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Recombinant Newcastle disease viruses with targets for PCR diagnostics for rinderpest and peste des petits ruminants

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ABSTRACT

Since February 1st 2011, rinderpest (RP) has been officially declared eradicated worldwide. National authorities have been requested to destroy all their RP related materials. Nonetheless, their national reference laboratories performing real time reverse transcription polymerase chain reaction assays (PCR diagnostics) need RP positive control samples, since some countries still prefer to maintain diagnostic capability for RP for several reasons. In the future, a similar situation will arise for peste des petits ruminants (PPR) as the ambition has been expressed to eradicate PPR. Anticipating on this, we intended to perform qualified PCR diagnostics without use of infectious RPV or PPRV. Therefore, Newcastle disease virus (NDV) with small RNA inserts based on RPV or PPRV sequences were generated and used as positive control material. Recombinant NDVs (recNDVs) were differentially detected by previously established PCR diagnostics for RPV or PPRV. Both recNDVs contain a second PCR target showing that additional targets in NDV are feasible and would increase the diagnostic sensitivity by use of two PCR assays. RecNDV with small PCR targets is not classified as RPV or PPRV containing material, and can be used to mimic RPV or PPRV. Using these recNDVs as virus positive material contributes to the ambition of worldwide eradication, while qualified PCR diagnostics for these OIE-listed diseases remains operational.

Rinderpest virus (RPV) and peste des petits ruminants virus (PPRV) form distinct virus species within the genus *Morbillivirus* (*Paramxyoviridae* family, order of *Mononegavirales*). Other virus species within the *Morbillivirus* genus are measles virus, canine distemper virus, cetacean morbillivirus, phocine distemper virus, and unclassified morbillivirus. RPV and PPRV cause severe disease in large and small ruminants, respectively, and are notifiable diseases according to the World Organization for Animal Health (OIE). Since February 1st, 2011, RP has been declared officially eradicated worldwide, and is the first eradicated disease of livestock following eradication of the human disease smallpox in 1980. In line with the global eradication of RP, international bodies like the Food and Agriculture Organization of the United Nations now promote reduction of the number of institutions holding and working with infectious RPV. For this, countries have been requested to destroy infectious RPV as well as RP associated materials, or to ship these materials to so-named Rinderpest Holding Facilities. Notwithstanding the worldwide eradication of RP, however, qualified diagnostic capability for RP should remain operational for an unknown time period for several reasons.

Following the success of RP eradication, the ambition has been expressed to eradicate PPR in the coming decades. Currently, PPRV is still circulating in many countries and is of risk for PPR-free countries. Well-validated diagnostics, preferably Differentiating Infected from VAccinated individuals (DIVA) diagnostics compatible with PPR DIVA vaccine, will accelerate control and eradication of PPR. This also implies infectious PRRV as positive control in diagnostic assays, such as virus isolation, immunofluorescence assays, serum neutralization assays and real time reverse transcription polymerase chain reaction assays (PCR diagnostics). On the other hand, veterinary laboratories should strengthen their level of biosecurity with regard to hold infectious PPRV in proportion to the level of risk incurred by the country. This is a dilemma for diagnostic laboratories, national reference laboratories, institutions and countries contributing to control of PPR through surveillance and monitoring programs, while they share the ambition to eradicate PPR. Here, a non-PPRV positive control for PCR diagnostics is very welcome.

Newcastle disease virus (NDV) is also a member of the *Paramyxoviridae* family but forms a separate virus species within the *Avulavirus* genus consisting of virus strains varying from non-virulent (lentogenic) to virulent (velogenic) virus strains. The natural hosts of

Abbreviations: DIVA, differentiating infected from vaccinated individuals; ND, Newcastle disease; OIE, Office International des Epizooties (World Organisation for Animal Health); PCR, polymerase chain reaction; PPR, peste des petits ruminants; RP, rinderpest; SPF, specific pathogen free; TCID, tissue culture infective dose

