Title: Generation of a recombinant Newcastle disease virus expressing two foreign genes for use as a multivalent vaccine and gene therapy vector

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ABSTRACT

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Generation of a recombinant Newcastle disease virus expressing two foreign genes for use as a multivalent vaccine and gene therapy vector

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Newcastle disease virus (NDV) is an enveloped, non-segmented, negative-stranded RNA virus and has been classified as avian paramyxovirus serotype 1 (APMV-1) in the genus Avulavirus of the family Paramyxoviridae [1]. NDV is an avian pathogen that can cause local infections in humans, however, there is no current data to suggest that NDV can be transmitted human-to-human [2, 3]. The NDV genome consists of approximately 15.2 kb and contains six transcriptional units, encoding the nucleocapsid protein (NP), phosphoprotein (P), matrix protein (M), fusion protein (F), hemagglutinin-neuraminidase protein (HN), and large polymerase protein (L), in that order [4]. The genomic RNA, together with the NP, P, and L proteins, form the ribonucleoprotein complex (RNP), which serves as the active template for transcription and replication of the viral genome [5, 6].

Since reverse genetics technology was first used to rescue infectious NDV from recombinant cDNA in 1999 [7, 8], many NDV clones have been developed and used as vectors to express foreign genes for vaccine or gene therapy purposes [9-15]. Most of these NDV vectors express only a single foreign gene from an additional independent transcription unit (ITU) that is inserted between other native viral transcription units in the NDV genome [15]. Recently we
developed a novel approach to express a foreign gene from within native viral transcription units using an internal ribosomal entry site (IRES). In this case the red fluorescence protein (RFP) gene was expressed from a second open reading frame (ORF) located in all six native transcriptional units, separately, using an IRES [16]. The addition of the second ORF did not significantly affect viral replication, and the level of foreign gene expression could be regulated by inserting the second ORF into different native viral transcription units.

In this study, we combined the ITU and IRES approaches to develop a multi-foreign gene expression vector. The NDV LaSota (LS) strain-based infectious clone, pLS/aMPV-C G [17], was used as a backbone to construct a recombinant cDNA clone containing both RFP and GFP genes (Figure 1). First, the ORF of the aMPV-C G gene in the pLS/aMPV-C G clone was replaced with the GFP ORF, amplified from the pAAV-hrGFP plasmid (Agilent Technologies, Santa Clara, CA), using an In-Fusion® PCR cloning kit (Clontech, Mountain View, CA), which resulted in the pLS/GFP subclone. Second, the RFP gene, amplified from the pCMV-Ds-Red-Express plasmid (Clontech), was cloned downstream of the IRES sequence in pIRES-hrGFP-2a vector (Clontech). Finally, the IRES and the RFP ORF sequences were amplified and cloned downstream of the NDV F ORF in the pLS/GFP vector as a 2nd ORF in the NDV F transcription unit using the In-Fusion® PCR Cloning Kit (Clontech). The resulting recombinant clone, designated as pLS/IRES-RFP/GFP, was amplified in Stbl2 cells at 30°C for 24 hours and purified using a QIAnprep Spin Miniprep kit (Qiagen, Valencia, CA).

After co-transfection of the pLS/IRES-RFP/GFP clone and the supporting plasmids, encoding for the NDV NP, P, and L proteins, into HEp-2 cells, the rescued the LaSota strain-based recombinant virus vectoring the RFP and GFP genes, designated as rLS/IRES-RFP/GFP, was subsequently amplified in SPF chicken embryonated eggs as described previously [18]. The rLS/IRES-RFP/GFP virus was purified and further propagated in SPF chicken embryonated eggs. The fidelity of the rescued rLS/IRES-RFP/GFP virus was confirmed by sequencing the isolated viral genome (data not shown).

To determine if the addition of the two foreign genes, RFP and GFP, affects the viral replication and pathogenicity of the rLS/IRES-RFP/GFP virus, the hemagglutination (HA), 50%
tissue culture infectious dose (TCID\textsubscript{50}), 50\% egg infectious dose (EID\textsubscript{50}), mean death time (MDT), and intracerebral pathogenicity index (ICPI) were examined \[19\]. As shown in Table 1, the recombinant virus appears to be slightly attenuated with a lower ICPI (0.0) in day-old chickens, longer MDT (>150hs) in embryos, and greater than a half-log reduction in viral yield in DF-1 cells compared to the parental LaSota strain. The viral yields of the recombinant virus grown in embryonated eggs, measured by EID\textsubscript{50} and HA, were comparable to the yields of the parental LaSota strain (Table 1). Overall, the replication rate of rLS/IRES-RFP/GFP appears to be similar when compared to the replication rate of LaSota in DF-1 cells (Fig. 2).

