Short Communication

A Y527A mutation in the fusion protein of Newcastle disease virus strain LaSota leads to a hyperfusogenic virus with increased replication and immunogenicity

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Newcastle disease is a highly contagious and economically important disease of poultry. Low-virulence Newcastle disease virus (NDV) strains such as B1 and LaSota have been used as live vaccines, with a proven track record of safety and efficacy. However, these vaccines do not completely prevent infection or virus shedding. Therefore, there is a need to enhance the immunogenicity of these vaccine strains. In this study, the effect of mutations in the conserved tyrosine residues of the F protein of vaccine strain LaSota was investigated. Our results showed that substitution of tyrosine at position 527 by alanine resulted in a hyperfusogenic virus with increased replication and immunogenicity. Challenge study with highly virulent NDV strain Texas GB showed that immunization of chickens with Y527A mutant virus provided 100 % protection and no shedding of the challenge virus. This study suggests that the strain LaSota harbouring the Y527A mutation may represent a more efficacious vaccine.

Newcastle disease virus (NDV) causes a highly contagious disease in chickens, resulting in severe economic losses to the poultry industry worldwide (Alexander, 2000; Samal, 2011). NDV isolates are categorized into three pathotypes – lentogenic (low virulence), mesogenic (intermediate virulence) and velogenic (high virulence) – based on their pathogenicity in chickens (Aldous & Alexander, 2001). Several lentogenic strains, such as LaSota and B1, are being used as live vaccines all over the world. Although these strains have been highly successful as live vaccines, they do not completely prevent virulent virus infection or shedding of virulent virus. Newcastle disease (ND) remains a major poultry disease problem in many parts of the world despite repeated vaccination. Therefore there is a need to enhance the immunogenicity and protectiveness of current live vaccines. Reverse genetics provides a powerful tool to increase the immunogenicity of current vaccine strains.

NDV belongs to genus Avulavirus within the family Paramyxoviridae, a family of enveloped, non-segmented, negative-strand RNA viruses. The genome of NDV contains six genes (3’-N-P-M-F-HN-L-5’), which encode seven proteins, namely nucleocapsid (N) protein, phosphoprotein (P), matrix (M) protein, fusion (F) protein, haemagglutinin–neuraminidase (HN) and RNA-dependent RNA polymerase (L). The envelope of NDV contains two transmembrane glycoproteins, the HN protein and the F protein. The HN protein is involved in attachment to host cell receptor and release of progeny virions. The F protein mediates fusion of the viral membrane to the cell plasma membrane, resulting in virus penetration, and also mediates fusion of adjacent cells to form syncytia (Samal, 2011). The NDV F protein is a trimer, and it is synthesized as an inactive precursor, F0 (66 kDa), which is post-translationally cleaved by host cell proteases into two disulfide-linked subunits, N-terminal F2 (12.5 kDa) and C-terminal F1 (55 kDa). The ability of the F protein to be cleaved by proteases is a major determinant of virulence in NDV and a prerequisite for virus entry and cell-to-cell fusion (Aldous & Alexander, 2001).

The NDV F protein is a class I fusion protein that has structural and functional characteristics related to those of the F protein of other paramyxoviruses (Morrison, 2003; Lamb & Parks, 2007). It is known that the structural features of the ectodomain of the F protein can have a major impact on fusion (Chen et al., 2001). Several reports have also indicated a role for the cytoplasmic tail (CT) of F protein in virus entry, F protein cleavage, and fusogenicity (Morrison, 2003; Lamb & Parks, 2007; Chen et al., 2001). It has been found in other enveloped viruses that tyrosine-containing signals, especially Y-X-X-aliphatic/aromatic consensus motifs, in the CT of viral membrane proteins are associated with the transport and delivery of proteins (Samal et al., 2013; Ball et al., 1997; Brewer & Roth, 1991; Weise et al., 2010). The NDV F CT is 31 aa long and its sequence is highly conserved among different
strains of NDV (Dolganiuc et al., 2003). We have previously shown that mutations in the two conserved tyrosine (Y524 and Y527) residues in the cytoplasmic domain of the F protein of moderately virulent NDV strain Beaudette C conferred a hyperfusogenic phenotype, increasing viral replication and pathogenicity (Samal et al., 2013). The mechanism by which the substitution of tyrosine residues causes hyperfusogenicity is not well known. It is possible that the substitution may have conferred increased efficiency of synthesis or transport to the cell surface, resulting in higher levels of F protein surface expression that in turn mediated increased cell-to-cell fusion. Alternatively, the substitution might have caused increased incorporation of F protein into virus particles or effects on the fusion process.