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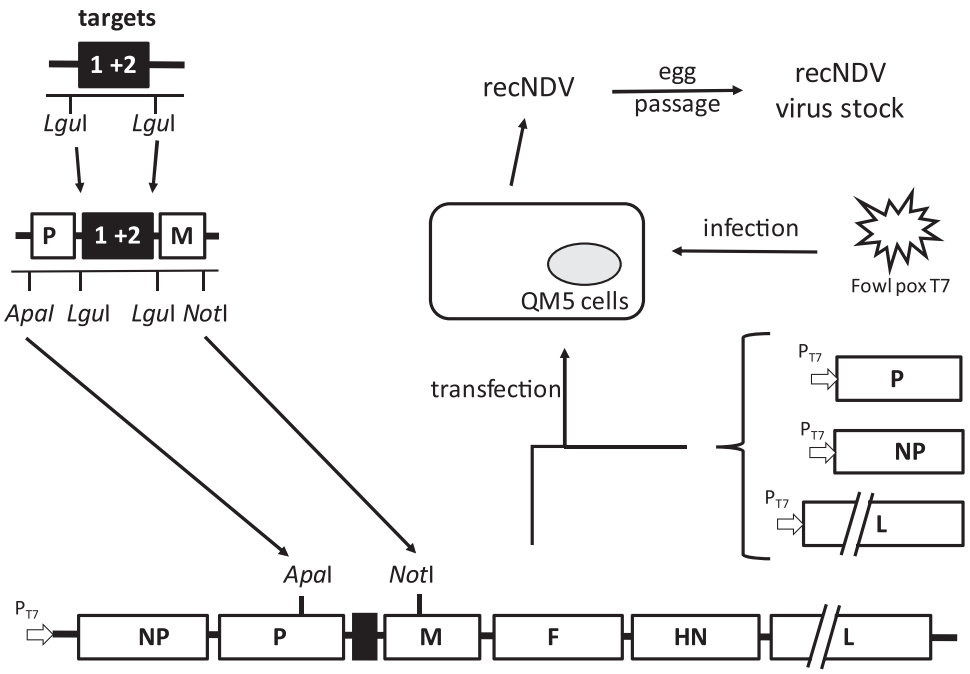

Fig. 1. Schematic overview of the rescue of recombinant Newcastle disease virus (recNDV). Rescue of recNDV using reverse genetics has been described (Peeters et al., 1999). cDNA encompassing the insert was cloned in the correct orientation in a shuttle plasmid, and subsequently recloned in full length cDNA of lentogenic NDV strain LaSota. Full length cDNA and expression plasmids of L, NP and P were transfected to fowlpox T7 infected QM5 cells. RecNDV was rescued and subsequently passed twice in eggs to prepare virus stocks.

NDV are avian species and NDV does not cause viremia in ruminants. Further, NDV is completely harmless for humans and has a long history as broad-spectrum oncolytic agent in humans (National Cancer Institute on Newcastle disease virus as a treatment for cancer, 2018). Reverse genetics for NDV has been developed to express foreign genes (Peeters et al., 1999; Zhao and Peeters, 2003), including NDV vectored vaccines for important pathogens of livestock and humans (Carnero et al., 2009; Duan et al., 2015; Kortekaas et al., 2010; Nakaya et al., 2001).

Similarly, slightly modified full length cDNA of lentogenic NDV strain LaSota, named pNDFL2 (Kortekaas et al., 2010), and helper plasmids pCIneo-NP, pCIneo-P and pCIneo-L (Peeters et al., 1999) were used to rescue recombinant NDVs (recNDVs) with PCR targets (Fig. 1). Two small cDNAs each with two PCR targets of RPV or PPRV, respectively, and separated by a small randomized spacing sequence were synthesized by Genscript Corporation Piscataway, NJ, USA (Fig. 2). One PCR target was based on in-house PCR tests for RPV or PPRV. The second PCR target was based on the signature primer and probe binding sites relevant to previously published PCR tests for RPV (Carrillo et al., 2010) and PPRV (Batten et al., 2011), respectively. The total foreseen RNA inserts were adjusted to multiples of six nucleotides according to the rule-of-six for packaging of the NDV genome (Peeters et al., 2000), and were 360 or 300 base pairs in length (Fig. 2). *Lgu*I cDNA fragments were recloned in the correct orientation in a shuttle plasmid, and the appropriate *Apa*I-*Not*I fragments were subsequently cloned in pNDFL2 according to standard cloning procedures (Peeters et al., 1999; Zhao and Peeters, 2003) (Fig. 1).

