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# Parenteral vaccination of mammalian livestock with Newcastle disease virus-based vector vaccines offers optimal efficacy and safety

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**Newcastle disease virus (NDV) is an avian virus that is being evaluated as a vaccine vector for the delivery of foreign genes in mammals. The use of NDV as a vaccine vector in these species offers two major advantages. First, NDV is highly attenuated in mammals, rendering its use inherently safe. Second, mammals lack pre-existing NDV immunity, which minimizes the risk of vaccination failure. NDV-vector vaccines are generally administered to mammals via the respiratory route. We recently showed that intramuscular vaccination with NDV-based Rift Valley fever virus (RVFV) vaccines provides complete protection in mice and induces neutralizing antibodies in sheep and cattle, the main target species of RVFV. Here, we discuss the use of NDV as a vaccine vector for applications in mammalian livestock with an emphasis on the vaccination route. We also report the results of novel experiments that underscore our notion that vaccination via a parenteral route is more effective than immunization via the respiratory route.**

**Key words:** vaccine, vector vaccine, vaccine safety, humoral immunity, vaccination route, intramuscular, subcutaneous, paramyxovirus, newcastle disease virus, rift valley fever virus

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## NDV-based Vector Vaccines

Newcastle disease causes severe economic losses in the poultry industry. The causative agent, Newcastle disease virus (NDV) belongs to the Avulavirus genus of the Paramyxoviridae family. The NDV genome is a negative-sense RNA molecule that encodes six structural proteins: nucleoprotein (NP), phosphoprotein (P), matrixprotein (M), fusionprotein (F), hemagglutinin-neuraminidase (HN) and RNA-dependent RNA polymerase (L). Two non-structural proteins, V and W, are expressed from alternative reading frames in the P gene.[1] The V protein functions as a species-specific alpha/beta interferon antagonist,[2,3] whereas the function of the W protein remains unclear. The viral polymerase initiates at a single promoter and copies each gene into a separate mRNA using short gene-end and gene-start transcription signals that flank each gene.

NDV strains are classified into three pathotypes depending on their virulence in chickens: velogenic (high virulence), mesogenic (medium virulence) and lentogenic (low virulence). An important virulence determinant is the amino acid sequence of the protease cleavage site in the F protein,[4] whose cleavage is essential for infectivity.[5,6] Velogenic and mesogenic strains contain multiple basic amino acid residues at this position which together function as a recognition sequence for ubiquitous subtilisin-like proteases. However, lentogenic strains contain fewer basic amino acids at this position and as a result cleavage of their F proteins depends on trypsin-like proteases, which are confined to the respiratory and intestinal tract. Consequently, only mesogenic and velogenic strains are capable of causing systemic infection. Lentogenic strains are commonly used worldwide for the control of NDV in poultry.

The availability of an NDV reverse-genetics system has opened up ways to use NDV as a vaccine vector for the control of other diseases in poultry as well as mammals.[4,7,8] The system (**Fig. 1**) includes a plasmid encoding a full-length antisense copy of the viral RNA and expression plasmids that provide the NP, P and

