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Validation of a Real-Time RT–PCR Method to Quantify Newcastle Disease Virus (NDV) Titer and Comparison with Other Quantifiable Methods

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A method for the rapid detection and quantification of Newcastle disease virus (NDV) produced in an animal cell culture-based production system was developed to enhance the speed of the NDV vaccine manufacturing process. A SYBR Green I-based real-time RT–PCR was designed with a conventional, inexpensive RT–PCR kit targeting the *F* gene of the NDV LaSota strain. The method developed in this study was validated for specificity, accuracy, precision, linearity, limit of detection (LOD), limit of quantification (LOQ), and robustness. The validation results satisfied the predetermined acceptance criteria. The validated method was used to quantify virus samples produced in an animal cell culture-based production system. The method was able to quantify the NDV samples from mid- or late-production phases, but not effective on samples from the early-production phase. For comparison with other quantifiable methods, immunoblotting, plaque assay, and tissue culture infectious dose 50 ($TCID_{50}$) assay were also performed with the NDV samples. The results demonstrated that the real-time RT–PCR method is suitable for the rapid quantification of virus particles produced in an animal cell-culture-based production system irrespective of viral infectivity.

Keywords: Method comparison, method validation, Newcastle disease virus (NDV), real-time RT–PCR (RRT–PCR), virus quantification

Newcastle disease (ND) is an infectious viral disease that affects most birds, including chickens. Because chickens make up the largest proportion of the poultry industry worldwide, ND can result in massive economic losses in many countries [1]. The Newcastle disease virus (NDV) is an avian paramyxovirus type I serotype of the genus *Avulavirus* belonging to the family *Paramyxoviridae* [2, 9]. The NDV was identified approximately 80 years ago and the genomic structure and functions of the NDV proteins have been elucidated by many researchers [6, 14, 15, 19, 20, 23, 29]. The NDV is an enveloped virus and has a linear, single-stranded RNA genome of negative polarity. The RNA genome has a size of ~15,200 nucleotides and contains six structural genes that encode, in order from 3' to 5' on the genomic RNA, the nucleocapsid protein, phosphoprotein, matrix protein, fusion protein, hemagglutinin-neuraminidase, and RNA-dependent RNA polymerase [16]. NDV strains are usually classified into three categories on the basis of their pathogenicity in chickens: velogenic (high virulence), mesogenic (moderate virulence), and lentogenic (low virulence) [4]. The pathogenicity of NDV is determined primarily by cleavage of the F protein by host cellular proteases [6, 20].

With the development of reverse genetics, the integration of certain foreign gene(s) into the NDV genome has been possible [20]. Recombinant NDV strains containing foreign gene(s) can be used for development of viral vaccines. Vaccines using recombinant NDV have been developed for severe acute respiratory syndrome [7, 31] and avian influenza [11]. In addition, because of oncolytic characteristics of the NDV, which can infect and lyse cancer cells specifically [23, 26], the NDV is also used in cancer research related fields.

Although it is expected for the applications of recombinant NDV to be on humans, the production of NDV is still dependent on the traditional egg-based production system. However, efforts to produce NDV in the animal cell-culture-based production system, which is much safer than the egg-based production system, have been undertaken. For future applications in clinical uses, and development of therapeutic drugs and viral vaccines, the animal cell-culture-based production system should be used to comply with the required Good Manufacturing Practice/Good Laboratory Practice regulations.

For the production of viral vaccines, measuring the exact titer of virus is important to make a vaccine of the

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correct dosage. The plaque and tissue culture infectious dose 50 ($TCID_{50}$) assays are commonly used methods to quantify virus titer. However, these methods are time consuming (~1 week) [5], and not very cost effective. Compared with these traditional methods, a real-time PCR method has many advantages, including rapidity, quantifiable measurement, higher sensitivity, higher specificity, and ease of standardization [24]. In many researches, real-time RT–PCR methods have already been developed to detect and quantify NDV [10, 12, 17, 21, 28, 30]. However, most of these methods are just focused on diagnosis of specimens from NDV-infected birds and not validated with validation criteria including specificity, linearity, and so on. The method validation is necessary to make high confidential results in most bioassays. Only the validated method can be used widely as a formally admitted one.

In this study, we developed a rapid and inexpensive method to detect and quantify NDV in the ND vaccine manufacturing process, as there is no real-time RT–PCR method for this purpose yet. To ensure the reliability of the method, we performed validation of the method. For future applications, we tried to apply the method for quantification of NDV produced from the animal cell culture-based production system rather than the egg-based production system. To describe the advantages and limitations of the developed method, it was compared with other virus quantifiable methods.

