Development of a Method to Evaluate Influenza Virus RNA-dependent RNA Polymerase Activity Through Real-time Reverse Transcription Polymerase Chain Reaction

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Methodology

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Abstract

Background: The development of an influenza RNA-dependent RNA polymerase (RdRp) inhibitor is required; therefore, a method for evaluating the activity of influenza RdRp needs to be developed. The current method uses an ultracentrifuge to separate viral particles and quantifies RdRp activity with radioisotope-labeled nucleosides, such as ^{32}P -GTP. This method requires special equipment and radioisotope management, so it cannot be implemented in all institutions. We have developed a method to evaluate the activity of RdRp without using ultracentrifugation and radioisotopes.

Results: RdRp was extracted from viral particles that were purified from the culture supernatant using magnetic beads. A strand-specific real-time reverse transcription polymerase chain reaction (RT-PCR) method was developed based on the reverse transcription, using tagged primers. Real-time PCR using the tag sequence as the forward primer and a segment-specific reverse primer ensured the specificity for quantifying the mRNA of segments 1, 4, and 5. The temperature, reaction time, and Mg^{2+} concentration were determined to select the optimum conditions for *in vitro* RNA synthesis by RdRp, and the amount of synthesized mRNAs of segments 1, 4, and 5 was determined with a detection sensitivity of 10 copies/reaction. In addition, mRNA synthesis was inhibited by ribavirin triphosphate, an RdRp inhibitor, thus indicating the usefulness of this evaluation method for screening RdRp inhibitors.

Conclusion: This method makes it possible to analyze the RdRp activity even in a laboratory where ultracentrifugation and radioisotopes cannot be used. This novel method for measuring influenza virus polymerase activity will further promote research to identify compounds that inhibit viral RNA synthesis.

Background

The influenza virus is an enveloped negative-strand RNA virus, and the viral particles contain eight genomic RNA segments encoding viral proteins [1, 2]. Each RNA segment contains a ribonucleoprotein (RNP) with a heterotrimeric RNA polymerase complex containing the viral acidic polymerase protein (PA), polymerase basic protein 1 (PB1), polymerase basic protein 2 (PB2), and nucleoprotein (NP) [2]. Viral RNPs (vRNPs) are involved in the transcription of viral genes and replication of the viral RNA genome in infected cells.

Although neuraminidase inhibitors, such as oseltamivir and zanamivir, are one of the mainstream treatments for influenza, an epidemic of neuraminidase inhibitor-resistant viruses has been reported [3]. Baloxavir marboxil, which has been in clinical use since 2018 in Japan, the United States, and other countries, potently and selectively inhibits the cap-dependent endonuclease within the polymerase PA subunit of influenza A and B viruses [4]. However, after treatment with baloxavir marboxil, amino acid substitutions at position 38 in the influenza virus PA (PA/I38T) subunit have occasionally been observed in pediatric and adult patients (23% and 10%, respectively). It has been reported that the susceptibility of the PA/I38T mutant virus to baloxavir marboxil has reduced by approximately 50-fold [5].

T-705 (favipiravir) and ribavirin have been reported as effective influenza virus RNA-dependent RNA polymerase (RdRp) inhibitors in *in vitro* and *in vivo* experiments [6, 7]. These compounds are analogs of nucleosides and interrupt RNA synthesis through viral RdRp. Humans do not have RdRp but they do have DNA-dependent RNA polymerase and DNA-dependent DNA polymerase. Animal experiments to assess the

teratogenicity of favipiravir using four separate species, mice, rat, rabbits and monkeys, demonstrate delayed development or embryonic death in the first trimester of pregnancy [8]. For this reason, the clinical use of favipiravir in women of reproductive age comes with a strong warning. Therefore, it is necessary to develop an inhibitor that can specifically inhibit influenza RdRp without affecting RNA and DNA synthesis in infected hosts.

A method for evaluating RdRp activity is important to screen for viral RdRp inhibitors. In a previously reported method, the virus is purified through density gradient ultracentrifugation [9–11]. However, this step is time consuming and the purification process is complicated. In addition, a polymerase reaction is performed *in vitro* using a radioisotope-labeled nucleoside, such as ^{32}P -GTP, and the synthesized RNA is determined with a scintillation counter. Even though this method is capable of determining even a very small amount of RNA, the use of a radioisotope is a safety concern.

