An improved method for the rescue of recombinant Newcastle disease virus

Pheik-Sheen Cheow¹, Tiong Kit Tan², Adelene Ai-Lian Song¹, Khatijah Yusoff^{1,3} & Suet Lin Chia^{1,4,*}

ABSTRACT

Reverse genetics has been used to generate recombinant Newcastle disease virus with enhanced immunogenic properties for vaccine development. The system, which involves co-transfecting the viral antigenomic plasmid with three helper plasmids into a T7 RNA polymerase-expressing cell to produce viral progenies, poses a great challenge. We have modified the standard transfection method to improve the transfection efficiency of the plasmids, resulting in a higher titer of virus progeny production. Two transfection reagents (i.e., Lipofectamine and polyethylenimine) were used to compare the transfection efficiency of the four plasmids. The virus progenies produced were quantitated with flow cytometry analysis of the infectious virus unit. The modified transfection method increased the titer of virus progenies compared with that of the standard transfection method.

METHOD SUMMARY

The standard transfection method was modified by changing the time to perform the co-transfection to produce viral progenies from the transfected mammalian cell line, which improved the titer of virus progenies. The method of viral titer quantification by infecting the cancer cell line was simple and time saving.

KEYWORDS

Newcastle disease virus • polyethylenimine • reverse genetics • transfection • virus titration

¹Department of Microbiology, Faculty of Biotechnology & Biomolecular Sciences, Universiti Putra Malaysia, 43400 UPM Serdang, Selangor, Malaysia; ²MRC Human Immunology Unit, Weatherall Institute of Molecular Medicine, University of Oxford, OX3 9DS, Oxford, UK; ³Malaysia Genome Institute, Jalan Bangi, 43000 Kajang, Selangor, Malaysia; ⁴Institute of Bioscience, Universiti Putra Malaysia, 43400 UPM Serdang, Selangor, Malaysia; *Author for correspondence: suetlin@upm.edu.my

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Newcastle disease virus (NDV) is a single-stranded, negative-sense, nonsegmented RNA virus [1]. A recombinant NDV (rNDV) can be generated from a cloned cDNA using reverse genetics. The cDNA of the full-length antigenome of the virus, with plasmids encoding for ribonucleoprotein (RNP), are co-transfected into T7 RNA polymerase-expressing cells [2]. The transcription and translation of the RNP in the cells will result in the production of viral progenies, which can then be propagated in embryonated eggs.

The success of the rNDV rescue depends on the efficiency of the transfections of four plasmids (one for full-length cDNA and three for RNP produced collectively by individual plasmids encoding the *NP*, *P* and *L* genes) into a cell. Several attempts have been carried out to either generate cells that constitutively express the RNP or to reduce the number of plasmids to be transfected [3,4]. Both efforts are complicated and time-consuming.

Lipofectamine, a cationic lipid, is commonly used for the transfection of the plasmids into cells. Other transfection reagents, such as cationic polymers (polyethylenimine [PEI]) and inorganic compounds, have also been used for this purpose. However, to the best of our knowledge, no transfection reagents other than Lipofectamine have been used to rescue rNDV. Therefore, it is unclear whether this has not been done or because it was not successful, failed to be reported. Nevertheless, PEI has been used to transfect plasmids for the rescue of influenza virus, a segmented negative-sense RNA virus [5]. Here, our aim was to compare the transfection efficiency of Lipofectamine™ 3000 (Invitrogen, MA, USA) and PEI with optimized transfection protocol for the rescue of rNDV.

MATERIALS & METHODS

Plasmids

pCITE(GFP), a plasmid that is driven by the T7 promoter, was used to monitor T7 polymerase activity in the cells. Plasmid pOLTV5(rAF-GFP), which encodes for the complete antigenome of NDV strain AF2240-I [6], with a green fluorescent protein (GFP), was used to rescue the recombinant NDV (rNDV). Plasmids pCI-neo(NP), pCI-neo(P) and pCI-neo(L), respectively, harboring the *NP*, *P* and *L* genes serve as helper plasmids for the rescue of the virus.

Cell culture

A Baby Hamster Kidney (BHK)-derived mammalian cell line stably expressing T7 RNA polymerase, known as BSR T7/5 [7], was used in this study. The cell was maintained in Glasgow's Minimum Essential Medium (Sigma-Aldrich, NY, USA) supplemented with 10% newborn calf serum (Himedia, India), 1% tryptose phosphate and 1% Minimum Essential Medium (MEM) nonessential amino acids. Colorectal cancer cell lines, HT29 cells were maintained in Dulbecco's Modified Eagle Medium (Sigma-Aldrich) supplemented with 10% fetal bovine serum (Sigma-Aldrich). All cell lines were cultured at 37°C in a humidified atmosphere with 5% CO₂.

Optimization of PEI

Prior to transfection, the cytotoxicity of 25-kDa linear PEI (PolySciences, PA, USA) was determined by MTT assay [8]. BSR T7/5 cells (1×10^5) were seeded in 24-well plates and subjected to PEI treatment at various concentrations. The cell viabilities were measured after 48 h of PEI treatment. The highest PEI concentration, with at least 80% relative cell viability, was chosen for subsequent transfections. The PEI/DNA ratio (w/w) optimization was performed by adding various PEI/DNA ratio complex into the cells and fluorescence was determined by flow cytometry 48 h post transfection.