Materials and Methods

Cells and Viruses

DF-1 (chicken embryo fibroblast) (ATCC; American Type Culture Collection, CRL12203, Manassas, VA, USA) and Vero (African green monkey kidney) (ATCC, CCL-81) cells were maintained in minimum essential medium (MEM)-α (GIBCO, Burlington, ON, Canada) supplemented with 10% and 5% fetal bovine serum (FBS) (PAA Laboratories, Etobicoke, ON, Canada), respectively. The lentogenic NDV strain, LaSota, was provided from DaeSung Microbiological Labs. Co., Ltd (Uiwang, Korea). The NDV LaSota was originally propagated in embryonated eggs from specific pathogen-free chickens by the DaeSung Microbiological Labs. Allantoic fluid in the embryonated eggs where the NDV LaSota propagated was collected and centrifuged at 16,000 ×*g* for 30 min at 4°C to remove egg debris. After centrifugation, the supernatant was filtrated with a 0.2-μm syringe filter (Sartorius Stedim Biotech GmbH, Goettingen, Germany). The NDV titer of the purified allantoic fluid was determined by plaque assay and used as a seed virus for further experiments. The genomic RNAs of the influenza virus, Japanese encephalitis virus, and hepatitis A virus, and the genomic DNA of the hepatitis B virus were kindly provided by Dr. Chung-Keel Lee of the Korea Food and Drug Administration.

NDV Production in Animal Cell Culture-Based Production System

DF-1 cells were seeded in 6-well plates at a density of 2.5×10^5 cells per well. Two days later, the cell density was measured by the trypan blue exclusion method as previously described [3]. Then, the cell monolayer was infected with the NDV LaSota strain at a multiplicity of infection of 0.01 and incubated at 37°C in virus production medium (basal MEM-α containing 400 ng/ml trypsin) for 4 days. Supernatants were harvested at 12 h intervals and centrifuged at 16,000 ×*g* for 10 min at 4°C to remove cellular debris.

Isolation of Viral Genomic RNA

NDV LaSota in allantoic fluids provided by the DaeSung Microbiological Labs and the harvested NDV LaSota samples produced in the animal cell culture-based production system were used for viral genomic RNA isolation. The RNA genome of NDV LaSota was extracted using a NucleoSpin RNA Virus kit (MACHEREY-NAGEL GmbH, Duren, Germany) according to the manufacturer's instructions.

Preparation of RNA Standard of NDV LaSota

The amount of NDV LaSota genomic RNA was quantified spectrophotometrically using a NanoDrop1000 (Thermo Fisher Scientific, Wilmington, DE, USA). The number of copies of the viral genome was calculated using the following formula [17]:

$$Y\ (\text{molecules}/\mu l)=X\ (g/\mu l)/(\text{nucleotide length}\times340)\times6.02\times10^{23}$$

The NDV LaSota genomic RNA was diluted to 4×10^7 copies/μl and 10-fold serial dilutions of the genomic RNA were made. The standard curve was derived from the crossing point (C_p) values obtained using serially diluted RNA samples.

Real-Time RT–PCR

Real-time RT–PCR was performed in two steps. First, 1 μl of LaSota genomic RNA was converted into cDNA using the Maxime RT–PCR Premix Kit (iNtRON Biotechnology, Seongnam, Korea). The reverse transcription was performed at 45°C for 30 min using 0.5 μM of the LaSotaF primer (Table 1), and samples were then incubated at 95°C for 5 min to inactivate reverse transcriptase. Second, 0.5 μM of the LaSotaR primer, 5% DMSO, and 1 μl of 20× SYBR Green I Nucleic Acid Gel Stain (Cambrex Bio Science Rockland, Inc., Rockland, ME, USA) were added to the cDNA. The final reaction volume was 20 μl. This real-time PCR mixture was

Table 1. Primer sequences and the characteristics of the NDV LaSota amplicon generated by SYBR Green I-based real-time RT–PCR.