The co-expression of the RFP and GFP proteins from rLS/ IRES-RFP/GFP infected DF-1 cells at 24 h post-infection was examined by fluorescence microscopy at 100 x magnification (Nikon, Eclipse Ti, Melville, NY) \[17\]. As shown in Fig. 3, both GFP (Fig. 3b) and RFP (Fig. 3c) expression was observed. After merging both fluorescent images (Fig.3d), GFP and RFP co-localized to the same infected cells as seen by NDV induced viral cytopathic effects (CPE) observed under bright field (Fig. 3a). It is notable that there are a few infected cells that express GFP but not RFP that most likely can be attributed to the different promoters that drive the expression of the reporter genes. The GFP gene is inserted into the NDV genome as its own ITU and therefore should be expressed readily. However, the RFP gene is inserted as a second ORF downstream of an IRES inside a native transcription unit. The expression of a foreign gene from an IRES appears to be slightly inconsistent as several cells expressing GFP but not RFP can be seen.

Several NDV based recombinant viruses expressing a foreign gene have been developed and evaluated as vaccine candidates in clinical trials resulting in varying levels of the host’s immune response and protection against the targeted pathogen \[17, 20-31\]. Although the host’s response to vaccination and, ultimately, a protective immunity is influenced by many factors, antigenicity and expression efficiency of the foreign antigen are undoubtedly the most important. In most reported NDV vaccine candidates, the recombinant vectors only express one foreign gene through an independent transcription unit. The insufficient immune response and lack of protection against a targeted pathogen conferred by these vectored vaccine candidates suggest that a single antigen from the pathogen or its expression level from the vector may not be
adequate. Alternatively, two or more antigenic components from a pathogen may be required to achieve an improved or complete protection against challenge of said pathogen [32-34]. In this study, we have shown that it is possible to express two foreign genes simultaneously from a single vector that could be used to deliver the necessary antigenic components from one pathogen to induce a stronger immune response and improve the protective efficacy.

Studies that express two foreign genes from a single NDV vector to be used as experimental vaccine candidates have been developed resulting in observed improvements in protective efficacy against pathogenic challenge [33-35]. However, these foreign antigens were expressed through two additional ITUs in the NDV genome. Previous studies have demonstrated that the addition of an ITU in the viral genome downregulates viral gene transcription downstream of the inserted ITU that may subsequently attenuate viral replication and pathogenicity and, ultimately, decrease foreign gene expression [36-40]. Whereas, the NDV vector developed in this study expresses one foreign gene through an additional ITU and the other through an IRES as a second ORF within a native transcription unit, which usually does not notably affect viral replication efficiency [16]. More importantly, expression of the second foreign gene, downstream of an IRES, could be regulated by selecting an insertion site in the NDV genome relative to the desired expression level to meet the needs of multivalent vaccines and anticancer therapy [16].

Multivalent vaccination is the preferred practice in the poultry industry to protect the birds from common infectious diseases with little handling as possible. One of the advantages in administering multivalent vaccines is the reduction in vaccination costs, especially for large producers. However, a general concern is that the combination of live-recombinant vaccines with live-attenuated viruses may interfere with the other’s replication in the host and compromise protective efficacy [41, 42]. The NDV vector developed in this study containing two foreign genes could express viral antigens from two different pathogens and be used as a trivalent vaccine against NDV and the other two targeted diseases. In this case, this trivalent vaccine is one isolated replicating virus population with regulated foreign gene expression that should not interfere with itself nor induction of the host’s immune response [43].

Several NDV based recombinant vectors expressing cytokines or anticancer factors have been evaluated as anticancer agents in tumor models [44-49]. Again, most of these NDV recombinant vectors express only a single cytokine or anticancer factor and varying levels of
therapeutic effects were achieved in clinical trials. It would be interesting if a combination of two or more cytokines or anticancer factors expressed from a single NDV vector, through the ITU and IRES approaches, improved the efficacy of potential anticancer therapies.