In this study, we developed a novel method for the evaluation of RdRp activity for screening of RdRp inhibitors that do not use density gradient ultracentrifugation or radioisotopes.

Materials And Methods

Cell culture

Madin-Darby canine kidney (MDCK) cells were grown in Eagle's minimum essential medium (MEM; FUJIFILM Wako Pure Chemical Industries, Ltd., Osaka, Japan) containing 7 % fetal bovine serum (FBS; Biowest SAS, Nuaille, France) and 1 % antibiotics (penicillin and streptomycin; FUJIFILM Wako).

Viruses

Influenza virus A/Puerto Rico/8/34 (PR8, H_1N_1) was used in this study. For cell infection, the virus was diluted in serum-free MEM supplemented with 0.4 g/L bovine serum albumin (FUJIFILM Wako) and adsorbed to cells at a multiplicity of infection (MOI) of 0.001 for 1 h at 37°C. The inoculum was then removed and replaced with FBS-free Dulbecco's modified Eagle medium (DMEM; FUJIFILM Wako) supplemented with 4 g/L bovine serum albumin and acetyltrypsin (2 $\mu\text{g}/\text{mL}$; Sigma-Aldrich, St. Louis, MO, USA) for 24 h.

Purification of the influenza virus RdRp

Influenza virus RNA polymerase was purified from viral culture supernatants using Viro-Adembeads (Ademtech, Pessac, France) according to the manufacturer's instructions [12]. Briefly, 500 μL of a virus solution (4.8×10^6 FFU/mL) was adsorbed onto magnet beads by mixing at 900 rpm for 20 min at 20°C. The tube was placed on the magnet for 1 min, the supernatant was removed, and the beads were washed with phosphate-buffered saline (PBS). After that, 70 μL of the polymerase elution buffer (50 mM Tris-HCl pH 8.0, 0.1 M KCl, 5 mM MgCl_2 , 2 mM DTT, 1000 U/mL RNase inhibitor, and 0.25 % Triton N-101) was added, and the mixture was incubated at 30°C for 30 min to extract the RdRp.

In vitro transcription with purified RdRp

The *in vitro* transcription was conducted using a standard RNA synthesis reaction buffer [7, 10, 11, 13], which contained 50 mM Tris-HCl (pH 8.0), 0.1 M KCl, 5 mM MgCl_2 , 2 mM DTT, 1000 U/mL RNase inhibitor, 0.25%

Triton N-101, 500 μ M ATP, 500 μ M CTP, 500 μ M UTP, 500 μ M GTP, 200 μ M ApG dinucleotide, and 20 μ L purified RdRp. The reaction was performed at 37°C for 30 min in a final volume of 50 μ L.

Evaluation of RdRp activity through real-time reverse transcription polymerase chain reaction (RT-PCR)

RdRp activity was evaluated using RT-PCR. mRNA specific RT primers (Table 1) for segments 1 (PB2), 4 (HA), and 5 (NP) of the PR8 strain were designed by modifying a previously reported protocol [14]. To prepare cDNA from the viral RNA produced through the aforementioned RdRp reaction, each tagged-specific RT primer and the ReverTra Ace qPCR RT Kit (TOYOBO, Osaka, Japan) were mixed as follows: 5 μ L of a mixture containing 4 μ L of the RdRp reaction product and 1 μ L of the 5 pmol tagged-specific RT primer. The mixture was heated at 65°C for 10 min, immediately cooled on ice for 5 min, and then heated again at 60°C for 5 min. 5X RT buffer (containing reaction buffer, $MgCl_2$, and dNTPs), RT enzyme mix (containing RT enzyme and RNase inhibitor), and nuclease-free water were added to the RdRp product and primer mixture to a total reaction volume of 10 μ L. Real-time quantitative PCR (qPCR) was performed on an ABI PRISM 7500 Fast Real-time PCR system using the Thunderbird SYBR qPCR mix (TOYOBO) and the primer sets for each segment mRNA (Table 1). Each 20 μ L of the PCR mixture contained 2 μ L of a ten-fold dilution of the cDNA and 0.3 μ M of forward and reverse primers. The amplification conditions were 95°C for 1 min, 40 cycles at 95°C for 15 s, 60°C for 30 s, and 72°C for 35 s. Copy numbers were estimated from a calibration curve obtained using serial 10-fold dilutions (10^9 , 10^8 , 10^7 , 10^6 , 10^5 , 10^4 , 10^3 , and 10^2) and 10 copies of the quantified standard sample as the template. Standard samples were created by inserting the respective amplification range sequences into the pEGFP-N1 plasmid.