The effect of tyrosine mutations on immunogenicity of the modified Beaudette C strain could not be determined because this virus was highly virulent, causing death of all infected chickens. Conserved tyrosine residues in the CT of the F protein of NDV play an important role in the fusogenicity of the virus, but the role of each tyrosine residue may vary among strains of NDV. Therefore, it was necessary to determine the effect of tyrosine mutations on virus replication and immunogenicity in the context of a low-virulence strain. The low-virulence strain LaSota was chosen for this study because this strain is widely used as a live vaccine throughout the world.

The construction of plasmid pNDV carrying the full-length antigenome cDNA of the NDV strain LaSota has been described previously (Huang et al., 2001). We used site-directed mutagenesis to introduce individual amino acid substitutions into cDNA of the F gene of strain LaSota. Tyrosine residues at positions 524 and 527 were changed to alanine individually and in combination. The mutated gene was then inserted into a full-length cDNA clone of the LaSota antigenome (Fig. 1a). The modified vaccinia virus strain Ankara (MVA) expressing T7 RNA polymerase was kindly provided by Dr Bernard Moss (NIH, Bethesda, MD, USA). The full-length clones were transfected into HEp2 cells, and mutant viruses were recovered as previously described (Huang et al., 2013). The CT mutant viruses were named rLas-524, rLas-527, and rLas-524+527, and the parental virus rLas-parent. The F genes from recovered viruses were sequenced to confirm the mutations. To assay genetic stability, the recovered CT mutant viruses were passaged in 9-day-old specific-pathogen-free (SPF) chicken embryos five times. From each passage, total RNAs were isolated from NDV-infected allantoic fluid of 9-day-old SPF chicken embryos, using TRIzol reagent (Invitrogen). Reverse transcription PCR (RT-PCR) was performed using the Thermoscript RT-PCR kit (Invitrogen) with specific forward and reverse primers to amplify the F gene. The amplified cDNA fragments were then sequenced using the BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems) in an ABI 3130xl genetic analyser to confirm the presence of the introduced mutations in the passaged viruses.

Plaque morphology of the recovered viruses showed that rLas-527 produced slightly larger plaques than parental and other CT mutant viruses (Fig. 1b). Syncytium formation was quantified by counting the number of nuclei in fused cells (Kohn, 1965). Briefly, Vero cells in six-well plates were infected with each virus at an m.o.i. of 0.1. Cells were maintained in 2 % minimal essential medium at 37 °C under 5 % CO₂. At 24 h post-infection (p.i.), the medium was removed and the cells were washed with PBS, fixed with methanol for 20 min at room temperature, and then stained with haematoxylin and eosin. The fusion index of each mutant virus was calculated by observing 10 fields per well in duplicate. The fusion index is the ratio of the total number of nuclei to the number of cells in which these nuclei are present (i.e. the mean number of nuclei per cell). This assay showed that rLas-527 produced more cell fusion than rLas-parent and other CT mutant viruses (Fig. 2a).

Multicycle growth kinetic studies were done in DF-1 cells and in 9-day-old embryonated chicken eggs. Briefly, DF-1 cells grown in six-well plates were infected with each mutant virus, in duplicate, at an m.o.i. of 0.001. After 1 h of adsorption, the cells were washed with PBS and overlaid with Dulbecco’s modified Eagle’s medium containing 2 % FBS and 10 % fresh allantoic fluid at 37 °C. A sample of the supernatant medium was collected and replaced with an equal volume of fresh medium every 8 h until 64 h p.i. Similarly in 9-day-old embryonated chicken eggs, the eggs were inoculated with 100 p.f.u. of each virus, and allantoic fluids were harvested at different time points (24, 48 and 72 h). Virus titres were quantified by TCID₅₀ on DF-1 cells by the endpoint method (Reed & Muench, 1938). The Y527A mutant virus showed increased virus replication in vitro. The replication of the Y527A mutant virus was increased at least 0.5 log₁₀ compared with the parental strain, and 2 log₁₀ compared with the Y524A mutant at 24 h p.i. in DF-1 cells (Fig. 2b). A slight increase in titre was also observed in embryonated chicken eggs (Fig. 2c). The increased virus replication may be the result of the hyperfusogenic phenotype of the virus, which helps the mutant virus to spread from cell to cell more efficiently, thereby increasing virus replication.

