1 **Evaluation of a novel rapid TRC assay for the detection of influenza using nasopharyngeal swabs**

2 **and gargle samples**

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27

28   **Abstract**

29   We evaluated a novel transcription-reverse transcription concerted reaction (TRC) assay that can detect

30   influenza A and B within 15 min using nasopharyngeal swab and gargle samples obtained from patients

31   with influenza-like illness, between January and March, 2018, and between January and March, 2019.

32   Based on the combined RT-PCR and sequencing results, in the nasal swabs, the sensitivity and specificity

33   of TRC for detecting influenza were calculated as 1.000 and 1.000, respectively. In the gargle samples, the

34   sensitivity and specificity of TRC were 0.946 and 1.000, respectively. The TRC assay showed comparable

35   performance to RT-PCR in the detection of influenza viruses.

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39   All authors meet the ICMJE authorship criteria.

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Introduction

   The transcription-reverse transcription concerted reaction (TRC) method is a combination of direct

and rapid isothermal RNA amplification and real-time identification using an intercalation-activating

fluorescence (INAF) probe [1,2]. In Europe, Japan, and Vietnam, TRC ready-to use regents have been

used for the diagnosis of tuberculosis [3], nontuberculous mycobacterial infections, *Chlamydia* infection,

gonorrhea, and mycoplasma pneumonia. Recently, a novel TRC assay that can detect influenza A and B

within 15 min was developed [4,5]. In this study we evaluated the efficacy of the novel rapid TRC assay

for detecting influenza viruses in nasopharyngeal swab and gargle samples obtained from patients with

influenza-like illness.

Materials and Methods

Ethics

   This study was approved by the ethics committee of Nagasaki University Hospital (approval numbers:

17121822 and 18111919) and was registered at the UMIN Clinical Trials Registry (reference numbers:

UMIN000032395 and UMIN000034545). Written informed consent for participation in and publication of

this study was obtained from all participants before sample collection.

59   *Study design*

60   A prospective observational study was conducted in period 1, between January and March, 2018, and period

61   2, between January and March, 2019. Patients who visited or were hospitalized at the Department of

62   Respiratory Medicine, Japanese Red Cross Nagasaki Genbaku Hospital, with influenza-like illness (ILI)

63   [6] were included in this study. Patients were excluded if they were administered anti-influenza agents

64   within one month prior to the day on which they were sampled. Nasopharyngeal swabs were collected from

65   patients in both periods using two swabs; one was used for antigen testing using silver amplification

66   immunochromatography (FUJI DRI-CHEM IMMUNO AG Cartridge FluAB, Fujifilm, Kanagawa, Japan)

67   [7] at Nagasaki Genbaku Hospital and the other was stored at −20 °C until further analysis. Gargle samples

68   were collected from patients in period 2. For gargle sampling, the patients gargled their throat with 20 mL

69   distilled water for 10 sec. The physicians determined the clinical diagnosis with history taking, physical

70   findings, and results of the influenza antigen test. All information, such as clinical report forms and results

71   of the TRC and RT-PCR, was summarized and analyzed at Nagasaki University Hospital.

72

73   *Sample analysis*

74   We performed a rapid TRC assay based on the protocol described in Japan-patent (JP,2017-195871, A)

75   at Nagasaki University Hospital. In summary, the procedure was performed as follows. A nasopharyngeal

76   swab or gargle swab was mixed in 1 mL extraction buffer containing surfactant and incubated at 52 °C for

77   1 min. Thirty microliters of the sample was mixed with dry reagent containing enzymes, substrates, primers,

78   and INAF probes. The mixture was incubated at 46 °C and the fluorescence was monitored. RT-PCR and

79   sequencing were performed as a gold standard at Tosoh Corporation based on the Influenza Diagnosis

80   Manual [8–10]. Total RNA was isolated from 140 µL of TRC extraction buffer mixed with a

81   nasopharyngeal swab or 140 µL of a gargle specimen using the Qiagen RNeasy kit. RT-PCR was performed

82   using the One Step PrimeScript™ RT-PCR Kit (TAKARA BIO, Shiga, Japan). TRC assay and RT-PCR

83   were performed independently, and the results of them were combined at Nagasaki University Hospital

84   after all analysis was completed. If the results of the TRC and RT-PCR were different, the samples were

85   analyzed by sequencing.