Figure 1. Optimization of BSR T7/5 cells for two parameters, cytotoxicity of polyethylenimine and polyethylenimine/DNA ratio. (A) Relative cell viability of BSR T7/5 after treatment with various PEI concentrations (μg/ml). The cells were treated with PEI at 24 h post seeding and the relative cell viability was determined after 48 h of treatment. Cell viability of BSR T7/5 decreased gradually when treated with increasing PEI concentration. The maximum PEI concentration that showed 80% cell viability was 6 μg/ml. (B) Percentage of BSR T7/5 cells emitting fluorescence when transfected with pCITE(eGFP) in either forward or reverse and different PEI/DNA ratios were implemented. The plasmid was transfected at 24 h post seeding and different PEI mass were added to keep the PEI/DNA ratio at 1:0.3, 1:0.8, 1:1.5, 1:3 and 1:5. The highest fluorescent percentage was observed for both forward and reverse transfection by using 1:1.5 (PEI/DNA) ratio. PEI: Polyethylenimine.

Moreover, either forward or reverse transfection was tested to determine the best transfection procedure. Forward transfection was performed by adding the PEI/DNA complex after 24 h post seeding, whereas reverse transfection was performed by adding PEI/DNA complex immediately after the cells were seeded.

Evaluation of transfection efficiency

Cells were trypsinized 48 h post transfection. Detached cells were centrifuged at 4000×g for 5 min at 4°C and the resulting cell pellet was resuspended in MACS buffer (1× PBS with 5% fetal bovine serum and 2 mM EDTA). Cells were then analyzed for fluorescence expression using flow cytometry (Novoexpress, ACEA Biosciences, CA, USA).

Rescue of recombinant NDV

Two transfection reagents (TRs), PEI and Lipofectamine 3000, were studied to compare the rescue efficiency of rNDV from cDNA. In the rescue system using Lipofectamine 3000, the plasmid cocktail (full-length NDV antigenome and the three helper plasmids) was mixed with the TR to form TR/DNA complex according to the instructions of the manufacturer. In the rescue system using PEI, the previously mentioned optimized protocol and TR: DNA ratio (w/w) was used. The mixture was incubated for 15 min at room temperature and then it was added to the BSR T7/5 cells. The culture medium containing the virus was harvested 4 days post transfection.

Quantification of viral titer

HT29 cells were seeded (1×10^5 cells/well) and incubated for 24 h, followed by infection of the diluted virus. The plate was incubated at 37°C for 1 h, with 15-min interval rocking. Then, the infectious media was discarded and complete media was added to the cells. Cells were harvested and paraformaldehyde-fixed to determine the percentage of fluorescent cells at 12 h post infection. Based on the fluorescence percentile, the virus titer was calculated as infectious unit/ml (IFU/ml) as shown in the following equation:

$$\text{Titer (IFU/ml)} =$$

$$\frac{\text{Number of cells plated} \times (\% \text{ of GFP positive cells}) \times \text{Dilution factor}}{\text{Volume of infectious media (ml)}}$$

Figure 2. Viral rescue using different types of transfections and transfection reagents in a 24-well plate. (A) Forward transfection, Lipofectamine™ 3000. (B) Reverse transfection, Lipofectamine 3000. (C) Forward transfection, PEI. (D) Reverse transfection, PEI. The fluorescence emitted by the virus were observed using a fluorescence microscope and with 100× magnification. Rescue of recombinant Newcastle disease virus was repeated in three independent biological replicates (each with three independent technical replicates). PEI: Polyethylenimine.

RESULTS & DISCUSSION

Several transfection parameters were optimized to obtain the optimum condition for co-transfection. Cytotoxicity of PEI is a prime concern for PEI-mediated gene transfer [9,10]. It was found that PEI at 6 μg/ml (total volume 0.5 ml in a 24-well plate) resulted in 81% relative cell viability (Figure 1A). In addition, we also investigated the effect of PEI/DNA ratios (w/w) and types of transfection on the transfection efficiency. Prior to the addition to cells, PEI (3 μg) was mixed with different masses of pCITE(eGFP) (μg) in tubes at prefixed ratios. It was found that the highest percentage ►

of cells emitting fluorescence was observed at a PEI/DNA ratio of 1:1.5 in both forward and reverse transfection (Figure 1B). The percentage gradually reduced at a higher PEI/DNA ratio. It is interesting to note that the optimal ratio obtained (6 µg of PEI: 9 µg of DNA/ml) was different compared with that of other studies [11,12] using Chinese hamster ovary cells. Higher DNA concentration was used in this study because low DNA concentration led to few or undetectable fluorescing cells (data not shown). The percentage of cells emitting GFP in this study was also relatively low (<30%) compared with that in Chinese hamster ovary cells, which can be as high as 70–80% [11]. This suggests that different cell type plays an important role in this case.

