Paramyxovirus-based production of Rift Valley fever virus replicon particles

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Replicon-particle-based vaccines combine the efficacy of live-attenuated vaccines with the safety of inactivated or subunit vaccines. Recently, we developed Rift Valley fever virus (RVFV) replicon particles, also known as nonspreading RVFV (NSR), and demonstrated that a single vaccination with these particles can confer sterile immunity in target animals. NSR particles can be produced by transfection of replicon cells, which stably maintain replicating RVFV S and L genome segments, with an expression plasmid encoding the RVFV glycoproteins, Gn and Gc, normally encoded by the M-genome segment. Here, we explored the possibility to produce NSR with the use of a helper virus. We show that replicon cells infected with a Newcastle disease virus expressing Gn and Gc (NDV-GnGc) were able to produce high levels of NSR particles. In addition, using reverse genetics and site-directed mutagenesis, we were able to create an NDV-GnGc variant that lacks the NDV fusion protein and contains two amino acid substitutions in, respectively, Gn and HN. The resulting virus uses a unique entry pathway that facilitates the efficient production of NSR in a one-component system. The novel system provides a promising alternative for transfection-based NSR production.

INTRODUCTION

Rift Valley fever virus (RVFV) causes recurrent outbreaks among ruminants and humans across the African continent, several islands off the coast of southern Africa and the Arabian Peninsula. The virus is mainly transmitted by Aedine and Culicine mosquito vectors. The tri-partite viral genome consists of a small (S), medium (M) and large (L) RNA genome segment of negative polarity (Elliott, 1990, 1997). The S segment encodes a nucleoprotein (N) and a non-structural protein named NSs, which is the main virulence factor by acting as an antagonist of host innate immune responses (Billecocq et al., 2004; Bouloy et al., 2001; Ikekami et al., 2009a, b; Muller et al., 1995). The M segment encodes a glycoprotein precursor (GPC) that is co-translationally cleaved into Gn and Gc (Kakach et al., 1988), which are the two major structural glycoproteins involved in virus–cell attachment and membrane fusion. The M segment additionally encodes a 78 kDa protein of unknown function that is incorporated into virions in the mosquito vector (Weingartl et al., 2014) and a non-structural protein (NSm) with anti-apoptotic properties (Terasaki et al., 2013; Won et al., 2007). The L segment encodes an RNA-dependent RNA polymerase responsible for transcription and replication.

Recently, we and others developed the first generation RVFV replicon particles, also known as nonspreading RVFV (NSR) (Dodd et al., 2012; Kortekaas et al., 2011). These particles are able to infect cells, followed by genome replication and protein expression but, due to the absence of the genes encoding the structural glycoproteins Gn and Gc, are incapable of forming progeny particles. Their inherent safety greatly facilitates studies on RVFV entry and fusion (de Boer et al., 2012a, b). Remarkably, NSR induces a protective immune response in mice and sheep after a single immunization (Kortekaas et al., 2011, 2012; Oreshkova et al., 2013). Very recently, we also showed that NSR is a potent vaccine vector. A single intranasal or intramuscular immunization of mice with NSR expressing the haemagglutinin protein of influenza, from the NSs location of the S-segment, provided full protection against a lethal dose of influenza virus (Oreshkova et al., 2014).

NSR is produced by transfection of baby hamster kidney (BHK) cells, that stably maintain replicating L and S
genome segments (replicon cells), with an expression plasmid encoding RVFV Gn and Gc (Kortekaas et al., 2011). Although the transfection-based production of NSR particles is relatively efficient, our laboratory is exploring alternative systems for the cost-effective industrial-scale production of NSR vaccines without the need for transfection or replicon cell lines. In this study, we evaluated whether a paramyxovirus helper virus expressing the RVFV glycoproteins could be used to produce NSR. The results show that a previously developed Newcastle disease virus (NDV) expressing Gn and Gc (NDV-GnGc) was able to efficiently package RVFV L and S genome segments into progeny NSR. Moreover, an NDV-GnGc mutant virus is described that employs a unique entry mechanism, thereby enabling NSR production in a one-component system.