Primers	Sequences (5'–3')	Genomic region[a]	Amplicon size (bp)	Average T_m (observed range, °C)
LaSotaF	5'-TAC AAC AGG ACA TTG ACC ACT TTG CTC AC-3'	4,793–4,821 (*F* gene)	299	84.26 (83.42~84.81)
LaSotaR	5'-TGC ATC TTC CCA ACT GCC ACT GC-3'	5,069–5,091 (*F* gene)		

[a]Prototype strain NDV LaSota (GenBank Accession No. AF077761).

transferred to a well in a 384-well plate suitable for use with the LightCycler 480 (Roche Applied Science, Mannheim, Germany). The real-time PCR conditions were 45 cycles of 95°C denaturation for 10 s, 65°C annealing for 10 s, and 72°C extension for 10 s. The specificity of amplification was determined by melting curve analysis that consisted of 95°C denaturation for 1 min, 65°C annealing for 5 min, and heating to 95°C at the rate of 0.11°C/s. The melting temperature (T_m) of melting curves and C_p values were calculated using the LightCycler 480 software.

Plaque Assay

Vero cells were seeded in 12-well plates at a density of 2.0×10^5 cells per well. After 24 h, Vero cells were infected with 50 μl of 10-fold serial dilutions of the virus samples and incubated for 1 h to allow for viral adsorption. The cells were then covered with a basal MEM-α agar overlay containing 0.02% (w/v) DEAE-dextran, 0.1% (w/v) glucose, 0.7% (w/v) Bactoagar (BD Biosciences, Sparks, MD, USA), 30 mM $MgSO_4$, and 4 μg/ml trypsin (GIBCO, Burlington, ON, Canada). Since the NDV LaSota is a representative lentogenic strain, addition of trypsin in the agar overlay was essential [13, 18]. The cells were incubated at 37°C for 3 days. Two days after virus infection, a 0.03% (w/v) neutral red agar overlay was added to each well to stain viable cells. All chemicals were purchased from Sigma-Aldrich Chemie GmbH (Steinheim, Germany) unless otherwise stated.

Tissue Culture Infectious Dose 50 ($TCID_{50}$) Assay

DF-1 cells were seeded in 96-well plates at a density of 2.0×10^4 cells per well. After 24 h, virus samples were serially diluted with basal MEM-α containing 3 μg/ml trypsin and the DF-1 cells were infected with 100 μl of 10-fold serial dilutions of the virus samples. The cells were incubated at 37°C for 4 days. The cytopathic effect on cells in each well was observed using light microscopy (Olympus CK40, Japan). The $TCID_{50}$ values were calculated by the Reed-Müench method [5].

Immunoblotting

A rabbit polyclonal antibody against the F protein of the NDV LaSota strain was obtained from AbFRONTIER (Seoul, Korea). Culture supernatants harvested at 12 h intervals were resolved by SDS–PAGE, and viral proteins were transferred to a polyvinylidene fluoride (PVDF) membrane. The membrane was blocked in TTBS (Tris-buffered saline with Tween-20) containing 5% (w/v) skim milk for 1 h, and probed with antibody to the NDV F protein. Horseradish peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) and an enhanced chemiluminescence solution (iNtRON Biotechnology) were used to detect the F protein.

Statistical Analysis

All statistical analyses were performed with the MINITAB Release 14 software. All null hypotheses (H_0) were tested using a 99% confidence interval (significance level, α=0.01) unless noted otherwise.

RESULTS AND DISCUSSION

There have been many reports on methods of detecting and determining a pathotype of NDV strains by using the real-time RT–PCR. However, the systematic validation has not yet been performed for these methods. Moreover, no reports have introduced practical application of the method to NDV produced in the animal cell culture-based production system. Therefore, in this study, we tried to develop and validate a real-time RT–PCR method for rapid quantification of NDV produced in an animal cell culture-based production system and compare it with other quantifiable methods to discuss its advantages and limitations.

The F protein of lentogenic NDV strains is usually not cleaved well by host proteases. Therefore, addition of extracellular proteases such as trypsin is required to produce NDV LaSota in the animal cell culture-based production system. In general, the mesogenic and velogenic NDV strains have an advantage, as the give higher yields without addition of extracellular proteases. However, since these NDV strains can cause serious damage to bird population and conjunctivitis in humans, their use is strictly restricted to the facilities that have specific safety equipment by regulatory policy. The commercial ND vaccines are mainly manufactured with lentogenic strains like LaSota or B1 by using the egg-based production system to comply with regulatory policy. Therefore, a lentogenic strain, NDV LaSota, was used in this study.

A quantifiable real-time RT–PCR method to determine NDV titer was validated for specificity, linearity, accuracy, precision, limit of detection, limit of quantification, and robustness.