Table 1
Primer sequences for quantitative real-time PCR

Target	Purpose	Prime name	sequences (5' to 3')
Segment 1 mRNA	Reverse transcription	PR8 seg1 mRNA tag	CCAGATCGTTCGAGTCGTTTTTTTTTTTTTTTTTAAACTATTCTGA
	Realtime PCR	mRNA tag	CCAGATCGTTCGAGTCGT
		PR8 seg1 mRNA Re	GGAGATATGGGCCAGCATTA
Segment 4 mRNA	Reverse transcription	PR8 seg4 mRNA tag	CCAGATCGTTCGAGTCGTTTTTTTTTTTTTTTTTCTCATATTTCT
	Realtime PCR	mRNA tag	CCAGATCGTTCGAGTCGT
		PR8 seg4 mRNA Re	GGGCAATCAGTTTCTGGATGTGTTCT
Segment 5 mRNA	Reverse transcription	PR8 seg5 mRNA tag	CCAGATCGTTCGAGTCGTTTTTTTTTTTTTTTTTCTTTAATTGTC
	Realtime PCR	mRNA tag	CCAGATCGTTCGAGTCGT
		PR8 seg5 mRNA Re	CGATCGTGCCTTCCTTTG

Results

Primer design and standard curve for mRNA segments 1, 4, and 5

To quantify the copy numbers of mRNA of segments 1, 4, and 5 in the PR8 strain, we designed primers specific for each segment (Table 1) by modifying the primer sequences previously reported by Kawakami et al., and calibration curves for each segment mRNA were constructed (Fig. 1a, b, and c). Each calibration curve was linear in the range of 10^1 – 10^9 copies in the reaction solution. The linear correlations of the calibration curves in this range were $r = 0.999$ in segment 1, $r = 0.998$ in segment 4, and $r = 0.999$ in segment 5 (Fig. 1a, b, and c, Table 2).

Table 2

Validation parameters for mRNA quantification of segments 1, 4, and 5 by strand-specific real-time RT-PCR using tagged primers.

Target		sensitivity (copies)	Linear regression			
			Slope	Intercept	Amplification efficiency (E%)	R ²
segment 1	mRNA	10 ¹	-3.2232	38.833	100	0.9979
segment 4	mRNA	10 ¹	-3.1873	38.24	100	0.9967
segment 5	mRNA	10 ¹	-3.2491	39.379	100	0.9972

Effect of the polymerase reaction time on influenza virus mRNA synthesis

The optimum conditions for the RdRp reaction were examined. After the RdRp reaction for 0, 15, 30, 60, and 120 min, mRNA production was maximal at 120 min in segment 1 ($7.8 \pm 0.39 \times 10^3$ copies in reaction), maximal at 60 min in segment 4 ($3.6 \pm 0.23 \times 10^3$ copies in reaction), and maximum at 60 min in segment 5 ($1.1 \pm 0.01 \times 10^4$ copies in reaction) (Fig. 2a, b, and c). Therefore, the RdRp reaction time was 30 min that corresponds to half of the maximum reaction rate for each segment.

Influenza virus mRNA synthesis at various temperatures

The optimum temperature for the RdRp reaction was examined in the range of 4–42°C. The highest activity was observed in segment 1 at 30–37°C (Fig. 3a), and in segments 4 and 5, mRNA production increased in a temperature-dependent manner between 15°C and 37°C (Fig. 3b and c). In all segments, RdRp activity decreased under high temperature conditions of 42°C (Fig. 3a, b, and c). Therefore, the reaction temperature of RdRp was set at 37°C.