RESULTS

Production of NSR with NDV-GnGc

To evaluate whether RVFV L and S genome segments can be packaged by NDV expressing Gn and Gc of RVFV, we infected BHK-Rep2 cells (Kortekaas et al., 2011) with NDV-GnGc (Kortekaas et al., 2010). BHK-Rep2 cells are BHK-21 cells that constitutively maintain replicating L and S genome segments of RVFV strain 35/74 (Kortekaas et al., 2011). One day post-infection, supernatants were evaluated for the presence of progeny NSR. As a positive control, BHK-Rep2 cells were transfected with pCAGGS-M, which is known to facilitate NSR production (Kortekaas et al., 2011). Supernatants of both infected and transfected wells contained up to 10^7 TCID₅₀ ml⁻¹ of NSR progeny (Fig. 1). Uninfected and non-transfected negative control cells did not produce any NSR progeny. These results show that BHK cells maintaining replicating RVFV L and S genome segments can efficiently be infected by NDV-GnGc and subsequently produce progeny NSR.

Use of NDV-GnGc-ΔF replicons to produce NSR

Although NDV-GnGc is a potent helper virus for the production of NSR, the production of NDV-GnGc, due to the absence of a polybasic cleavage site in the F protein, depends on embryonated chicken eggs (Dortmans et al., 2011; Kortekaas et al., 2010). To circumvent the use of embryonated chicken eggs for the production of NDV-GnGc and to preserve optimal safety, we constructed an NDV-GnGc replicon virus that can efficiently grow in cell culture. Using NDV reverse genetics (Peeters et al., 1999; Römer-Oberdörfer et al., 1999), we deleted the entire F gene from NDV-GnGc thereby generating NDV-GnGc-ΔF (Fig. 2a, b, d). In addition, a QM5 cell line expressing the F protein of NDV strain Herts/33 (de Leeuw et al., 2005) (QM5-F₇₅₃) was constructed (Fig. 2c). The F₇₅₃ fusion protein contains a polybasic cleavage site facilitating infection in various cell types. As expected, NDV-GnGc-ΔF was unable to grow on QM5 cells but able to grow on QM5-F₇₅₃ cells, resulting in the formation of NDV-specific syncytia (Fig. 2e). Titres of NDV-GnGc-ΔF reached up to 10^7 TCID₅₀ ml⁻¹. Infection of BHK-Rep2 cells, at different m.o.i., with NDV-GnGc or NDV-GnGc-ΔF showed that NDV-GnGc-ΔF is equally efficient in the production of progeny NSR as NDV-GnGc (Fig. 2f).

Stability of NDV-mediated NSR production

To investigate whether NDV-assisted NSR production can be stable over several cell passages, we passaged BHK-Rep2 cells infected with NDV-GnGc-ΔF several times and monitored virus titres in the culture media. In two independent experiments, a rapid decrease in NSR titres

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Fig. 1. Transfection- and infection-mediated NSR production. BHK-Rep2 cells stably maintaining RVFV L and S genome segments were transfected with pCAGGS-M or infected with NDV-GnGc at an m.o.i. of 0.5. One day post-transfection or -infection, supernatants were collected and titrated on BHK-21 cells to determine the level of NSR progeny. TCID₅₀ values were determined 2 days post-infection by evaluation of eGFP expression. Bars represent means and standard errors of three experiments.
in the supernatants of sequentially passaged cells was observed (data not shown), whereas in one experiment, NSR titres detected in the supernatant of passaged infected cells remained relatively stable over three cell passages (Fig. 3). Remarkably, starting from cell passage 2, mild cytopathic effects (CPE) were noted. CPE increased in time, and after cell passage 3 all cells died. This suggested that NDV-GnGc-DF had gained the ability to spread to a certain extent. To evaluate whether the supernatant of cell passage 3 contained infectious NDV-GnGc-ΔF besides infectious NSR, we inoculated fresh BHK-21 cells with the collected supernatant. The induction of CPE and the presence of NDV antigens in these cells confirmed the presence of small amounts of infectious NDV-GnGc-ΔF. Interestingly, evaluation of supernatants of passages 4–7 revealed a remarkably stable ratio of NSR and infectious NDV-GnGc-ΔF particles of about 1000 : 1. Probably, limited amounts of infectious NDV-GnGc-ΔF are sufficient for high-level NSR production. From here on the combination of high-level infectious NSR particles and low-level infectious NDV-GnGc-ΔF particles is referred to as NSRNDV (Fig. 3).