Specificity

Since commercially developed one-step real-time RT–PCR kits are relatively expensive, a conventional RT–PCR kit whose price is one-third that of the one-step real-time RT–PCR kit was used in this study. In addition, SYBR Green I was used as a fluorescent dye because of its low cost. There are many types of probes labeled with fluorescent molecule, such as Molecular beacon, Taqman probe, FRET probe, and Scorpion probe for the real-time PCR. These probes can represent a specific amplification of target sequence, but are relatively more expensive than SYBR Green I. On the other hand, since the SYBR Green I dye has an intrinsic characteristic of random double-stranded DNA binding, it is important to inhibit the formation of primer dimers to eliminate a possible overestimation in amplification of the target sequence.

The specificity of the real-time RT–PCR method depends mainly on the primer sequence, which is designed to be specific to the target virus. The detailed genomic locations, sequences of the primers used here, as well as characteristics of the amplicon generated by the real-time RT–PCR method, are shown in Table 1. In addition, after cDNA synthesis and inactivation of reverse transcriptase, 5% (v/v) DMSO was added to the reaction mixture to prevent formation of primer dimers. To evaluate the specificity of

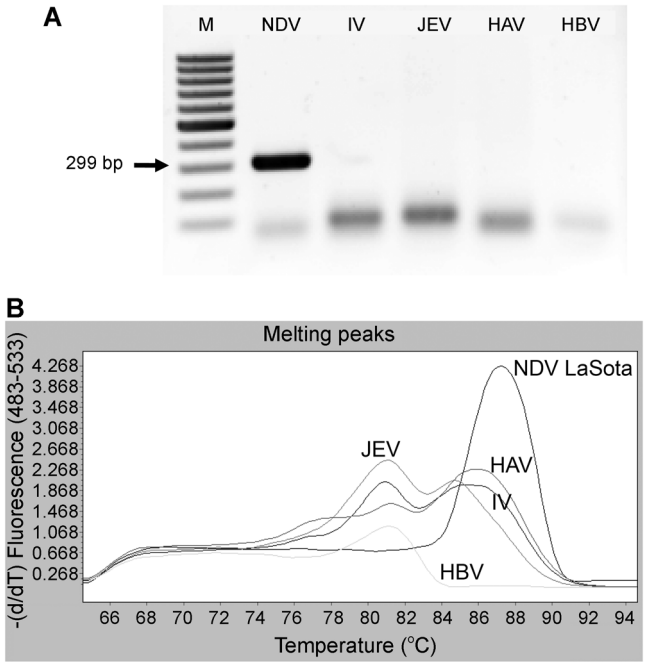

Fig. 1. The specificity of a real-time RT–PCR.
A. End-products of the real-time RT–PCR were visualized after separation on an agarose gel. Only the NDV showed specific amplification. **B**. Melting curve analysis of real-time RT–PCR products. Only the NDV showed a single, high-temperature melting peak (T_m>84°C). NDV: NDV LaSota strain; IV: Influenza virus; JEV: Japanese encephalitis virus; HAV: Hepatitis A virus; HBV: Hepatitis B virus.

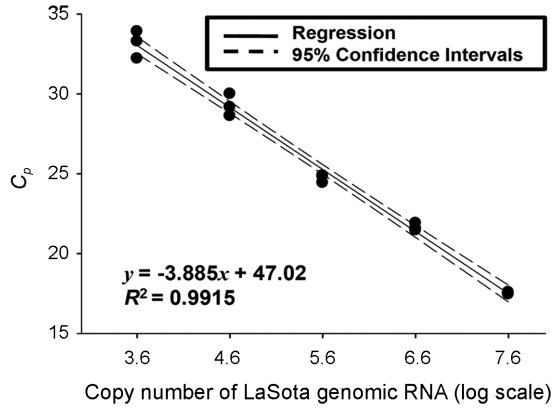

Fig. 2. Analysis of linearity.
The standard curve for the real-time RT–PCR used for quantifying NDV LaSota genomic RNA was generated from the C_p values plotted against 10-fold serially diluted genomic RNA samples (4×10^7 to 4×10^3 copies). Triplicate samples were used for the assay. $R^2\geq0.99$.

the method, various viral genomes were also tested together. Influenza virus (IV) containing a genome composed of several negative-sense single-stranded RNAs, Japanese encephalitis virus (JEV) and hepatitis A virus (HAV) containing a positive-sense single-stranded RNA genome, and the hepatitis B virus (HBV) containing a DNA genome were used for comparison. A 299-bp fragment was specifically amplified from the NDV LaSota, but not the other viruses (Fig. 1A). In melting peak analysis, only the PCR product from the NDV LaSota showed a single, high-temperature melting peak without peaks originated from the formation of primer dimers, whereas the PCR products from the other viruses showed low temperature melting peaks consistent with formation of primer dimers or nonspecific amplification (Fig. 1B). This indicated that the specific amplification of target sequence had occurred exclusively from the NDV LaSota genome.