Effect of Mg²⁺ concentration in RNA polymerase reaction

Generally, the polymerase reaction changes depending on the Mg²⁺ concentration in the reaction solution. In segment 1, almost no mRNA was produced up to 4 mM MgCl₂, but mRNA production increased rapidly at 5 mM MgCl₂ (Fig. 4a). Segments 4 and 5 showed concentration-dependent production of mRNA at 3 mM to 5 mM of MgCl₂ (Fig. 4b and c). CaCl₂ did not affect RdRp activity (data not shown). The reaction solution containing 5 mM MgCl₂ synthesized 17.8- (Fig. 4a), 23.5- (Fig. 4b), and 55.8-fold (Fig. 4c) more mRNA in segments 1, 4, and 5, respectively, than in the reaction solution without MgCl₂. From these results, the MgCl₂ concentration in the RdRp reaction solution was fixed at 5 mM.

Effect of ApG

The addition of dinucleotide ApG has been reported to significantly accelerate influenza virus mRNA synthesis in the RdRp reaction [10, 15]. Due to the lack of ApG, an initiator of mRNA synthesis, in the RdRp reaction solution, the amount of mRNA synthesis in segments 1, 4, and 5 was markedly reduced to 7.9%, 9.2%, and 3.8%, respectively (Fig. 5a, b, and c).

The inhibitory effect of the RNA polymerase inhibitor ribavirin triphosphate (RTP)

To investigate whether the method developed in this study to determine RdRp activity might be useful to evaluate RdRp inhibitory compounds, the inhibitory effect of ribavirin triphosphate (RTP), which has been reported to inhibit influenza virus RdRp, was analyzed. In segment 1, 200 μM and 300 μM of RTP inhibited mRNA synthesis by 46.4% and 47.9%, respectively, compared to that of the control (Fig. 6a). In segments 4 and 5, mRNA production was inhibited in a concentration-dependent manner by RTP addition. At 500 μM of RTP in the reaction solution, mRNA production was inhibited in segments 4 and 5 by 86.7% and 91.5%, respectively. This inhibitory effect of RTP in influenza virus RdRp activity did not differ from those previously reported [16, 17] (Fig. 6b and c).

Discussion

We have established a novel method for measuring the RdRp activity of influenza viruses using RT-PCR. In current methods [15, 18], the amount of mRNA synthesized is quantified using ^{32}P -labeled nucleoside triphosphates (GTP or UTP). Therefore, only the total number of eight-segment mRNAs is calculated. However, in the method we developed, the mRNA of each segment can be analyzed individually, and the copy number of the mRNA produced can be quantified through RT-PCR.

In this study, mRNAs of segments 1, 4, and 5 were examined, but by designing the primer using the primer design reported by Kawakami et al. [14] as reference, the amount of cRNAs and vRNAs of each of the eight segments present in influenza virus might be also quantified.

Viral particles were purified from the culture supernatants of infected cells. Although ultracentrifugation is commonly used to prepare influenza virus polymerase solutions, the method is time consuming and unsuitable for screening. In this experiment, we collected influenza viral particles using magnetic beads. Sakudo et al. have shown through immunochromatography that these beads can efficiently capture influenza viruses in cell culture media [12]. Using this method, it is possible to collect viral particles more easily and quickly than that using ultracentrifugation; therefore, viral particles with high RdRp enzyme activity can be collected. However, as these beads may adsorb various viruses through electrostatic interactions, they are not specific for influenza virus, leading to contamination with other components from the cell culture medium. In our case, the virus polymerase activity was not affected by contamination with the culture medium.

The reaction time for polymerase activity was the same as previously reported [19]. The optimum temperature for this method was 37°C (Fig. 3), but some previous studies have reported the use of 30°C [7, 13, 20, 21] Even though studies have reported the use of 37°C [22, 23], it is unclear why 37°C was optimum for our method.

Regarding the concentration of Mg in the RNA synthesis reaction buffer, the polymerase activity increased (Fig. 4) at Mg concentrations similar to those previously reported [7, 11, 15]. However, the amplification of each segment increased sharply at 3 or 4 mM MgCl₂ and showed little increase at concentrations below 3 mM (Fig. 4). Zhang et al. showed a similar rapid increase, although the border concentrations were slightly different [24].

The presence of ApG, a specific dinucleotide primer, increased mRNA production by approximately 10-fold in each segment, but mRNA was synthesized to a certain extent even in its absence (Fig. 5).