**Next-generation sequence analysis of NSRNDV**

To evaluate possible nucleotide changes in the genomes of NDV-GnGc-ΔF and NSR in NSRNDV resulting from a putative adaptation to the BHK cells, we sequenced the RNA isolated from NSRNDV passage 7 (Fig. 3) using Illumina-based next-generation sequencing. The analysis,
with a minimal coverage of 200, revealed that none of the genome segments of either NDV-GnGc-ΔF or NSR contained insertions or deletions. Moreover, there was no evidence of recombination events. Nevertheless, as shown in Table 1, several single nucleotide polymorphisms (SNPs) were detected in both the NSR and NDV-GnGc-ΔF genomes. NSR contained six SNPs and NDV-GnGc-ΔF contained four SNPs present in ≥50% of sequence reads. The non-synonymous SNPs in NSR did not give clues about the mechanism responsible for the changed phenotype. In contrast, the non-synonymous SNPs detected in NDV-GnGc-ΔF in the Gn (Y303→S) and HN (I192→M) proteins could have effects on virus entry, fusion and subsequent spread.

Y303→S in Gn and I192→M in HN are responsible for in vitro spreading of NDV-GnGc-ΔF

To investigate a possible role of the amino acid substitutions in HN and Gn in the NDV-GnGc-ΔF genome in the in vitro spreading phenotype of NSR<sub>NDV</sub>, we decided to construct single and double site-directed NDV-GnGc-ΔF mutants (Fig. 4a, d). All NDV-GnGc-ΔF site-directed mutants were viable and able to grow efficiently in QM5-FHerts cells up to titres of 10<sup>7</sup> TCID<sub>50</sub> ml<sup>−1</sup> (Fig. 4b). Remarkably, NDV-GnGc-ΔF-HN* and NDV-Gn*Gc-ΔF-HN* containing the Y303→S substitution in QM5-FHerts cells (Fig. 4c).

Infection of BSR cells with the site-directed mutants, grown on QM5-FHerts cells, showed a significant variation in plaque formation (Fig. 4c). NDV-GnGc-ΔF predominantly infected single cells, whereas NDV-Gn*Gc-ΔF and NDV-Gn*Gc-ΔF-HN* containing the Y303→S substitution in

### Table 1. Genetic changes in NSR<sub>NDV</sub> detected by next-generation sequencing

<table>
<thead>
<tr>
<th>Segment</th>
<th>SNP</th>
<th>Frequency (%)</th>
<th>Region</th>
<th>aa change</th>
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<tbody>
<tr>
<td>RVFV-SeGFP</td>
<td>T10C</td>
<td>50</td>
<td>3’UTR</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>C11A</td>
<td>100</td>
<td>3’UTR</td>
<td>–</td>
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<td>A1604T</td>
<td>100</td>
<td>5’UTR</td>
<td>–</td>
</tr>
<tr>
<td>RVFV-L</td>
<td>G1181A</td>
<td>100</td>
<td>Pol gene</td>
<td>G388E</td>
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<tr>
<td></td>
<td>A1602C</td>
<td>100</td>
<td>Pol gene</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>T6325C</td>
<td>100</td>
<td>5’UTR</td>
<td>–</td>
</tr>
<tr>
<td>NDV-GnGc-ΔF</td>
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<td>100</td>
<td>Gn gene</td>
<td>Y303S*</td>
</tr>
<tr>
<td></td>
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<td>HN gene</td>
<td>I192M</td>
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<tr>
<td></td>
<td>T14476C</td>
<td>90</td>
<td>L gene</td>
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Passage 7 culture supernatant is used as RNA template (Fig. 2). SNPs present in ≥50% of sequence reads are shown. UTR, untranslated region, Pol, polymerase. *Counted from the fourth methionine of the M-segment.
Gn were able to spread to neighbouring cells. Interestingly, BSR cells infected with the double site-directed mutant NDV-Gn*Gc-ΔF-HN* showed large virus plaques. Passage of supernatants to fresh cells revealed that NDV-Gn*Gc-ΔF and NDV-Gn*Gc-ΔF-HN* are able to produce limited amounts of infectious progeny in BSR cells.