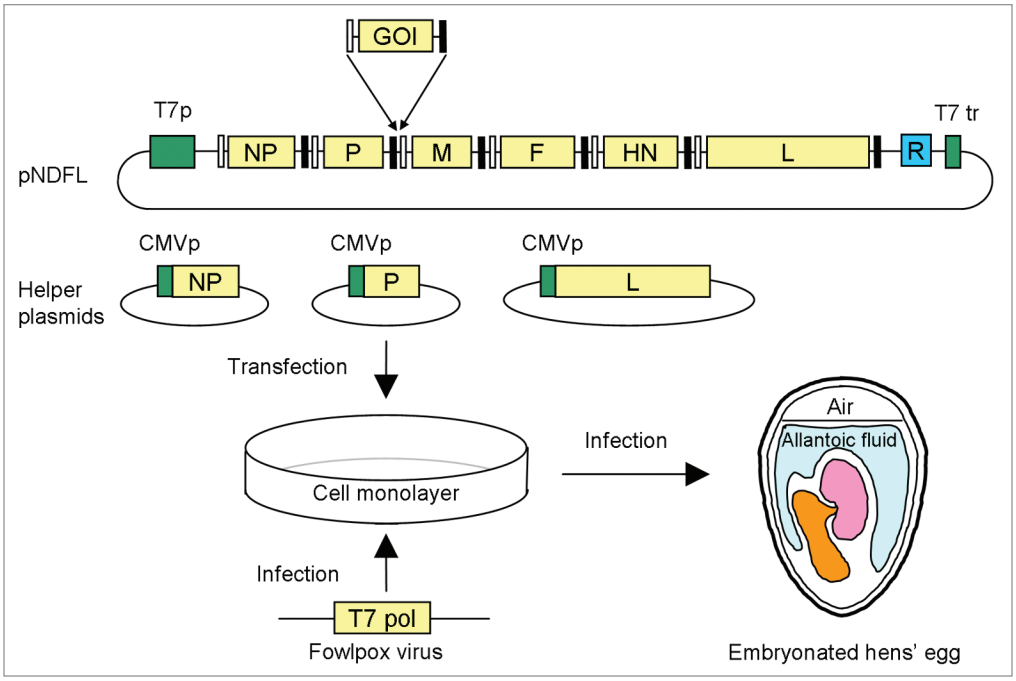


**Figure 1.** Construction of recombinant NDV strains. Protein coding regions (yellow bars) of foreign genes of interest (GOI) containing suitable additional gene-start and gene-end transcription regulation sequences are inserted between the P and M genes of plasmid pNDFL encoding the complete antigenomic RNA of NDV strain LaSota. The gene-start and gene-end transcription signals flanking each gene are shown as white and black boxes, respectively. The NDV genome can be transcribed from this plasmid using T7 promoter (T7 p) and terminator (T7 tr) sequences and a self-cleaving ribozyme site (R) that ensures generation of the correct 5' end. Infectious rNDV is generated by infection of cells with a fowlpox virus to supply T7 polymerase and subsequent transfection with pNDFL and three helper plasmids that encode NP, P and L proteins under control of the CMV promoter (CMVp). Infectious rNDV is further propagated on embryonated hens' eggs.

L proteins. Expression of the viral RNA is driven by T7 polymerase that is either supplied by a recombinant pox virus[4] or a stable cell line.[7] After rescue of small amounts of virus from transfected cells efficient propagation of the virus can be performed in embryonated hens' eggs. By introducing foreign genes flanked by dedicated gene-start and gene-end transcription signals in the full-length cDNA, recombinant NDV-based vectors can be produced.[9]

## Effect of Administration Route on Vaccine Efficacy

NDV-vectored vaccines as well as other types of paramyxovirus-based vaccine vectors are generally administered via the respiratory route, by which they generally show good immunogenicity.[10-15] A combined intranasal/intratracheal (IN/IT) route is most often used. Importantly, DiNapoli et al.[12] recently demonstrated that delivery to the lower respiratory tract is required for effective immunization of non-human primates with NDV-based vector vaccines. From these studies, it was concluded that NDV replicates poorly in the upper respiratory tract of primates due to the relatively low temperature at this site, compared to the body temperatures of birds, the natural hosts of NDV.[12] The main interest of the authors of this work, is to develop human vaccines for emerging pathogens such as the SARS coronavirus.[11] For application of NDV-based vector vaccines in humans, delivery to the lower respiratory tract can indeed be realized by using innovative nebulizers. However, for mass applications in livestock, the use of nebulizers is not a practically feasible approach. We were therefore interested in evaluating immune responses elicited by IN administration only. Furthermore, regarding the hypothesis of DiNapoli et al.[12] we also considered the possibility that delivery of NDV by parenteral immunization (i.e., administration by any route other than the alimentary or respiratory tract) could provide the virus with the optimal temperature for replication, resulting in improved immunogenicity.

Our laboratory is evaluating NDV as a vaccine vector for the control of RVFV in livestock. We use the lentogenic LaSota strain for the production of these vaccines.[4] In a first study, calves were immunized via either the intranasal (IN) or the intramuscular (IM) route with a recombinant NDV that produces the RVFV Gn protein. Surprisingly, antibody responses against both the vector and the Gn protein were detected after IM delivery but not after delivery via the IN route.[16] In a subsequent study, we showed that immunization via the IM route with a recombinant NDV that produces both the RVFV Gn and Gc proteins protects mice against RVFV challenge infection. Importantly, a single vaccination with this vaccine was sufficient for the induction of neutralizing antibodies in sheep, the main target species of RVFV.[17] Thus, IM inoculation with our NDV-based experimental RVFV vaccines elicits high levels of antibodies in both sheep and cows and elicits protective immunity in mice.[16,17]

It was, however, unexpected to find that IN inoculation of calves did not result in