The pathogenicity of parental and CT mutant viruses was determined by the mean time to death (MDT) test in 9-day-old SPF embryonated chicken eggs and by the intracerebral pathogenicity index (ICPI) test in 1-day-old SPF chicks (Alexander, 1989). Briefly, for the MDT test, a series of 10-fold dilutions of infected allantoic fluid (0.1 ml) were inoculated into the allantoic cavities of five 9-day-old eggs per dilution and incubated at 37 °C. The eggs were examined once every 8 h for 7 days, and the time of embryo death was recorded. The MDT was determined as the mean time (h) for the minimum lethal dose of virus to kill all the inoculated embryos. The criteria for classifying the virulence of NDV isolates are: <60 h, virulent strains; 60 to 90 h, intermediate or moderately virulent strains; and >90 h, avirulent strains. The MDT
values of rLas-524, rLas-527, rLas-524+527 and rLas-parent are 98, 90, 95 and 100 h (Table 1), indicating that the mutant viruses are avirulent for chicken.

For the ICPI test, 0.05 ml of a 1:10 dilution of fresh infective allantoic fluid for each virus was inoculated into a group of ten 1-day-old SPF chicks via the intracerebral route. At each observation, the birds were scored as follows: 0 if normal, 1 if sick, and 2 if dead. The ICPI is the mean score per bird per observation over the 8 day period. Highly virulent velogenic viruses give values approaching 2, and avirulent or lentogenic strains give values at or close to 0. The ICPI values of rLas-524, rLas-527, rLas-524+527 and rLas-parent are 0.10, 0.20, 0.04 and 0.11 (Table 1). Mesogenic ND vaccine strains, such as Roakin, Mukteswar and Komarov, all have two pairs of basic amino acids at the F0 cleavage site and ICPI values of around 1.40. These vaccines are used as live vaccines primarily in countries where ND is endemic. In the USA, the NDV strains with ICPI values ≥ 0.7 are virulent and reportable. The OIE Biological Standards Commission similarly recommended that ND vaccines should have an ICPI < 0.7 (OIE, 2009). This means that infection of birds with rLas-527 or other CT mutant viruses would fall within the intended definition of ND vaccination.

The immunization and challenge studies were performed in chickens at our US Department of Agriculture-approved enhanced biosafety level 3 (BSL-3+) containment facility. Two-week-old SPF chickens obtained from Charles River Laboratories were randomly assigned to five treatment groups of ten birds each. All birds were housed in separate poultry isolation chambers with ad libitum access to feed and water. The birds were vaccinated via the intranasal route with 200 µl 1 x 10⁶ EID₅₀ (50% egg-infective dose) rLas-parent, rLas-524, rLas-527, rLas-524+527 or PBS. All birds were observed daily for 10 days for any vaccine-related clinical signs. Oral and cloacal swabs were collected on day 3 post-vaccination to monitor the vaccine virus shedding. Blood was collected at 1, 2, 3 and 4 weeks post-immunization (PI) for analysis of NDV antibody response by haemagglutination inhibition (HI) assay (Samal, 2011). At 4 weeks PI all the groups were challenged by the occulo-nasal route with virulent NDV strain Texas GB at 100 x chicken LD₅₀ of 1 x 10⁴ TCID₅₀ per bird. All birds were observed for 10 days for clinical signs (death, paralysis and torticollis) of neurotropic NDV. All the birds that had been immunized with parental rLas or CT mutant viruses were completely protected from virulent Texas GB challenge virus without any clinical signs. In contrast, all the birds in the unvaccinated control group died within 3 days after challenge. In order to determine shedding of the challenge virus, oral and cloacal swabs were collected on days 3, 5 and 7 post-challenge from all chickens and injected into the allantoic cavity of 9-day-old SPF embryonated chicken eggs after antibiotic treatment. The presence of virus in the swabs was confirmed by

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**Fig. 1.** (a) Top: schematic diagram of the NDV genome. Bottom: black boxes, fusion peptide cleavage site; dark grey boxes, heptad repeats (HR); light grey box, cytoplasmic tail (CT); horizontal line shade, transmembrane (TM) domain. (b) Plaque size and morphology of rLasota and its CT-mutant viruses.
The HI titre induced in chicken by Y527A mutant virus was at least 2 log higher than that induced by the rLas-parent virus at 7 days PI and reached a plateau at 4 weeks PI (Fig. 2d). Our results showed that Y527A mutation increased the immunogenicity of LaSota virus. This is remarkable considering the fact that the LaSota strain is a highly immunogenic vaccine, and any further increase in

Table 1. Pathogenicity of rLasota and its CT mutants in embryonated eggs and chicks

<table>
<thead>
<tr>
<th>Virus</th>
<th>ICPI</th>
<th>MDT (h)</th>
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<tbody>
<tr>
<td>rLas-524</td>
<td>0.10</td>
<td>98.00</td>
</tr>
<tr>
<td>rLas-527</td>
<td>0.20</td>
<td>90.00</td>
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<tr>
<td>rLas-524+527</td>
<td>0.04</td>
<td>95.00</td>
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<tr>
<td>rLas-parent</td>
<td>0.11</td>
<td>100.00</td>
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haemagglutination assay of the allantoic fluid 3 days after injection into eggs.