We subsequently investigated the feasibility of using the NDV-Gn*Gc-ΔF and NDV-Gn*Gc-ΔF-HN* viruses to produce NSR. BHK-Rep2 cells were infected with the different site-directed mutants (grown on QM5-FHerts cells) and the levels of NSR progeny in the supernatants were determined. The results show that none of the mutations

![Fig. 4. Construction and evaluation of NDV-GnGc-ΔF site-directed mutant-mediated NSR production. (a) Schematic presentation of the genomes of NDV-GnGc-ΔF, NDV-Gn*Gc-ΔF, NDV-GnGc-ΔF-HN* and NDV-Gn*Gc-ΔF-HN*. (b) Growth curve of the site-directed mutants in QM5-FHerts cells. (c) DAPI staining of QM5 and QM5-FHerts cells and IPMA of BSR cells infected with the site-directed mutants. The mutants grown on BSR cells were also evaluated for the presence of infectious progeny by testing supernatants of infected BSR cells on fresh BSR cells. (d) Confirmation of F deletion in the NDV-GnGc-ΔF site-directed mutants genomic RNA isolated from virus grown on QM5-FHerts cells and determined by conventional RT-PCR using primers 5 and 6 (Table S1). (e) Site-directed mutant-mediated NSR production. BHK-Rep2 cells were infected with the site-directed mutants and 2 days post-infection the level of NSR progeny in the supernatant (P0, first bar). The supernatant of P0 was subsequently passaged to fresh BHK-21 cells. These cells were washed twice at 6 h post-infection, and at 4 days post-infection the level of NSR progeny was determined (P1, second bar). Bars represent means and standard errors of three experiments. (f) Stability of NDV-Gn*Gc-ΔF-HN*-mediated NSR production. BSR cells were infected with NDV-Gn*Gc-ΔF-HN* and each 2–3 days post-infection, supernatants were passaged to fresh cells. Levels of progeny NSR and NDV-Gn*Gc-ΔF-HN* were determined after each passage by titration using eGFP as a readout for NSR and CPE as a readout for NDV-Gn*Gc-ΔF-HN*. At the passages indicated with an asterisk (*) an additional cell passage was performed. Bars represent means and standard errors of three experiments.](image-url)
had a significant effect on the transient NDV-GnGc-ΔF-mediated production of NSR (Fig. 4e, first bars). However, passage of the supernatants of infected BHK-Rep2 cells to fresh wild-type BHK cells resulted in a rapid decrease of NSR production when using NDV-GnGc-ΔF, NDV-Gn*Gc-ΔF or NDV-GnGc-ΔF-HN* (Fig. 4e). By contrast, incubation of cells with passaged supernatant of NDV-Gn*Gc-ΔF-HN*-infected BHK-Rep2 cells again resulted in NSR progeny. Altogether, these results indicate that the autonomous production of NSRNDV, as observed previously (Fig. 3), is mediated by two amino acid substitutions in NDV-GnGc-ΔF in respectively HN and Gn.

**NSRNDV production in a one-component system**

To investigate whether NSRNDV could be produced repeatedly, we passaged supernatants of NDV-Gn*Gc-ΔF-HN*-based NSRNDV in BSR cells. The titres of NSR and NDV-Gn*Gc-ΔF-HN* were determined by monitoring eGFP expression and CPE, respectively. Of note, immunoperoxidase monolayer assays (IPMA) demonstrated that CPE correlated with Gn expression from the NDV genome. When no complete CPE was observed after initial infection, due to lower levels of infectious NDV-Gn*Gc-ΔF-HN*, cells instead of supernatants were passaged. Ratios between NSR and NDV in each passage of NSRNDV varied between 1 : 100 and 1 : 1000 (Fig. 4f). Altogether, these results indicate that NSRNDV production is relatively stable upon passage and can be produced in a one-component system (Fig. S1, available in the online Supplementary Material).

**Spread of NDV-Gn*Gc-ΔF-HN* is mediated by RVFV Gn and Gc**

To investigate whether RVFV Gn and Gc are involved in the cell entry of NDV-Gn*Gc-ΔF-HN*, we incubated the virus with serial dilutions of a RVFV neutralizing serum. As a control, we evaluated neutralization of NDV-GnGc and NDV-GnGc-ΔF. In these experiments, infection by NDV-GnGc or NDV-GnGc-ΔF was not neutralized, whereas infection by NDV-Gn*Gc-ΔF-HN* was completely prevented by serum diluted up to 540 times (Fig. 5). Altogether, these results strongly suggest that infectivity of NDV-Gn*Gc-ΔF-HN* depends on the RVFV glycoproteins.