RTP inhibits RdRp because it is mistakenly taken up during mRNA synthesis as it is similar in structure to GTP and stops mRNA synthesis [16, 17]. Initially, when the GTP concentration was the same as that of ATP, CTP, and UTP, 100 µM RTP had no inhibitory effect, and 500 µM RTP only inhibited segment 1 by 57.1% and segment 4 by 47.1% (data not shown). Therefore, in this experiment we lowered GTP concentration to show the inhibitory effect of RTP. The inhibitory effect of 100 µM RTP was observed resulting in 20.8% inhibition for segment 1, 39.2% for segment 4, and 44.5% for segment 5 (Fig. 6). In segments 4 and 5, there was no effect of the GTP concentration on mRNA production, and mRNA production decreased depending on the RTP concentration (Fig. 6b and c). However, in segment 1, mRNA production was significantly reduced by the dilution of GTP, even without RTP. Therefore, the concentration-dependent inhibition of RTP could not be confirmed in segment 1 (Fig. 6a). The concentration of GTP is considered important for the synthesis of segment 1 mRNA.

Since the amount of mRNA synthesis was reduced in the absence of ApG or magnesium, and the inhibition of synthesis by RTP was confirmed, this experimental method proved useful at evaluating the activity of RdRp of influenza viruses.

Conclusions

This novel method for measuring influenza virus polymerase activity will further promote research to identify compounds that inhibit viral RNA synthesis.

Abbreviations

HA: hemagglutinin; MOI: multiplicity of infection; NP: nucleoprotein; PA: acidic polymerase protein; PB1: polymerase basic protein 1; PB2: polymerase basic protein 2; qPCR: quantitative PCR; RdRp: RNA-dependent RNA polymerase; RTP: ribavirin triphosphate; RNP: ribonucleoprotein; RT-PCR: reverse transcription polymerase chain reaction; vRNPs: viral ribonucleoprotein particles.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent to publication

Not applicable.

Availability of data and material

All data generated or analyzed during this study are included in this published article.

Competing interests

The authors declare no competing financial interests.

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Author's contributions

YH performed the experiments and analyzed the data. YH and MS designed the experiments and wrote the manuscript. YI conceived and supervised the study. All authors read and approved the final manuscript.

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Figures

Figure 1

Standard curve for segments 1 (a), 4 (b), and 5 (c). A standard curve was generated by plotting the threshold cycle (Ct). Ten-fold serial dilution (101_109 copies/μL) of standard DNA were used to generate a standard curve.

Figure 2

Effect of the reaction time on the influenza virus mRNA synthesis. The RNA polymerase reaction for influenza virus was performed under regular reaction conditions except the reaction time. (a) Segment 1 mRNA, (b)

segment 4 mRNA, and (c) segment 5 mRNA. Data are presented as mean $\pm$ standard deviation ($n = 3$).

Figure 3

Influenza virus mRNA synthesis at various temperatures. (a) Segment 1 mRNA, (b) segment 4 mRNA, and (c) segment 5 mRNA. Data are presented as mean $\pm$ standard deviation ($n = 3$).

Figure 4

Effect of Mg²⁺ concentration in the RNA polymerase reaction on influenza virus mRNA synthesis. The RNA polymerase reaction for influenza virus was performed under regular reaction conditions except the concentration of MgCl₂. The RNA polymerase reaction mixture was incubated for 30 min at 37 °C. (a) Segment 1 mRNA, (b) segment 4 mRNA, and (c) segment 5 mRNA. Data are presented as mean $\pm$ standard deviation ($n = 3$).

Figure 5

Effect of ApG on influenza virus mRNA synthesis. The reduction of each mRNA synthesis, (a) segment 1 mRNA, (b) segment 4 mRNA, and (c) segment 5 mRNA, was analyzed with the RNA polymerase reaction without the specific dinucleotide primer ApG. Data are presented as mean $\pm$ SD ($n = 3$). *, $p < 0.05$; **, $p < 0.001$; ***, $p < 0.0001$

Figure 6

The inhibitory effect of the RNA polymerase inhibitor ribavirin triphosphate (RTP) on influenza mRNA synthesis. (a) Segment 1 mRNA, (b) segment 4 mRNA, and (c) segment 5 mRNA. Various concentrations of RTP (0_500 μ M) were added to the polymerase reaction solution. Since RTP is used as a GTP inhibitor, the GTP concentration in this experiment was set to 5 μ M. Data are presented as mean $\pm$ SD ($n = 3$).