Since the NDV was produced from a cell culture-based production system, it is hard to confirm removal of host cell-derived DNA and RNA completely. Although the manufacturer guarantees that most of the host cell-derived DNA and RNA are removed during viral RNA preparation process with the NucleoSpin RNA Virus kit, it is necessary to investigate the effect of residual host cell-derived DNA and RNA on amplification of the NDV LaSota *F* gene. As shown in Supplementary Fig. 1, the host cell-derived DNA and RNA had no effect on amplification of the NDV LaSota *F* gene. Massive amounts of host cell-derived DNA and RNA showed no amplification with the NDV LaSota *F* gene-specific primer set. Therefore, residual host cell-derived DNA and RNA have no interference on the amplification of the NDV LaSota *F* gene fragment from the NDV viral RNA genome.

Linearity

The NDV LaSota RNA genome was serially diluted from 4×10^7 to 4×10^3 copies and the samples were used as templates. The C_p values at each dilution were collected in triplicate and used to prepare a standard curve. The derived standard curve showed that the correlation coefficient, R^2, is 0.9915, higher than the 0.99 acceptance criterion in the range of 4×10^7 to 4×10^3 genomic RNA copies (Fig. 2). According to the regression analysis performed using the MINITAB software, the linear regression was highly significant (p-value<0.001) at the 99% confidence level (α=0.01). Analysis of the residuals satisfied normality, independence, and equal variance (data not shown).

Accuracy

Accuracy is the measure of how closely an experimentally measured value agrees with the true value of the sample [25], usually determined by percent recovery, which is the measured or actual value divided by the true value. In this assay, the acceptance criterion was 80~120% recovery of the viral genome, which is commonly used in bioassays. The NDV LaSota RNA genomes serially diluted from 4×10^7 to 4×10^3 copies were used as templates. The actual values were calculated from the standard curve (Fig. 2) with measured C_p values. The percent recoveries were calculated as shown in Table 2 and Supplementary Table 1.

Table 2. The validation results of the real-time RT–PCR method to quantify NDV: accuracy and precision.

Copy number of LaSota genomic RNA	Accuracy (Recovery (%)[a])	Precision – intra-assay (CV (%)[b])	Precision – inter-assay (inter-day) (CV (%))	*F*-value[c]	*p*-value[d]
4e+7	100.1	1.0	2.13	0.88	0.502
	99.7				
4e+6	97.9	1.8	2.41	1.06	0.449
	99.2				
4e+5	101.8	2.5	3.65	0.17	0.848
	103.8				
4e+4	102.8	0.6	3.01	0.83	0.518
	91.7				
4e+3	98.1	1.2	2.09	2.56	0.225
	105.8				

[a]Recovery (%)=actual value/true value×100.
[b]CV (%): Coefficient of variation=(SD of C_p/Mean of C_p)×100.
[c]*F*-value is less than $F_{(2,3;\,0.01)}$=30.82, indicating that there is no difference between days.
[d]*p*-value> α (α=0.01 at 99% confidence level), indicating that there is no difference between days.

Since the recoveries are in the range of 91~106%, they all satisfied the acceptance criterion for the assay.

Precision

Precision is an indicator of the repeatability and reproducibility of a method under normal conditions [25]. First, the intra-assay precision was tested to determine the repeatability; that is, the variation between replicate samples in the same experimental set (*e.g.*, in the same tube or plate). In this study, the same experimental set means that all intra-assays were performed in the same 384-well PCR plate at one time. The real-time RT–PCR was performed with templates of NDV LaSota RNA genomes serially diluted from 4×10^7 to 4×10^3 copies in triplicate. The results are shown in Table 2 and Supplementary Table 2. The acceptance criterion for repeatability was set to a coefficient of variation (CV) less than 3% as previously described [24]. As shown in Table 2 and Supplementary Table 2, the repeatability criterion was satisfied since all the CV values were less than 2.5%, indicating that the intra-assay variation was very limited.