**NSRNDV induces a protective immune response in mice**

To investigate whether NSRNDV is equally effective as an NSR vaccine produced by transfection, we performed a vaccination challenge experiment in mice. Mice were either vaccinated with NSR (10⁵.8 TCID₅₀), NSRNDV (containing 10⁵.8 TCID₅₀ NSR and 10⁵.8 TCID₅₀ NDV-GnGc-ΔF) or NDV-GnGc-ΔF (10⁵.8 TCID₅₀) and challenged 3 weeks post vaccination. As expected, the low amount of NDV-GnGc-ΔF alone was unable to provide protection and the challenge virus disseminated in high amounts to the brain and liver (Fig. 6). By contrast, NSR- and NSRNDV-vaccinated mice were completely protected from a lethal RVFV challenge dose without any virus disseminating to the organs. Protection could be correlated with high levels of neutralizing antibodies, which were detected before challenge infection (Fig. 6b). In conclusion, NSR produced by the NDV-Gn*Gc-ΔF-HN* helper virus is able to protect mice against a lethal challenge dose.

**DISCUSSION**

In previous work, we demonstrated that NSR is highly effective in preventing viraemia and clinical disease in the natural target species. Our laboratory is currently developing NSR production systems that are cost-effective and scalable. Here, we evaluated the feasibility to produce NSR using an NDV to provide the RVFV structural glycoproteins Gn and Gc.

NDV-GnGc is able to induce a neutralizing immune response in mice and sheep (Kortekaas et al., 2010, 2012), strongly suggesting that Gn and Gc, when expressed from the NDV genome, are correctly processed and assembled. The ability of NDV-GnGc to complement the RVFV glycoproteins in the BHK-Rep2 cells was therefore not totally unexpected. Overall, the success of the NDV-based complementation is explained by a combination of factors: (i) high level expression and correct processing of GnGc from the NDV genome, (ii) no or low-level of competition between NSR and NDV genome replication, and (iii) the ability of NDV and NSR to infect the same cell. Probably, the highly similar nature of the NSR and NDV genomes, both negative-sense, single-strand RNA, does not result in unbalanced use of host cell machinery. We expect that similar methods may be successful for production of other bunyavirus replicons, although we envisage that efficiencies will vary between certain combinations of helper and replicon virus.

NDV-GnGc is based on lentogenic NDV strain LaSota and requires trypsin-like proteases for cleavage and subsequent activation of the F protein. Growth of lentogenic NDV is therefore most efficient in the allantoic cavity of embryonated eggs, where trypsin-like proteases are abundant (Goldhaft, 1980; Kortekaas et al., 2010). To circumvent the need for embryonated eggs, mesogenic or velogenic NDV strains can be used that contain a polybasic cleavage site in the F protein and thereby do not require trypsin-like proteases for infectivity. However, these viruses are not avirulent and are therefore not suitable for vaccine applications. As a safe alternative, we constructed an NDV-GnGc-ΔF replicon which can be amplified in a QM5 cell line constitutively expressing a fusogenic F protein. The ability to construct a cell line stably expressing high levels of a fusogenic F protein (Fig. 2c) may be partly related to the absence of the HN protein. Indeed, several reports show that F mainly induces syncytia and cell death in the presence of HN (de Leeuw et al., 2005; McGinnes et al.,
**Fig. 5.** Neutralization of NDV-GnGc, NDV-GnGc-ΔF and NDV-Gn*Gc-ΔF-HN*. NDV-GnGc, NDV-GnGc-ΔF and NDV-Gn*Gc-ΔF-HN* were incubated with serial dilutions of normal sheep serum or a sheep serum containing anti-RVFV antibodies. After 1.5 h, the virus–serum mixture was added to BHK-21 cells. Two days later, cells were fixed and evaluated for Gn expression using IPMA. Pictures (a) were taken from wells containing the 1 : 40 serum dilutions and neutralization titres (b) are expressed as the serum dilution able to block over 50 % of infectious particles. The detection limit was a serum dilution of 1 : 20.

**Fig. 6.** Vaccination challenge experiment NSRNDV in mice. (a) Survival curve of mice vaccinated with NSR, NSRNDV or NDV-GnGc-ΔF (NDV). Three weeks post-vaccination, mice were challenged with a lethal RVFV challenge dose. (b) RVFV-specific virus neutralization titres present in sera the day before challenge. Bars represent mean titres and standard errors of 10 animals per group. (c, d) RVFV dissemination into the livers (c) and brains (d) of challenged mice determined by quantitative (q)RT-PCR.
After observing that a mixture of NSR and NDV-GnGc- members of the Paramyxoviridae, including the respiratory syncytial virus replicon, several replicons have been developed for other the first report that describes the construction of an NDV et al. complex that is capable of facilitating cell entry of NDV-GnGc- activity. Our results are in full agreement with these decreased haemagglutinating activity and increased fusion (2011), who showed that the I192A mutation was previously described and characterized by Estevez A (Yoshizaki et al., 2000). Although to our knowledge this is the first report that describes the construction of an NDV replicon, several replicons have been developed for other paramyxoviruses, including the respiratory syncytial virus (RSV) (Malykhina et al., 2011) and Sendai virus (Yoshizaki et al., 2006). Probably, deleting and subsequently trans-complementing glycoproteins can be generally applied for members of the Paramyxoviridae.