Fig. 2. (a) Comparison of the fusogenicity and CPE of parental rLasota and the CT mutant viruses in Vero cells. Relative levels of fusion obtained for the CT mutants compared with parental rLasota virus are shown. The fusion index was calculated as the ratio of the total number of nuclei in multinuclear cells to the total number of nuclei in the field. Ten fields were counted for each virus. Fusion levels were normalized to parental rLasota as 100 %. (b) Growth kinetics of parental rLasota and CT mutants in chicken embryo fibroblast (DF-1) cells. Cells were infected at an m.o.i. of 0.001 of each virus, and the cell culture supernatant was collected at 8 h intervals for 64 h. The virus in the supernatant was titrated by plaque assay. (c) Growth kinetics of parental and CT mutant viruses in 9-day-old embryonated chicken eggs. For each virus, 30 eggs were injected with $10^5$ p.f.u. into the allantoic cavity and 10 eggs were chilled at 24 h intervals till 72 h. The virus titre in allantoic fluid was determined by TCID$_{50}$ assay in DF-1 cells. (d) Comparison of humoral immune response in chickens infected with parental rLasota and CT mutant viruses. Ten 2-week-old chickens in each group were immunized with $10^6$ p.f.u. of each virus. The antiserum was collected at weekly intervals for 4 weeks. The antibody titre against NDV was measured by haemagglutination inhibition (HI) assay. All virus titres are expressed as mean reciprocal log$_2$(titre) ± SE and antibody titres are expressed as mean reciprocal log$_2$(titre) ± SE. Statistical differences were calculated by two-tailed unpaired t-test, *$P<0.05$, **$P<0.01$ and ***$P<0.001$. 
immunogenicity is an added advantage for the virus as a live vaccine. It is particularly important for an NDV vaccine because NDV is a highly infectious and fast-replicating virus, and for the vaccine to be efficient it must induce the highest level of immune response possible. It is not known whether the increase in HI titre was due to increase in antibody production or whether more-specific HI antibodies were developed owing to this mutation. Our challenge virus shedding results showed that one of the birds immunized with rLas-parent was positive for virus shedding, whereas none of the birds in the CT mutant groups showed virus shedding (Table 2). These results indicate that the CT mutant viruses were more effective than parenteral rLasota in protection against virulent NDV challenge. However, the results obtained in this study could be just a coincidence, and a larger study is needed to confirm them.

The CT of paramyxovirus F protein has been shown to play an important role in modulating membrane fusion and hence is a significant determinant in the replication of these viruses (Branigan et al., 2006; Cathomen et al., 1998). The exact mechanism by which Y527A increases the fusogenicity is unclear. We think that Y527A mutation may increase the interaction of the F protein with viral proteins M and HN, which in turn may modulate the fusion process. For example, in parainfluenza virus 5 F protein, the CT was important in regulating fusion-pore formation (Dutch & Lamb, 2001). The single mutation Y524A seems to have a more pronounced effect on growth kinetics, plaque size and immunogenicity than the double mutation Y524A+Y527A. We predict that the Y524A mutation is located in the transmembrane region of the F protein, which may affect the incorporation of the F protein into the membrane. The Y527A mutation, at the first amino acid in the CT, somehow compensates for the incorporation of the F protein into the membrane and restores membrane fusion.

The results presented here indicate that the currently used vaccine strain LaSota can be modified to increase its replication and immunogenicity. We found that the Y527A substitution in the CT of the F protein of strain LaSota resulted in a hyperfusogenic virus with increased replication and immunogenicity. The increased replication of the rLas-527 strain will benefit the vaccine-manufacturing companies and reduce the cost of the vaccine for the poultry farmer. The increased immunogenicity of the rLas-527 strain will prevent replication and shedding of virulent virus. Although the increase in immunogenicity of rLas-527 seems minimal, considering the number of doses of ND vaccine used and the number of ND outbreaks that occur in the world, a slight increase in the immunogenicity of the current ND vaccine will have a significant effect on the control of ND. Our results suggest that the rLas-527 virus may be useful as an improved NDV vaccine, which will be highly beneficial to the poultry industry throughout the world.

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### References