86

87   *Statistical analysis*

88   All statistical analyses were performed with EZR version 1.41 (Saitama Medical Center, Jichi Medical

89   University, Saitama, Japan) [11], which is a graphical user interface for R (the R Foundation for Statistical

90   Computing, Vienna, Austria; version 3.6.3). Fisher's exact test was used to compare categorical variables,

91   and the statistical significance level was set at <0.05. The sensitivity (Se), specificity (Sp), positive

92   predictive value (PPV), and negative predictive value (NPV) of the TRC against the combined results of

93   the RT-PCR and sequencing were calculated with 95% confidence intervals (95% CI).

94

95   **Results**

96   *Patient characteristics*

97   During the study period, a total of 188 patients were evaluated, comprising 92 patients in period 1 and

98   96 patients in period 2. Patient characteristics are shown in Table 1. Of the patients, 95 (50.5%) visited a

99   hospital within 24 h of the onset of symptoms. The percentage of patients with fatigue was significantly

100   lower in period 1 (37.0%) than in period 2 (71.9%, P = <0.001). The influenza antigen test using silver

101   amplification immunochromatography detected influenza A and B in 38 (20.2%) and 39 (20.7%) patients,

102   respectively. The percentage of influenza A was significantly lower, while that of influenza B was

103   significantly higher, in period 1 than in period 2 (Table 1).

104

105   *Comparison of TRC and RT-PCR results*

106   The results of the RT-PCR and TRC are shown in Table 2. In the nasal swabs, influenza A and B were

107   detected using RT-PCR in 36 (19.1%) and 39 (20.7%) patients, respectively, and were detected using TRC

108   in 38 (20.2%) and 40 (21.3%) patients, respectively (Table 2). Of all patients, three tested negative for

109   influenza with RT-PCR, but positive with TRC. Influenza A was detected in two of these three patients and

110   influenza B was detected in one by sequencing. Based on the combined RT-PCR and sequencing results,

111   the Se, Sp, PPV, and NPV of the TRC were 1.000, 0.973, 0.962, and 1.000, respectively (Table 3).

112   In the gargle samples, influenza A was detected by RT-PCR and TRC in 37 (38.5%) and 35 (36.5%)

113   patients, respectively (Table 2). Of all patients in period 2, two tested positive for influenza A with RT-PCR,

114   but negative with TRC. Influenza A was detected in these two patients by sequence analysis. Based on the

115   combined RT-PCR and sequence analysis results, the Se, Sp, PPV, and NPV of the TRC in the gargle

116   samples were 0.946, 1.000, 1.000, and 0.967, respectively (Table 3).

117

118   *Comparison of results between nasopharyngeal swabs and gargle samples*

119   In the RT-PCR testing, five patients tested negative for influenza A in their nasopharyngeal swabs, but

120   tested positive for influenza A in their gargle samples (Fig. 1). The Se, Sp, PPV, and NPV of the RT-PCR

121   in the gargle samples were 1.000, 0.922, 0865, and 1.000, respectively. The Se, Sp, PPV, and NPV of the

122   TRC in the gargle samples were 0.912, 0.935, 0.886, and 0.951, respectively.

123

124   **Discussion**

125   The novel rapid TRC assay showed great sensitivity and specificity in nasopharyngeal swabbing in

126   both periods 1 and 2. Based on the results of the antigen test, period 1 was considered to be the influenza

127   B epidemic season and period 2 was considered the influenza A epidemic season (Table 1). The

128   sensitivity and specificity of the rapid TRC assay was 1.000 and 1.000, respectively, based on the

129   combined RT-PCR and sequencing results for both periods. There are several rapid RT-PCR assays for

130   detection of influenza, such as the ID Now influenza A & B 2 assay (ID Now), Cobas influenza A/B

131   nucleic acid test (Liat), and Xpert Xpress Flu assay (Xpert). The previous studies reported that the

132   sensitivity and specificity of these methods for detecting influenza A/B was 0.932 to 1.000/0.917 to 1000

133   and 0.977 to 1.000/0.976 to 0.998, respectively [12–14]. These results indicate that the performance of

134   the rapid TRC assay is comparable to that of rapid RT-PCR assays.