The SNP in Gn has not been reported previously. The SNP in both genomes. We decided to focus on the SNPs of NDV-GnGc- that were present in > 95 % of sequence reads and located in the glycoprotein genes, since these proteins are involved in receptor binding and fusion. The α-g transition in HN, resulting in amino acid change I192→M, was of particular interest, because this mutation was previously described and characterized by Estevez et al. (2011), who showed that the I192→M substitution decreased haemagglutinating activity and increased fusion activity. Our results are in full agreement with these observations and emphasize that paramyxovirus fusion activity is a dynamic process that can be influenced by subtle changes in the HN globular head (Porotto et al., 2012). We hypothesize that the HN mutation increases the binding capacity of the NDV virion to the cell surface due to prolonged receptor contact, although this remains to be confirmed experimentally.

The SNP in Gn has not been reported previously. The mutation does not significantly affect the infectivity of NSR. Titres of NSR produced by NDV-Gn*Gc-ΔF and NDV-Gn*Gc-ΔF-HN* were only slightly decreased compared to NSR titres produced by NDV-GnGc-ΔF. It is important to note that the Y303→S substitution in Gn is required for the F-independent infection of NDV-GnGc-ΔF and that infectivity of NDV-Gn*Gc-ΔF-HN* can be blocked efficiently by RVFV-specific neutralizing antibodies. By combining these two observations we hypothesize that Gn and Gc are incorporated in NDV-GnGc-ΔF virions as non-functional complexes that cannot mediate infectivity. However, our findings suggest that a single substitution in the Gn protein (Y303→S) results in a functional GnGc complex that is capable of facilitating cell entry of NDV-GnGc-ΔF virions in the absence of F complementation. Whereas NDV normally uses a class-I fusion protein (Chang & Dutch, 2012) to fuse at the plasma membrane, the ability of NDV-Gn*Gc-ΔF-HN* to autonomously replicate in tissue culture is apparently attributed to a switch from a class I to a class II pH-dependent fusion protein-mediated entry (Fig. 7). The mechanisms of entry of the NDV variants described in this study will be the subject of future research.

Wild-type RVFV induces strong CPE in various mammalian cell lines including the BHK and BSR cell lines used in this study. Remarkably, BHK-Rep2 cells, which stably maintain replicating RVFV L and S genome segments in the absence of a replicating M-segment, do not show signs of CPE. Most likely, RVFV-mediated cytotoxicity is caused by high-level expression of the M-segment-encoded glycoproteins Gn and Gc. In NSRNDV-infected cultures, CPE was observed as well, although this CPE was consistently less pronounced than CPE resulting from RVFV infection. Considering that CPE resulting from incubation of cells with NSRNDV is solely attributed to NDV-Gn*Gc-ΔF-HN*, CPE was used to determine titres of NDV-Gn-Gc-ΔF-HN* virions in NSRNDV preparations. Titres of NSR particles in NSRNDV preparations were determined by detection of eGFP, expressed from the S-segment of the NSR genome. Remarkably, multiple passages of NSRNDV revealed only minor variation in NSR progeny production, and ratios between NSR and NDV in NSRNDV preparations were relatively stable over time.

To efficiently propagate NSRNDV in vitro, relatively high m.o.i. infections are required. Low m.o.i. infections do not result in the production of progeny since this depends on co-infection of a cell with NSR and NDV. In vivo administration of NSRNDV will, due to the dilution effect, always result in low m.o.i. and consequently NSRNDV is unable to spread efficiently. This, together with the absence of the major virulence factor NSs, highlights the safety of the NSRNDV vaccine.

In conclusion, we show here that NDV-GnGc and especially the variant referred to as NDV-Gn*Gc-ΔF-HN* can be used for efficient production of NSR. This novel system could be further evaluated as a cost-effective alternative for transfection-based NSR production.

