducted are indicated by the numbers in Table 1. The type of method used (RT-qPCR or LAMP) and the specific combinations of test kits are also presented in Table 1.

The detection limit for each test was set as the NIID[4] (Table 2). The detection limit is 50 copies/reaction or less if the test takes more than one hour and 100 copies/reaction for rapid tests within 15 minutes to one hour.

The dried virus was dissolved in water or PBS and used as a standard solution (2600 copies/ml). The number of copies in each reaction tube when the stock solution was used for each test is listed in Table 1. For example, if the QIAamp Viral RNA Mini Kit (Qiagen) and One Step Primescript III RT-qPCR Mix (TAKARA BIO) were used as per the protocol, 3.0×10^4 copies were contained in one tube (reaction system) (Table 1). The amount in each reaction tube varies depending on the

Table 1 Accuracy management study of nucleic acid amplification tests at laboratories in Hiroshima Prefecture.

Laboratory Number	Category	Nucleic acid extraction kit	PCR reagent	Test Device	Quantity of virus to be detected (copies/reaction)	Quantity of virus introduced into a reaction* (copies/reaction)	Dilution to be detected	Standard specimen dilution rate			
								1-fold	10-fold	100-fold	1000-fold
9	RT-qPCR	QIAamp Viral RNA Mini Kit (QIAGEN)	One Step PrimeScript III RT-qPCR Mix (TAKARA BIO)	a	50	3.0×10^4	100-fold diliution	N.T.	**Positive**	**Positive**	**Positive**
12				b				N.T.	**Positive**	**Positive**	**Positive**
6								N.T.	**Positive**	**Positive**	**Positive**
15				a				N.T.	**Positive**	**Positive**	**Positive**
13			QuantiTect Probe RT-PCR (QIAGEN)	c				N.T.	**Positive**	**Positive**	Negative
14			TaqMan Fast Virus 1-Step Master Mix (Thermo Fischer Scientific)					N.T.	**Positive**	**Positive**	**Positive**
11		NucleoSpin RNA Virus (TAKARA BIO)	One Step PrimeScript III RT-qPCR Mix (TAKARA BIO)	a		3.9×10^4		N.T.	**Positive**	**Positive**	**Positive**
2		QIAamp Viral RNA Mini Kit (QIAGEN)	LightMix Modular SARS-CoV (COVID-19) (Roche Diagnostics)	d		6.1×10^4	1000-fold diliution	N.T.	**Positive**	**Positive**	**Positive**
10								N.T.	**Positive**	**Positive**	**Positive**
		NucleoSpin RNA Virus (TAKARA BIO)				7.8×10^4		N.T.	**Positive**	**Positive**	**Positive**
6		The 2019 Novel Coronavirus Detection Reagent Kit (Shimadzu)		b		1.3×10^4	100-fold diliution	N.T.	**Positive**	**Positive**	**Positive**
15				a				N.T.	**Positive**	**Positive**	**Positive**
13				e				N.T.	**Positive**	**Positive**	**Positive**
10								N.T.	**Positive**	**Positive**	**Positive**
5				a				N.T.	**Positive**	**Positive**	Negative
9		SARS-CoV-2 DirectDetection RT-qPCR Kit (TAKARA BIO)				2.1×10^4	100-fold diliution	N.T.	**Positive**	**Positive**	Negative
6				b				N.T.	**Positive**	**Positive**	Negative
15				a				N.T.	**Positive**	**Positive**	**Positive**
13				e				N.T.	**Positive**	**Positive**	**Positive**
11				a				N.T.	**Positive**	**Positive**	Negative
1	LAMP	QIAamp Viral RNA Mini Kit (QIAGEN)	Loopamp Novel Coronavirus 2019 (SARS-CoV-2) Detection Kit (Eiken Chemical)	f		6.1×10^4	100-fold diliution	N.T.	**Positive**	**Positive**	Negative
3								N.T.	**Positive**	**Positive**	Negative
1		Influenza Virus Extraction Reagent (Eiken Chemical)				6.5×10^2	1-fold dilution (no dilution)	**Positive**	**Positive**	Negative	N.T.
5								**Positive**	**Positive**	Negative	N.T.
4								**Positive**	**Positive**	**Positive**	N.T.
7								**Positive**	**Positive**	Negative	N.T.
8								**Positive**	**Positive**	Negative	N.T.

*The number of copies of the viral genome that enter the tube of each test when using the undiluted standard specimen.
The range of dilutions that should be reliably detected is shaded. N.T.: not tested.
a: LightCycler 480 II, 480 or 96 (Roche Diagnostics), b: Thermal Cycler Dice Real Time System II or III (TAKARA BIO), c: QuantStudio 3 or 5 (ThermoFisher Scientific)
d: cobas z480 (Roche Diagnostics), e: CFX96 touch or Deep Well (Bio Rad), f: LoopampEXIA (EIKEN).

Table 2 Detection limit required for the test method (genetic test reagent)

Time for reverse transcription and gene amplification	Detection limit	Test method (genetic test reagent)
1 hour and more	50 viral genome copies/reaction or less	• Methods confirmed by the National Institute of Infectious Diseases* • LightMix Modular SARS-CoV (COVID-19) (Roche Diagnostics) • The 2019 Novel Coronavirus Detection Reagent Kit (Shimadzu)
15 min to 1 hour	100 viral genome copies/reaction or less	• Loopamp Novel Coronavirus 2019 (SARS-CoV-2) Detection Kit (Eiken Chemical) • SARS-CoV-2 Direct Detection RT-qPCR Kit (TAKARA BIO)

*In the methods confirmed by the National Institute of Infectious Disease, QIAamp Viral RNA Mini Kit (QIAGEN) or NucleoSpin RNA Virus (TAKARA BIO) was used for nucleic acid extraction and One Step PrimeScript II RT-qPCR Mix (TAKARA BIO), QuantiTect Probe RT-PCR (QIAGEN), or Taqman Fast Virus 1-Step Master Mix (Thermo Fisher Scientific) for RT-qPCR.

combination of each test because the amount of sample solution used, the amount of purified RNA solution, and the amount provided for RT-qPCR differ depending on the test kit.