135   In the present study, the rapid TRC assay also showed great sensitivity and specificity for gargle

136   sampling. The rapid TRC assay and RT-PCR detected influenza in more patients from gargle samples

137   than from nasopharyngeal swabs. The sensitivity and specificity of the Rapid TRC assay were 0.946 and

138   1.000, respectively. The previous studies reported that the sensitivity of Xpert and Liat for gargle

139   sampling was 0.917 and 1.000, respectively, in comparison with in-house RT-PCR [15,16]. Although

140   there are no data on the specificity of rapid RT-PCR assays, the rapid TRC assay seems to be comparable

141   to rapid RT-PCR assays for gargle samples. In the diagnosis of influenza, nasopharyngeal swabbing is the

142   major sampling type, but these samples are difficult to obtain and the procedure is uncomfortable for

143   patients [17]. In addition, due to the novel coronavirus (SARS-CoV-2) pandemic, healthcare workers

144   must wear personal protective equipment when they obtain nasopharyngeal swabs. Therefore, it is vital to

145   develop a diagnostic method, such as the rapid TRC assay, using gargle samples, that is easy to perform,

146   non-invasive, material saving, and safe for healthcare workers [18].

147   There are some limitations in this study. First, the samples were obtained from one community hospital

148   in Nagasaki, which might have limited the generalizability of the findings. Second, we used an equipment

9

149   of the novel TRC assay under development. Accordingly, we are conducting a multicenter study for the

150   rapid TRC assay using production version. Third, the rapid TRC assay was not compared with other rapid

151   RT-PCR assays. Since these assays have not yet been approved by the Japanese Pharmaceuticals and

152   Medical Devices Agency, we will conduct a comparative study in the future.

153   In conclusion, the novel rapid TRC assay showed comparable performance to RT-PCR in the detection

154   of influenza viruses. In addition, because it could detect influenza viruses using gargle samples, the rapid

155   TRC assay could contribute to the diagnosis of influenza during the SARS-CoV-2 pandemic.

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254 **Figure legends**

255 **Fig. 1. Comparison of gargle sample and nasopharyngeal swab results.**

256 The results obtained from gargle samples and nasopharyngeal swabs during period 2 were compared.

257 Period 2 was between January 1 and March 31, 2019.

17

258   **Tables**

259   **Table 1. Patient characteristics.**

Characteristic	Overall (N=188)		Period 1 (N=92)		Period 2 (N=96)		P value
	N	(%)	N	(%)	N	(%)	
Age (average ± S.D)	50.5	± 19.4	51.3	± 19.5	49.6	±19.5	NS
Gender = female	103	(54.8%)	58	(63.0%)	45	(46.9%)	0.029
Underlying diseases	94	(50.0%)	48	(52.2%)	46	(47.9%)	NS
Time since onset of symptoms							
0–12 h	48	(25.5%)	23	(25.0%)	25	(26.0%)	NS
12–24 h	47	(25.0%)	23	(25.0%)	24	(25.0%)	NS
24–48 h	39	(20.7%)	17	(18.5%)	22	(22.9%)	NS
48–72 h	19	(10.1%)	7	(7.6%)	12	(12.5%)	NS
72+ h	26	(13.8%)	14	(15.2%)	12	(12.5%)	NS
Unknown	9	(4.8%)	8	(8.7%)	1	(1.0%)	0.017
Symptoms							
Fever	143	(76.1%)	66	(71.7%)	77	(80.2%)	NS
Cough	124	(66.0%)	60	(65.2%)	64	(66.7%)	NS
Fatigue	103	(54.8%)	34	(37.0%)	69	(71.9%)	<0.001

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Sore throat	100	(53.2%)	46	(50.0%)	54	(56.3%)	NS
Nasal discharge	99	(52.7%)	47	(51.1%)	52	(54.2%)	NS
Headache	70	(37.2%)	35	(38.0%)	35	(36.5%)	NS
Arthralgia	56	(29.8%)	23	(25.0%)	33	(34.4%)	NS
Myalgia	56	(29.8%)	22	(23.9%)	34	(35.4%)	NS
Diarrhea	11	(5.9%)	3	(3.3%)	8	(8.3%)	NS
Nausea	9	(4.8%)	3	(3.3%)	6	(6.3%)	NS

Results of influenza antigen test using silver amplification immunochromatography

Influenza A	38	(20.2%)	3	(3.3%)	35	(36.5%)	<0.001
Influenza B	39	(20.7%)	38	(41.3%)	1	(1.0%)	<0.001
Negative	111	(59.0%)	50	(54.3%)	60	(62.5%)	NS

S.D. = standard deviation; NS = not significant.