Using the optimized parameters, we proceeded with the transfection of plasmid cocktail into the BSR T7/5 cells to produce the rNDV harboring the GFP gene. Transfection using Lipofectamine 3000 was included for a comparison. The total mass of DNA added for transfection was 4.5 µg in a 0.5-ml culture media. Lipofectamine 3000 transfection successfully rescued rNDV for both forward and reverse transfection, whereas PEI was successful only once (with forward transfection) in a 24-well plate (Figure 2).

The experiment was then upscaled from a 24-well plate to a 6-well plate, as commonly described [2]. Therefore, the previously mentioned optimization parameters were scaled up accordingly. The transfections using both transfection reagents were successful as shown in Figure 3, albeit with inconsistent success rates (Table 1). This could be owing to the high plasmid DNA (pDNA) mass (22.5 µg) used in the transfection, which could lead to cytotoxicity. To investigate this, we reduced the total pDNA mass to 1.8 µg, a mass that has been optimized for Lipofectamine 3000 in most laboratories for the rescue of rNDV [2,13]. The success rate of transfection was 100% in forward transfection and approximately 90% in reverse transfection (Figure 4 & Table 1). However, viral rescue was not successful at all when PEI was used.

The culture media was harvested to detect the presence of viral progenies. Rather than using the standard plaque

Figure 3. Successful viral rescue using different types of transfections and transfection reagents in a 6-well plate. (A) Forward transfection, Lipofectamine™ 3000. (B) Reverse transfection, Lipofectamine 3000. (C) Forward transfection, PEI. (D) Reverse transfection, PEI. Cell seeding density: 5.05×10^5 cells/well, PEI mass (15 µg in 2.5 ml of media) and the total plasmid DNA (pDNA) mass was 22.5 µg (at a ratio of 1:0.4:0.2:0.2 for pOLTV5(rAF-GFP), pCI-neo(NP), pCI-neo(P), and pCI-neo(L)). rNDV-emitting fluorescence was observed via fluorescence microscope at 100× magnification. Rescue of rNDV was repeated in three independent biological replicates (each with three independent technical replicates).
PEI: Polyethylenimine; rNDV: Recombinant Newcastle disease virus.

assay, we developed a relatively simple and straight-forward method for the quantification of rNDV, which harbors a fluorescent reporter protein transgene in its genome and expresses the reporter protein during viral

replication. The titer of virus rescued from both forward and reverse transfection using Lipofectamine 3000 was calculated and is shown in Figure 5. Although not statistically significant, there is a trend of higher virus

Figure 4. Viral rescue using different types of transfections and transfection reagents with reduced DNA mass of 1.8 µg in a 6-well plate. (A) Forward transfection, Lipofectamine™ 3000. (B) Reverse transfection, Lipofectamine 3000. (C) Forward transfection, PEI. (D) Reverse transfection, PEI. All wells were observed at 100× magnification under a fluorescence microscope. Rescue of recombinant Newcastle disease virus was repeated in three independent biological replicates (each with three independent technical replicates).
PEI: Polyethylenimine.

Figure 5. Virus quantification was performed by infecting HT29 cells with culture media harvested from the well. $n = 9$ for forward transfection, whereas $n = 8$ for reverse transfection. The number of samples (n) was based on the attempts of previous successful rescues using Lipofectamine™ 3000 and $1.8 \mu\text{g}$ of total pDNA.

Table 1. Summary of successful viral rescue attempts from co-transfection of BSR T7/5 with either forward or reverse transfection and different total mass of pDNA.

Transfection	Forward	Reverse
22.5 μg of total pDNA		
Lipofectamine™ 3000	4/9, 44.4%	3/9, 33.3%
PEI	2/9, 22.2%	1/9, 11.1%
1.8 μg of total pDNA		
Lipofectamine 3000	9/9, 100%	8/9, 88.9%
PEI	0/9, 0%	0/9, 0%

pDNA: Plasmid DNA; PEI: Polyethylenimine.

titer using reverse transfection compared with forward transfection.

In conclusion, we have shown that the virus could be rescued in both transfection methods; however, reverse transfection prevailed the standard forward transfection with a twofold increase in virus titer, albeit insignificant statistically. Despite low success rates in transfection, PEI could be used to rescue NDV via reverse genetics. We have also managed to establish a more rapid and accurate method to quantitate infectious NDV titer compared with standard plaque assays and hemagglutination assays.

FUTURE PERSPECTIVE

To our knowledge, there are no reports describing the rescue of NDV using PEI as a transfection reagent for plasmids. The success of rescuing NDV in our system showed that PEI could be used as

an alternative to Lipofectamine. PEI reduces the cost of production for rescuing NDV in large scale. In addition, the newly established virus titration using flow cytometry provides rapid yet accurate quantification of the infectious GFP-expressing virus.

AUTHOR CONTRIBUTIONS

P-S Cheow conducted the experiments and prepared the manuscript. TK Tan, AA-L Song, K Yusoff and SL Chia designed the study. All authors reviewed and revised the manuscript.

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