**METHODS**

**Ethics statement.** The animal experiment was conducted in accordance with the Dutch Law on Animal Experiments (Wod, ID number BWBR0003081) and approved by the Animal Ethics Committee of the Central Veterinary Institute (permit number: 2013050).

**Cells and growth conditions.** All cell lines were routinely maintained at 37 °C and 5 % CO₂. BHK-21, BHK-Rep2 cells and BSR cells were grown in Glasgow Minimum Essential Medium (GMEM; Invitrogen) supplemented with 4 % tryptose phosphate broth (TPB; Invitrogen), 1 % non-essential amino acids (NEAA; Invitrogen), 5 % FBS (Bodinco) and 1 % penicillin–streptomycin (Invitrogen). QM5 cells were routinely grown in QM35 medium (Invitrogen) supplemented with 5 % FBS and 1 % penicillin–streptomycin. QM5-L cells were grown in the same medium as QM5 cells, supplemented with 0.5 mg Geneticin ml⁻¹ (G-418; Promega).

**Viruses.** RVFV strain 35/74 (Barnard, 1979) was grown and titrated on BHK cells. NDV-GnGc (Kortekaas et al., 2010) was grown in embryonated chicken eggs and titrated on QM5 cells. Viral titres were determined by serial dilution and expressed as TCID₅₀ using the Spearman–Kärber algorithm. In the absence of CPE or eGFP expression, viral infection was visualized by IPMA (see below).
Fig. 7. Model showing replication and processing of wild-type NDV (LaSota), wild-type RVFV, NDV-GnGc-ΔF and NDV-Gn*Gc-ΔF-HN* virions. NDV enters cells via fusion with the plasma membrane and progeny is formed by budding at the plasma membrane. Due to the absence of trypsin-like proteases, the F protein is not cleaved and progeny is not infectious. RVFV enters cells via the endosomal route and buds from the trans-Golgi compartments. Resulting progeny is infectious. NDV-Gn*Gc-ΔF, previously grown on F-complementing cells, enters cells via fusion with the plasma membrane. Progeny virus is not infectious due to the absence of the F protein. Unknown amounts of GnGc heterodimers are expected to be present in the viral envelope. NDV-Gn*Gc-ΔF-HN* virions follow the RVFV-specific entry route, via endosomes, where infectious progeny is formed at the plasma membrane due to the presence of mutations in HN and Gn.
Construction of a QM5 cell line stably expressing the F protein of the NDV Herts strain. QM5 cells were transfected with pCINeo-PEerts (de Leeuw et al., 2005) using jetPEI transfection reagents (Polyplus-transfection) according to the manufacturer’s instructions. Two days post-transfection, G-418 selection reagent was added to the growth medium at a concentration of 0.5 mg ml⁻¹. One week post-selection, individual clones (>50) were subcultured and finally screened for PEerts expression by IPMA. The clone with the most stable and homogeneous F expression after numerous passages was used for further studies and is from here on referred to as QM5-PEerts.

NDV reverse-genetics. The rescue of NDV virus by reverse genetics was performed as described previously (Peeters et al., 1999; Romer-Oberdörfer et al., 1999) with some minor modifications. Briefly, 6 x 10⁶ QM5-PEerts cells (per well of 6-well plates) were infected with a Fowlpox virus expressing 17 polymerase (FP-T7) (Chaudhry et al., 2007) in Opti-MEM containing 0.2 % FBS (Invitrogen). Two hours post FP-T7 infection, medium was replaced (Opti-MEM, 0.2 % FBS) and cells were transfected with pCINeo-NP, pCINeo-P, pCINeo-L and a plasmid encoding the complete cDNA of NDV strain LaSota (pNDFL) using jetPEI transfection reagents according to the manufacturer’s instructions. Three hours post-transfection, medium was replaced with QT-35 medium containing 5 % FBS and three days post-transfection supernatants were filtered (0.2 µm) to remove FP-T7 and used to inoculate fresh QM5-PEerts cells.

Construction of pNDFL-GnGc-ΔF. To delete the F gene in the infectious clone of NDV-GnGc, a fusion PCR strategy was conducted. Briefly, two PCR fragments were generated from pNDFL-GnGc (Kortekaas et al., 2010) that flank the F gene and contain about 20 nt overlap using primer pairs 1/2 and 3/4 (Table S1). The two purified fragments were subsequently used as template in a fusion PCR based on primers 1 and 4. The resulting fragment was digested with HpaI and SpeI and ligated to pNDFL-GnGc digested with the same enzymes. Plasmids were confirmed to have the expected sequences using Sanger sequencing. The construct was designed to comply with the rule-of-six (Peeters et al., 2000).