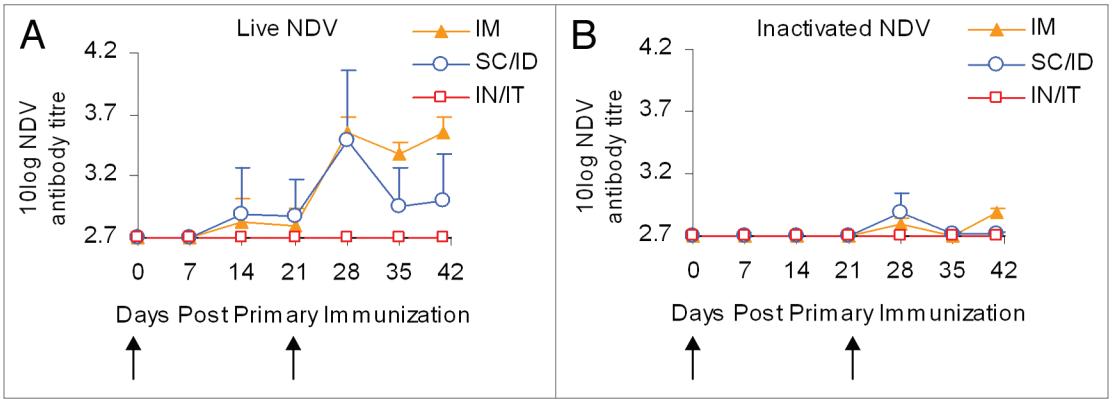


**Figure 2.** NDV-specific antibody responses in sheep inoculated with wildtype NDV strain LaSota via different immunization routes. Groups of 4 sheep (cross bred Texelaar x Swifter) were inoculated with live (A) or formalin-inactivated (B) NDV strain LaSota that was originally derived from the ATCC (VR-699). The virus was passaged three times in the allantoic cavity of 9- to 11-day-old embryonated hens' eggs and diluted to $10^7$ $TCID_{50}$/ml in PBS prior to administration via the IN/IT, SC/ID or IM route in a volume of 2, 1 or 2 ml, respectively. When two different inoculation routes were used (IN/IT or SC/ID), the dose was equally divided between the two inoculations. Vaccinations were performed on days 0 and 21 (arrows). Serum samples were analyzed for the presence of NDV specific antibodies by ELISA.[16] Geometric mean titres and standard deviation are shown. The Y-axis intercepts at 2.7 10log titre, corresponding to the lowest serum dilution analyzed (500-fold).

any detectable antibody response.[16] In the current study, we compared the immunogenicity of wildtype (non-recombinant) NDV strain LaSota when administered to sheep via a combined IN/IT route to two parenteral immunization routes: IM and a combined subcutaneous/intradermal (SC/ID) route. In addition, we compared the immunogenicity of live and inactivated NDV when delivered via these routes. Live NDV administered via the IM route elicited significantly higher ($p < 0.001$ at 28 days post primary immunization) antibody responses as compared to the IN/IT route (**Fig. 2A**). The antibody responses elicited after SC/ID immunization were more comparable to those obtained after IM immunization (**Fig. 2A**). Thus, in line with our previous studies, parenteral immunization with live NDV was more effective when compared to delivery via the respiratory route, in this case a combined IN/IT route.

Our results seem to contrast two earlier studies in which parenteral NDV administration was compared with delivery via the respiratory tract. In the first study, immunization of mice with an NDV vector vaccine via the IN route induced more effective protective immune responses as compared to intravenous (IV) or intraperitoneal (IP) immunization.[18] In another study, immunization with NDV-based vectors that either produce the SARS coronavirus spike protein or the HN protein of human parainfluenza virus type-3 elicited higher antibody titres when delivered via a combined IN/IT route than when the same vaccines were delivered via the SC route.[12] It is important to note, however, that these studies differ from our studies with respect to the parenteral vaccination routes (IV, IP or SC versus IM or SC/ID), the inoculated species (mice or non-human primates versus cow or sheep) as well as the NDV strain used. Additional studies are clearly required to further elucidate the immune responses elicited by NDV inoculation via different routes in mammals.

The NDV-based RVFV vaccines described in our previous studies were produced in embryonated hens' eggs. The allantoic fluid of these eggs contained RVFV Gn and Gc proteins,[16,17] which could have contributed to the elicited immune responses independent of virus replication and de novo protein production. To gain insight into the role of injected proteins in the antibody response induced by our vaccines, we compared the antibody responses induced by live NDV with those elicited by formalin-inactivated NDV. The results obtained from this experiment (*cf.* **Fig. 2A and B**) underscore the notion that NDV propagation is essential for high immunogenicity in sheep and thereby show that co-injected protein is of little, if any, influence to the antibody response elicited.