In summary, we developed a novel NDV vector co-expressing the GFP and RFP reporters through a combination of the ITU and IRES approaches in this study. The recombinant NDV vectoring the two foreign genes was slightly attenuated when compared to its parental virus. There was no discernible interference with viral replication rates or expression of the two foreign genes. Our results suggest that the NDV LaSota strain-based two-foreign gene expression vector is reliable and may be used in the development of multivalent vaccines against veterinary viral diseases, and may also have potential applications in human and veterinary gene therapy or anticancer treatments.

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Conflict of interest: The authors declare that they have no conflicts of interest.

REFERENCE


Table 1. Biological assessments of the rLS/IRES-RFP/GFP and parental LaSota viruses

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<th>Virus</th>
<th>MDT&lt;sup&gt;a&lt;/sup&gt;</th>
<th>ICPI&lt;sup&gt;b&lt;/sup&gt;</th>
<th>HA&lt;sup&gt;c&lt;/sup&gt;</th>
<th>EID&lt;sub&gt;50&lt;/sub&gt;&lt;sup&gt;d&lt;/sup&gt;</th>
<th>TCID&lt;sub&gt;50&lt;/sub&gt;&lt;sup&gt;e&lt;/sup&gt;</th>
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<tr>
<td>LaSota</td>
<td>110hs</td>
<td>0.15</td>
<td>1024</td>
<td>2.37×10&lt;sup&gt;9&lt;/sup&gt;</td>
<td>9.88×10&lt;sup&gt;8&lt;/sup&gt;</td>
</tr>
<tr>
<td>rLS/IRES-RFP/GFP</td>
<td>&gt;150hs</td>
<td>0</td>
<td>512</td>
<td>2.37×10&lt;sup&gt;9&lt;/sup&gt;</td>
<td>1.76×10&lt;sup&gt;8&lt;/sup&gt;</td>
</tr>
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<sup>a</sup> MDT: Mean death time assay in embryonated chicken eggs.
<sup>b</sup> ICPI: Intracerebral pathogenicity index assay in day-old chickens.
<sup>c</sup> HA: Hemagglutination assay.
<sup>d</sup> EID<sub>50</sub>: 50% egg infective dose assay in embryonated chicken eggs.
<sup>e</sup> TCID<sub>50</sub>: 50% tissue infectious dose assay in DF-1 cells.
Fig. 1. Schematic representation of pLS/IRES-RFP/GFP construction. The ORF of the aMPV-C G gene in the pLS/aMPV-C G clone was replaced with the GFP ORF, amplified from the pAAV-hrGFP plasmid (Agilent Technologies, Santa Clara, CA), using an In-Fusion® PCR cloning kit (Clontech, Mountain View, CA), resulting in a subclone pLS/GFP. The RFP ORF, amplified from the plasmid pCMV-DsRed-Express (Clontech), was cloned downstream of the IRES sequence in the pIRES-hrGFP-2a vector (Clontech). Subsequently, the IRES and the RFP ORF sequences were amplified and cloned downstream of the NDV F ORF in the pLS/GFP vector using an In-Fusion® PCR Cloning Kit (Clontech). The NDV Gene End and Gene Start signal sequences and the RFP and GFP sequences are underlined. The direction of the T7 promoter is indicated by a bold black arrow. HDVRz and T7φ represent the site of the Hepatitis delta virus ribozyme and the T7 terminator sequences, respectively.
Fig. 2. Growth curve analysis comparing the viral replication of the rLS/IRES-RFP/GFP and parental LaSota viruses. DF-1 cells were infected with each virus separately at an MOI of 0.01. Every 12 h post-infection, virus lysates were harvested and subsequently, viral titers were determined by TCID₅₀ titration for each time point in DF-1 cells in triplicate. The mean titer of each time point of the two viruses is expressed in log₁₀ TCID₅₀/ml.
**Fig. 3.** Detection of RFP and GFP protein expression of the rLS/IRES-RFP/GFP virus by fluorescence microscopy. DF-1 cells were infected with rLS/IRES-RFP-GFP at an MOI of 0.01. At 24 h post-infection, infected cells were examined and viral CPE and corresponding fluorescence from the same field were digitally photographed at 100X magnifications with bright field (a) and GFP (b) and RFP (c) specific filter combinations (Nikon, Eclipse Ti, Melville, NY). The green and red fluorescent images (b and c) were merged (d).