IPMA. IPMAs were performed as described previously with some minor modifications (de Boer et al., 2010). Briefly, infected cell monolayers were fixed with 4 % (w/v) paraformaldehyde for 15 min, washed with PBS and permeabilized with 1 % Triton in PBS (5 min). After washing with washing buffer [PBS, 0.05 % (v/v) Tween 20], cells were incubated for 30 min with blocking buffer (PBS, 5 % horse serum). Subsequently, primary antibody incubations were performed for 1 h at 37 °C in blocking buffer. Expression of F protein was detected with monoclonal antibody 8E12A8C3 (de Leeuw et al., 2005), of Gn protein with monoclonal antibody 4-39-cc (Keegan & Collett, 1986) and of Gc protein with a polyclonal rabbit antiserum against the Gc ectodomain (de Boer et al., 2012). The resulting fragment was digested with HpaI and SpeI and ligated to pNDFL-GnGc digested with the same enzymes. Plasmids were confirmed to have the expected sequences using Sanger sequencing. The construct was designed to comply with the rule-of-six (Peeters et al., 2000).

Next-generation sequencing. A volume of 6 ml cell culture supernatant of NSRNDV passage 7 was concentrated to 125 µl using an Amicon Ultra 30K centrifugal filter (Merck). Subsequently, 400 µl Trizol LS (Sigma-Aldrich) was added to the concentrate and RNA was isolated using a Direct-zol RNA Miniprep kit (Zymo Research) according to the manufacturer’s instructions. Libraries were prepared using a ScriptSeq v2 RNA-Seq Library Preparation kit (Epicoence Biotehnologies). Quality and quantity of the library were determined using a Bioanalyzer with a High Sensitivity DNA kit (Agilent Technologies) and a Qubit dsDNA HS Assay kit (Life Technologies). Cluster generation and paired-end 250 bp sequencing of the libraries was subsequently performed with an illumina MiSeq V2 instrument. Raw sequencing data were trimmed and subjected to quality control using in-house scripts. Virus-specific reads were mapped against the reference sequences using Bowtie2 (Langmead & Salzberg, 2012) and local-sensitive mapped reads were visualized using Tablet (Milne et al., 2013).

Site-directed mutagenesis of NDV-GnGc-ΔF. Site-directed mutants of NDV-GnGc-ΔF were constructed using a QuikChange II XL Site-Directed Mutagenesis kit (Agilent Technologies) in combination with standard cloning techniques using pNDFL-GnGc-ΔF as a template. Primers used (7–10) are listed in Table S1. Site-directed-mutant infectious clones were confirmed to have the expected sequence using Sanger sequencing.

In vitro neutralization of NDV-GnGc variants. Virus (m.o.i. 0.05) was incubated with serial dilutions of a convalescent sheep serum containing RVFV antibodies or a negative control serum for 1.5 h in complete GMEM medium and subsequently added to 200 000 BHK cells per well of a 24-well plate. Two days post-inoculation, infected cells were visualized using IPMA.

Vaccination and challenge of mice. Six-week-old female BALB/cAnCrl mice (Charles River Laboratories) were divided into 4 groups of 10 mice, kept in type III filter-top cages under BSL-3 conditions, and allowed to acclimatize for 6 days. At day 0, mice were vaccinated intramuscularly (thigh muscle) with either medium (Mock), NSR-Gn (Oreshkova et al., 2013) (10³.8 TCID₅₀), NSRNDV (NSR: 10³.8 TCID₅₀; NDV: 10³.8 TCID₅₀) or NDV-GnGc-ΔF (10³.8 TCID₅₀) in 0.1 ml medium. RVFV-specific neutralization titres in sera were determined one day prior to challenge, using a previously developed RVFV neutralization assay (Kortekaas et al., 2011). Virus dissemination to the livers was evaluated by quantitative reverse-transcriptase (qRT)-PCR as described previously (Kortekaas et al., 2012). Briefly, RNA was isolated from 200 µl 10 % (w/v) organ suspension using an RNaseasy 96 kit (Qiagen) according to the manufacturer’s instructions. A volume of 5 µl purified RNA was used as PCR template and RVFV-specific RNA copy numbers in the 5 µl RNA sample were determined using an in vitro synthesized RNA standard.

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REFERENCES