## Vaccine Safety

Although the immunogenicity of NDV-based vector vaccines depends on virus replication in the inoculated mammal, spread in these unnatural hosts was previously shown to be highly restricted or even absent.[10,11,19] In accordance with these findings, accidental NDV infections of mammals are rare and if these do occur they mostly remain subclinical.[19] The attenuation of NDV in mammals is primarily caused by the species-specificity of the interferon antagonist function of the V protein.[2] Consequently, spread of both lentogenic and mesogenic NDV in mammals is highly restricted and the use of both pathotypes as vaccine vectors for application in mammals is therefore considered acceptable with respect to safety for the inoculated mammal.[19] The dependence of lentogenic strains on trypsin-like proteases for infectivity makes it more difficult to grow these viruses in tissue culture. Since the introduction of foreign genes generally results in further attenuation of the viruses, it is sometimes challenging to produce recombinant lentogenic viruses with large foreign gene inserts. Therefore, vector vaccines are often based on mesogenic strains or lentogenic strains with modified F cleavage sites that confer trypsin independence. The safety for the domesticated poultry industry must, however, also be taken into consideration when applying

mesogenic strains as vaccine vectors in the field. Since the detection of NDV that contains a polybasic cleavage site in the field could result in the pre-emptive culling of poultry in fear of an NDV outbreak, it is highly preferred to use true lentogenic strains for applications in livestock.

Although the use of lentogenic NDV in mammals is considered highly safe, we determined whether NDV strain LaSota was capable of systemic spreading in the inoculated sheep of the current experiment. Heparinised blood samples were collected and evaluated for the presence of infectious NDV at 1, 3, 6, 8, 10 and 13 days post primary immunization. In none of the sheep infectious NDV was detected, indicating that the virus did not spread systemically. At these same time points we could also not detect infectious NDV in nasal fluids, which was only analyzed for sheep inoculated by the IN/IT route. These results are consistent with similar findings of others who were unable to detect infectious NDV in nose-throat swabs and tracheal lavages of monkeys that were inoculated via the combined IN/IT route with recombinant NDV strains, with the exception of one sample of one monkey that contained a very low NDV titre in nasal fluid.[10] In another experiment, low levels of NDV were detected in lung tissues of monkeys.[10] Finally, aerosol NDV administration to non-human primates resulted in only low levels of NDV shedding in respiratory secretions.[20] From the results of current and previous studies it can be concluded that spread to the environment is possible when the respiratory route is used for vaccination. Furthermore, it is plausible to assume that this risk is higher when a mesogenic strain is used. However, administration of a lentogenic strain via a parenteral route decimates this theoretical risk of spread to the environment. Moreover, if spillover of a lentogenic vaccine vector from mammals to poultry or other birds would ever incidentally occur, this could elicit protective immunity against NDV, something that can hardly be considered a negative effect.

When it comes to safety, it is also important to establish the risk of genetic exchange between vaccine and field strains. In general, gene exchange between nonsegmented negative-strand RNA viruses is a rare event.[21] This is in contrast to the frequent genetic exchange observed between negative-strand RNA viruses with segmented genomes, such as influenza virus[22] and positive-strand RNA viruses such as picornaviruses.[23] Although rare, there are three recent examples that suggest the occurrence of recombination between live NDV vaccine strains that are used for the vaccination of poultry and NDV field strains.[24,25] Therefore, Han et al.[26] expressed their concern that the use of NDV vector vaccines in mammals would result in untoward recombination events. However, we fully support the criticism on this concern published by Collins et al.[27] because of the following reasons. First, although genetic exchange between vaccine strains and field strains sporadically occurs, it is highly unlikely that such a recombination event would ever result in a novel virus of higher virulence than the virus that is already circulating. Second, it is important to note that any possible recombination event would require efficient replication of the vaccine virus in the inoculated animal. As detailed above, vaccination of mammals with NDV-based vector vaccines does not result in viremia nor systemic infection, rendering the chance of any recombination event negligible in these species. Third, other live virus vaccines based on nonsegmented negative-strand RNA viruses, such as measles virus, have been used world-wide for a long time without any reported adverse events attributable to genetic exchange involving a vaccine strain.

In summary, we conclude that application of NDV-based vector vaccines to mammalian livestock by a parenteral, preferably IM, route offers the advantages of optimal immunogenicity, optimal safety and high practical feasibility for mass applications in livestock.



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