Second, the inter-assay variation was tested to determine the reproducibility; that is, the variation between separate assays. Day-to-day variation with the test being performed by the same analyst, as well as analyst-to-analyst variations, can be tested [25]. In this study, day-to-day variation was tested for 3 days. The NDV LaSota RNA genomes serially diluted from 4×10^7 to 4×10^3 copies were used as templates in duplicate. The results are shown in Table 2 and Supplementary Table 3. Some CV values were slightly higher than the predetermined acceptance criterion of 3%. Considering that other bioassays have higher acceptance criteria (CV of 5% for intra-assay precision, 20% for inter-assay precision in a previous report [25]), the slightly higher CV values measured here are thought to be negligible. Since the average CV for inter-assay variation was within the limit of the acceptance criterion, the assay exhibited a high degree of precision. In addition, statistical analyses showed that the C_p values were not significantly different between days (see *F*- and *p*-values in Table 2 and Supplementary Table 3).

Limit of Detection (LOD) and Limit of Quantification (LOQ)

The NDV genomic RNA was serially diluted from 4×10^7 to 4×10^1 copies to determine the LOD and LOQ. The C_p values and end-products of the real-time RT–PCR are

Table 3. Summary of the real-time RT–PCR validation results.

Assay characteristics	Acceptance criteria	Validation results
Specificity	Negative and other virus tests are not reactive	Pass
Accuracy	80~120% (% recovery)	91~106% (% recovery)
Linearity	Correlation coefficient ≥ 0.99	R^2=0.9915
Repeatability	CV (%) ≤ 3%	CV (%) ≤ 2.5%
Reproducibility	*p*-Value > α (α=0.01 at 99% confidence level)	Pass
LOQ	Linearity	4×10^7~4×10^3 copies
LOD	Detection limit	~400 copies
Robustness	Not different	Pass

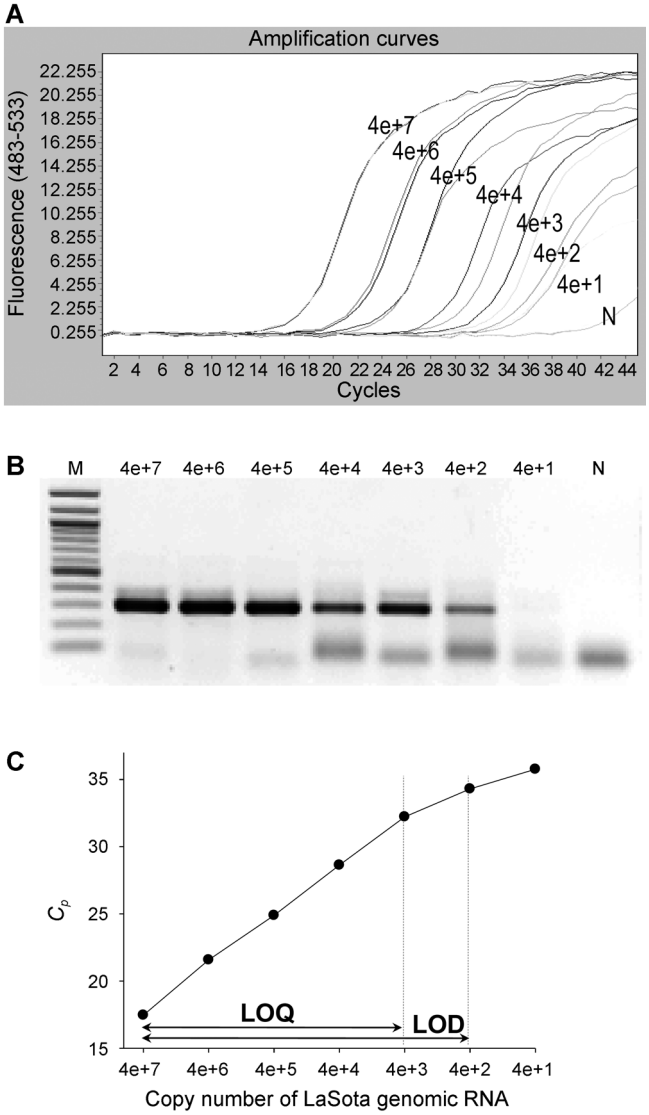

Fig. 3. The limit of detection and quantification of the real-time RT–PCR.