260

261 **Table 2. Results of RT-PCR and TRC.**

Item	Overall (N=188)		Period 1 (N=92)		Period 2 (N=96)		P value
	N	(%)	N	(%)	N	(%)	
RT-PCR in nasopharyngeal swab							
Influenza A	36	(19.1%)	4	(4.3%)	32	(33.3%)	<0.001
subtype H1	9	(4.8%)	0	(0.0%)	9	(9.4%)	0.003
subtype H3	27	(14.4%)	4	(4.3%)	23	(24.0%)	0.001
Influenza B	39	(20.7%)	39	(42.4%)	0	(0.0%)	<0.001
Negative	113	(60.1%)	49	(53.3%)	64	(66.7%)	NS
TRC in nasopharyngeal swab							
Influenza A	38	(20.2%)	4	(4.3%)	34	(35.4%)	<0.001
Influenza B	40	(21.3%)	40	(43.5%)	0	(0.0%)	<0.001
Negative	110	(58.5%)	48	(52.2%)	62	(64.6%)	NS
RT-PCR in gargle sample							
Influenza A	N/A		NCW		37	(38.5%)	N/A
subtype H1	N/A		NCW		11	(11.5%)	N/A
subtype H3	N/A		NCW		26	(27.1%)	N/A
Influenza B	N/A		NCW		0	(0.0%)	N/A

			59	(61.5%)	
Negative	N/A	NCW	59	(61.5%)	N/A

TRC in gargle sample

Influenza A	N/A	NCW	35	(36.5%)	N/A
Influenza B	N/A	NCW	0	(0.0%)	N/A
Negative	N/A	NCW	61	(63.5%)	N/A

NS = not significant; N/A = not applicable; NCW = not complied with.

262

263 **Table 3. Performance of TRC assay for detection of influenza.**

	Overall	Period 1	Period 2
Nasopharyngeal swab			
TP	78	44	34
TN	110	48	62
FP	0	0	0
FN	0	0	0
Se (95% CI)	1.000 (0.931-1.000)	1.000 (0.882-1.000)	1.000 (0.851-1.000)
Sp (95% CI)	1.000 (0.951-1.000)	1.000 (0.891-1.000)	1.000 (0.915-1.000)
PPV (95% CI)	1.000 (0.931-1.000)	1.000 (0.882-1.000)	1.000 (0.851-1.000)
NPV (95% CI)	1.000 (0.951-1.000)	1.000 (0.891-1.000)	1.000 (0.915-1.000)

Gargle samples

TP	35	N/A	35
TN	59	N/A	59
FP	0	N/A	0
FN	2	N/A	2
Se (95% CI)	0.946 (0.818-0.993)	N/A	0.946 (0.818-0.993)
Sp (95% CI)	1.000 (0.911-1.000)	N/A	1.000 (0.911-1.000)
PPV (95% CI)	1.000 (0.855-1.000)	N/A	1.000 (0.855-1.000)
NPV (95% CI)	0.967 (0.887-0.996)	N/A	0.967 (0.887-0.996)

Period 1 = between January 1 and March 31, 2018; period 2 = between January 1 and March 31, 2019;

TP = true positive; TN = true negative; FP = false positive; FN = false negative; CI = confidence

interval; N/A = not applicable.

264

RT-PCR					TRC			
	Nasopharyngeal swab					Nasopharyngeal swab		
		Pos	Neg				Pos	Neg
Gargle	Pos	32	5		Gargle	Pos	31	4
	Neg	0	59			Neg	3	58

Se, Sp, PPV, and NPV in gargle samples

Se	1.000	(0.842-1.000)	0.912	(0.763-0.981)
Sp	0.922	(0.827-0.974)	0.935	(0.843-0.982)
PPV	0.865	(0.712-0.955)	0.886	(0.733-0.968)
NPV	1.000	(0.911-1.000)	0.951	(0.863-0.990)

Fig. 1. Comparison of results in gargle samples with nasopharyngeal swabs
 The results obtained from gargle samples and nasopharyngeal swabs during period 2 were compared. Period 2 was between January 1 and March 31, 2019.