RecNDVs with the small RNA insert between the P gene (phosphoprotein) and M gene (matrix protein) of NDV were rescued (Fig. 1). Briefly, Quail fibrosarcoma cells (QM-5) were grown in Ford Dodge QT35 medium (Invitrogen, Carlsbad, CA, USA) containing 5% foetal calf serum (FCS) and 1% Pen Strep (10,000 units/ml, Invitrogen) to 60–80% confluence and infected with fowlpox recombinant virus expressing bacteriophage T7 DNA dependent RNA polymerase (Britton et al., 1996) for 1 h at 37 °C. Subsequently, monolayers were co-transfected with 1.0 µg of pNDFL2 derivative, 1.6 µg of pCIneo-NP, 0.8 µg of pCIneo-P and 0.8 µg of pCIneo-L using Fugene HD according to the instructions (Roche, Mannheim, Germany). Culture supernatant was harvested at 3–4 days post transfection and filtered through a 0.20 µm-pore-size filter. The insert in rescued recNDV was confirmed by conventional sequencing with appropriate sequence primers (not shown). A virus stock was prepared by two passages in eggs. For this, 9–11 days embryonated specific pathogen free (SPF) eggs were inoculated in the allantoic cavities and virus was harvested after 3–5 days. The first egg passage was filtered and 1000 times diluted prior to the second inoculation. Virus stocks were titrated on QM5 cells according to standard procedures. Infection foci were immunostained with anti-F monoclonal antibody 8E12A8C3 (Peeters et al., 1999), and virus titres were calculated (Reed and Muench, 1938). Typically, the first egg passage reached high virus titres of 10^{7-8} $TCID_{50}$/ml recNDV.

RecNDVs were studied on their use as alternative positive controls for PCR diagnostics for RPV and PPRV. Primers and probes of previously developed and validated in-house PCR tests for RPV and PPRV haven been synthesized by Eurogentec and Tib Molbiol, respectively. Primers and probes, including those of PCR tests previously published by others, are listed in Table 1. In-house PCR tests were used to study detection of viral genomic RNA in dilutions of both recNDVs (Fig. 3). Briefly, total nucleic acid was extracted from 200 µl using a MagNA Pure Compact Nucleic Acid Isolation Kit according to manufacturer's instructions (Roche), and was eluted in 100 µl water.

RPV RT-PCR mix: 20 µl total volume: 5 µl isolated RNA, 100 nM of each primer, 200 nM of each probe, 2.25 mM MnO_2, 7.5 µl LC master mix (Roche). 20 min 61 °C, 30 s 95 °C, 45 cycli (1 s 95 °C, 10 s 59 °C, 15 s

RPV insert:

gctcttcaagcaCCTGATGAAAGGACATGCCATATTTTGCGGGATTATCATCAACGGGTATCGGGACAGGCATGGAGGGAGCTGGCCACCgtttaagtcgggtacatatcagaagtagtt
ATGGGTGAACTGGCTCCTTATATGGTGATCTTAGAGAACTCAATCCAGAACAAATTCAGTGCAGGAGCATACCCCTGTTGTGGAGCTATGCTATGGGGGTCGGGGTTGATTAGAGAACTC
TATGGGGGGACTTAATTTCGGCAGATCTTATTTTGACCCTGCATATTTCAGGTgctcttc-300

PPRV insert:

gctcttcaagcAAGAGTTCAATATGTTGTTAGCCTCCATCTTAGCACAAGTTTGGATCCTCCTGGCCAAGGCGGTTACGGCACCGGATACGGCAGCTGACTCAGAACTGAGAAGGTGGGT
TAAATACACACAACAAAGGAGAGTGATTGGGGAAgtttaagtcgggtacatatcagaagtaGTTAGTATCCGCCTTGTTGAGGTAGTTCAAAGTACTAGGTCCCAGTCCGGGTTGACCTT
TGCATCACGTGGTGCTGATTTAGACAACGAGGCAGACATGTATTTTCAACTGAGGCGCCCTCGAGTGGAGGTAAGAAAAGGATCAACTGGTTTGAGAACAGAGAAATAATAGAgctcttc-360

PCR tests and in-house PCR tests are underlined and double underlined, respectively.

Fig. 2. Overview of RNA inserts in recNDVs. RNA inserts encompasses two PCR targets of RPV or PPRV, respectively. Flanking *Lgu*I sites are grey shaded. PCR targets are separated by a randomized spacer sequence. RPV and PPRV specific sequences and nonspecific sequences are presented by capitol and small symbols, respectively. Targets of previously published