A. Amplification curves obtained during real-time RT–PCR. **B**. Visualization of the end-products of the real-time RT–PCR after separation on an agarose gel. **C**. The C_p values were plotted against 10-fold serially diluted genomic RNA (4×10^7 to 4×10^1 copies). Amplicons were detected in samples with 4×10^2 copies or more copies on the agarose gel, but linearity was only satisfied over the range of 4×10^7 to 4×10^3 copies.

shown in Fig. 3. The target band was amplified from the samples containing 400 or more copies of the RNA genome (Fig. 3B), but the C_p values were only linear within the range of 4×10^7~4×10^3 copies (R^2=0.9948) (Fig. 3C). Therefore, the LOD and LOQ were determined as 4×10^2 and 4×10^3 copies, respectively.

As per our expectation, both LOD and LOQ were similar to those of other researches. These values almost coincided with those of other real-time RT–PCR methods for detecting and quantifying NDV or other RNA viruses in previous reports (LOD and LOQ of 10^2~10^4 copies [17, 24, 30]). However, unlike previous reports, much cheaper materials, such as SYBR Green I fluorescent dye and conventional RT–PCR kit, were used in this study. This is much more attractive for economical consideration.

Robustness

Robustness measures the ability to withstand small predictable (or even unpredictable) variations in the method and is an indicator of the reliability of the method during normal use [8].

Since the RT–PCR conditions were fixed, two parameters, the temperature and incubation time for the virus lysis procedure, which could be critical for preparation of the NDV LaSota RNA genome, were tested for robustness. The robustness assay results are shown in Supplementary Tables 4 and 5. The statistical analysis was performed by using a two-way ANOVA. The statistical results, *F*- and *p*-values, showed that the method could be robust enough to endure intentional small changes in the temperature or incubation time of virus lysis during isolation of the NDV LaSota RNA genome.

Application of the Real-Time RT–PCR Method and Comparison with Other Quantifiable Methods

The validation results for the real-time RT–PCR method are summarized in Table 3. The validated real-time RT–PCR method was used to quantify the NDV LaSota samples produced in the animal cell culture-based production system. The NDV LaSota was harvested from the production medium every 12 h. The C_p values were not measured until 36 h post infection (h.p.i) in the early production phase (Fig. 4A). This might be caused by the low efficiency of preparation process of nucleic acids from virus particles rather than sensitivity of the real-time RT–PCR method. In other research, the direct multiplex RT–PCR method for influenza virus without RNA purification showed no positive results at lower virus titers [27]. Therefore, when one evaluates the usefulness of the real-time PCR methods for quantification of virus, the preparation process of nucleic acids from the virus particles should be also considered importantly to evaluate the whole sensitivity of the real-time PCR methods. The measured C_p values continuously decreased as production time passed, indicating that the NDV LaSota genomic RNA continuously increased during the virus production. To compare this method with other quantifiable methods, the NDV LaSota samples were quantified by plaque and $TCID_{50}$ assays and by immunoblotting for the NDV envelope glycoprotein, the F protein (Fig. 4B–4D). The immunoblotting result showed a trend that was similar to the real-time RT–PCR method. The NDV envelope F protein increased continuously during the virus production. From the results of the real-time RT–PCR and immunoblotting, the virus particles increased