For the tests, the standard solutions were diluted to 1/10, 1/100, and 1/1000. The dilutions to be detected in light of the detection limit are shown in Table 1. In the previous example, a 100-fold dilution provides 3.0×10^2 copies in a reaction tube, which is the amount that should be detected. A 1000-fold dilution provides 3.0×10^1 copies that do not need to be detected.

The standard concentration for detection by RT-qPCR and LAMP was 100-fold diluted. An exception was the test using LightMix Modular SARS-CoV (COVID-19) (Roche Diagnostics), for which a 1000-fold dilution was used. In the LAMP method using the influenza virus extraction reagent (Eiken Chemical Co., Ltd.), the concentration of the undiluted solution should be determined because the test virus concentration decreased with dilution (Table 1).

The test results (positive and negative) are listed in Table 1. The column of dilutions to be detected is shaded. All results were positive in the 27 tests conducted in the 15 laboratories that participated in this study. All tests were positive at concentrations in the range that should be reliably detected (Table 1). In addition, there were 17 tests in which viral RNA was detected, even at a dilution of 1/10th of the concentration that should be reliably detected, indicating a sensitivity higher than the minimum required. These results confirmed that the accuracies of the SARS-CoV-2 nucleic acid detection tests conducted in laboratories in Hiroshima Prefecture were all at the required level.

DISCUSSION

Inactivated virus, for which morphology could not be observed by election morphology and culture was negative, including that the virus was not infectious, was used in standard specimens in this study. The amount of virus in each test should be considered when evaluating assays. For example, in the NucleoSpin RNA Virus kit (TAKARA BIO), RNA was extracted from 150 μL of a pharyngeal swab and made into a 50 μl solution. Subsequently, 5 μL of this solution was analyzed using the One Step PrimeScript III RT-qPCR Mix (TAKARA BIO). Because the amount of specimen placed in a reaction tube differs depending on the RNA extraction method, RT-qPCR, or LAMP method, the range to be detected was determined using these methods (Table 1).

There may be an error in measuring the amount of virus by the RT-qPCR method for standard specimens due to which viral RNA near the detection limit was not detected. However, RNA in many specimens was detected up to a 1/10 dilution of the detection limit. It was determined that there was no problem with test accuracy. In actual clinical specimens, the amount of virus detected may not be accurate, owing to the influence of foreign substances. It is possible to test for viruses in a contaminated model by adding viruses to pharyngeal swabs collected from non-COVID-19 individuals. However, in this study, priority was given to distributing identical specimens to multiple laboratories in Hiroshima Prefecture. Therefore, we decided to distribute relatively pure dried material derived solely from viral fluids. In addition, each laboratory was informed that it would be possible to test for the presence of foreign substances by adding them to the remaining standard samples after the prescribed tests.

A portion of SARS-CoV-2 RNA [Amplirun coronavirus RNA control (Vircell, Granada, Spain)] and RNA prepared by incorporating a part of SARS-CoV-2 RNA into an alphavirus AccuPlex SARS-CoV-2 Reference Material Kit (SeraCare Life Sciences, Milford, U.S.A.)] were used as positive controls for the SARS-CoV-2 test. Many other reference RNAs are commercially available, but none are full-length RNAs and may not react with some of the primer sets used in the test. A unique feature of the inactivated specimen used in this study is that it is SARS-CoV-2, which can be used as a positive control in all nucleic acid detection tests used in clinical SARS-CoV-2 tests.

In the future, when we start using a new test method, we can use standard specimens to confirm detection. The SARS-CoV-2 gene is mutated at a rate of 20–24 bases per year, and variants containing more mutations have recently been identified[9]. Some mutant strains have mutations in their primer-binding sites for testing[9]. We have also reported a mutant of ORF8 detected in a specimen collected in Hiroshima Prefecture[1]. In the future,

if a unique genetic mutation occurs in a certain region and may cause a difference in test sensitivity, a new standard specimen can be created by propagating the virus, as was performed to develop the standard specimens for this study. In addition, when a completely new infectious disease emerges, it is possible to respond quickly by applying the accuracy control method established in this study.

The same standard samples were distributed to COVID-19 testing laboratories in Hiroshima Prefecture for accuracy control. SARS-CoV-2 genomic RNA was detected at concentrations within the required detection range in all 27 laboratory tests. The inactivated specimens used in this study were safe for distribution and could be used as positive controls for all methods used. In the future, when SARS-CoV-2 mutates and the test method changes or new infections appear, the rapid distribution of positive controls is expected to contribute to the establishment of a reliable testing system.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest associated with this manuscript.

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AUTHOR CONTRIBUTIONS

ToN, MK, HiroO, and TS designed and conceived the study. KA, JT, HT, and HideO supervised the study. ToN, TaN, AH, and KK cultured the virus and prepared standard specimens. NaS, HK, KO, and NoS provided technical support and collected data, and TK, KK, and JT analyzed and interpreted the data. ToN and TS wrote the manuscript. All the authors have read and approved the manuscript.

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