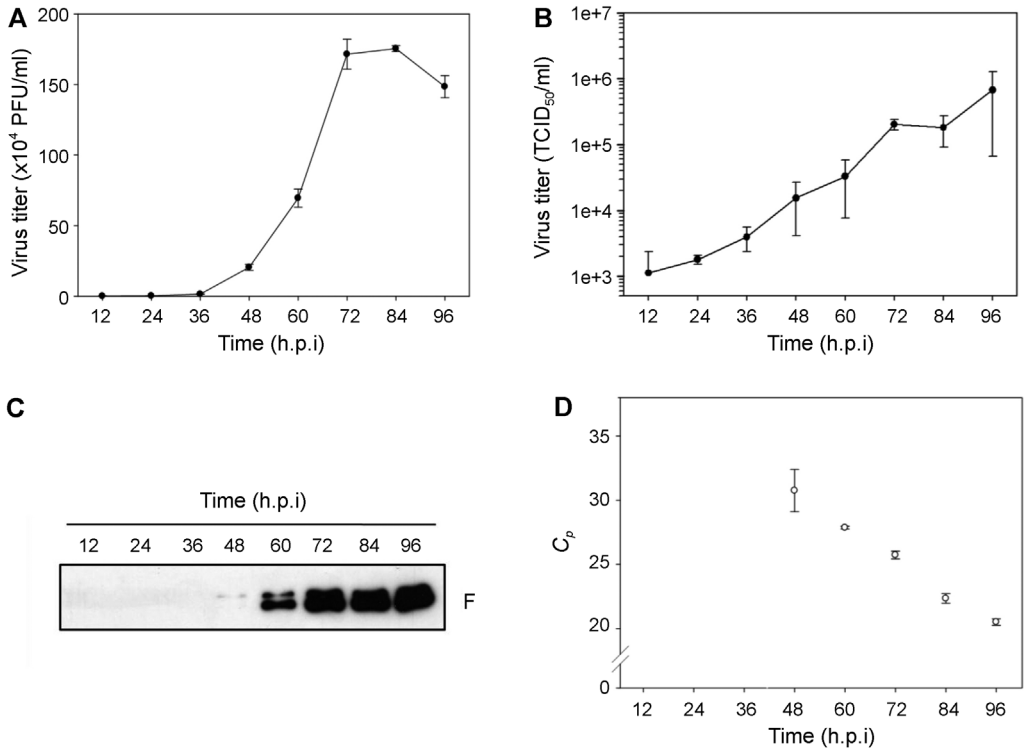

Fig. 4. Comparison of methods for quantification of the NDV.
A. The C_p values from the real-time RT–PCR method. **B**. Immunoblotting for the NDV envelope glycoprotein, F protein. **C**. Plaque assay result. **D**. Tissue culture infectious dose 50 ($TCID_{50}$) assay result. **A** and **B** represent total virus particles (both infective and non-infective), whereas **C** and **D** represent only the infective virus particles. h.p.i: hours post infection.

continuously during the period of virus production. In contrast, the results from the plaque and $TCID_{50}$ assays, which are the most commonly used methods in determining virus titers, showed somewhat different patterns. Unlike the former two methods, the latter two methods showed a decrease in virus titer in the late-production phase (84, 96 h.p.i), although the $TCID_{50}$ assay exhibited a large standard deviation, especially at 96 h.p.i. These results indicate that the infectious NDV particles are decreased in the late-production phase. Since these assays only detect the virus that has infectivity, the number of infectious virus particles decreased eventhough the number of total virus particles continued to increase in the late-production phase. This discrepancy among quantifiable methods is related to the stability of virus envelope glycoprotein, F and HN proteins. The denaturation of these proteins leads to the loss of infectivity of NDV. In our other experiment, the titer of the NDV LaSota stored at 37°C for 3 days was reduced by 10-folds compared with the NDV LaSota stored at 4°C or −20°C (data not shown). The viruses produced in the early-production phase might lose infectivity since their envelope glycoproteins are denatured in the late-production phase at the 37°C production temperature. This might cause the decreased virus titers in the plaque and $TCID_{50}$ assays, although the total virus particles were increased continuously in the late-production phase.

It requires one or two days to obtain the results of immunoblotting and at least one week for the plaque and $TCID_{50}$ assays from the preparation of cells to the final result. Considering the materials required for the latter two assays, such as cultured animal cells, FBS, and trypsin, the methods are not economically attractive. In contrast, the real-time RT–PCR method developed in this study not only uses relatively inexpensive materials but also takes a shorter time to produce final results, as the whole process from the preparation of the NDV LaSota genomic RNA to the final results can be completed within approximately 3 hours. However, as the method cannot distinguish between infectious and non-infectious virus particles and because of the relatively low efficiency of the preparation process of nucleic acids from the virus particles, the plaque and $TCID_{50}$ assays would still be more adequate when the quantification of infectious virus particles or viruses produced in the early-production phase is required.

In conclusion, we have developed a real-time RT–PCR method for the rapid detection and quantification of NDV LaSota strain produced in an animal cell culture-based production system. The method was validated for specificity, linearity, accuracy, precision, LOD, LOQ, and robustness. The validation results showed that the method satisfied predetermined acceptance criteria and it could be applied to the rapid quantification of the NDV LaSota strain.

Specifically, the method was well suited for the quantification of high amounts of virus particles produced in an animal cell culture-based production system, irrespective of viral infectivity. It is a simple, specific, economical, and rapid method.

Acknowledgments

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