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HN gene C-terminal extension of Newcastle disease virus is not the determinant of the enteric tropism

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Abstract The hemagglutinin-neuraminidase (HN) protein of Newcastle disease virus (NDV) plays an important role in virus pathogenicity and tissue tropism. Sequence analysis revealed that the HN gene of many asymptomatic enteric NDV strains encodes a larger open reading frame (616 amino acids, aa) with additional 39 aa at its C-terminus when compared with that (577 aa) of respirotropic NDV strains. Therefore, it has been suspected that the HN C-terminal extension may contribute to the enteric tropism. In the present study, we generated a NDV respirotropic strain LaSota-based recombinant virus with a HN C-terminal extension of 39 aa derived from an enterotropic NDV strain using reverse genetics technology. The biological characterization of the recombinant virus, rLS-HN-ex, showed that the HN C-terminal extension slightly attenuated the virus pathogenicity in embryonated eggs and in day-old chicks when compared to the parental LaSota virus. However, the HN C-terminal extension did not alter virus tissue tropism. In infected chickens, the recombinant virus was detected and re-isolated from the tracheal tissue, but not from the intestinal tissue, exhibiting a similar respirotropic tissue preference as its parental LaSota strain. These results demonstrated that the HN protein C-terminal extension of NDV is not the determinant of the virus enteric tropism.

Keywords Newcastle disease virus · HN gene C-terminal extension · Enteric tropism · Reverse genetics

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Introduction

Newcastle disease virus (NDV) is the causative agent of Newcastle disease (ND), one of the most important poultry diseases worldwide, affecting a wide variety of birds and causing significant economic losses to the poultry industry [1]. The severity of disease depends on the virus strain and the host species. On the basis of pathogenicity for chicken, NDV strains have been classified into lentogenic (low virulent), mesogenic (intermediate virulent), or velogenic (highly virulent) pathotypes [2]. Lentogenic viruses produce disease with little or no clinical signs, while mesogenic viruses can produce more severe disease and histological lesions. Velogenic strains can cause significant respiratory, neurological, and digestive tract pathology with high mortality [3]. Despite intensive vaccination programs implemented since 1950s, it appears that the threat NDV poses to commercial poultry has not diminished.

NDV belongs to the family Paramyxoviridae, subfamily Paramyxovirinae, in the genus *Avulavirus* [4], and possess a non-segmented negative-sense single-stranded RNA genome, which consists of approximately 15.2 kb nucleotides and contains six genes, encoding the nucleocapsid protein (NP), phosphoprotein (P), matrix protein (M), fusion protein (F), hemagglutinin-neuraminidase protein (HN), and large

polymerase protein (L), in the order 3′leader-NP-P-M-F-HN-L-5′trailer. The genome RNA together with NP, P, and L proteins forms the ribonucleoprotein complex (RNP), which serves as the active template for transcription and replication of the viral genome [5].

The HN protein of NDV is a multifunctional, type II integral membrane glycoprotein. It spans the membrane once and possesses three distinct domains: an N-terminal transmembrane domain, a stalk region, and a large C-terminal globular domain. Many studies have shown that the C-terminal globular domain contains the sites for receptor recognition, neuraminidase (NA) activity, and antibody binding, and plays an important role in viral replication cycle [6]. The globular domain is responsible for recognition and absorption of the virus to sialic acid containing receptors on cell surfaces [7]. The binding of globular domain to the receptor induces the conformational change on HN protein, which triggers the activation of the F protein and initiates membrane fusion [8]. In addition, it possesses NA activity, the ability to release sialic acids from the surface of virions and infected cells, preventing self-agglutination of viral particles during budding [9].

Due to the varying location of the termination codon within the HN protein C-terminus, NDV HN genes have demonstrated three different genotypes resulting in proteins of 571, 577, or 616 amino acids in length [10]. The HN proteins of 616 amino acids (additional 39 or 45 amino acids at C-terminal) appear to be a HN_0 precursor which needs to be processed into biologically active HN by removing 42 residues at its C-terminal [11–13]. In contrast, most NDV strains bearing a HN protein of 577 or 571 amino acids in length do not need the HN protein cleavage process to remove any residues for its functionality [14]. It is interesting to note that, Australian NDV isolates with the HN C-terminal extensions generally replicate in the intestine of the birds, although all of them exhibit a lentogenic phenotype at F protein cleavage site; however, those with a shorter HN protein were shown to be associated with summer respiratory disease and virus replication in the lungs and respiratory tract [15, 16]. Furthermore, the routinely used NDV enterotropic vaccine strains, such as Queensland V4, D26, Ulster, and PHY-LMV42, also possess the additional 39 amino acids at HN protein C-terminal when compared with the commonly used NDV respirotropic vaccine strains, such as B1 and LaSota (Fig. 1a). Thus, it has long been thought that the HN protein C-terminal extension may contribute to the enteric tropism of NDV [15].

In this study, we generated a recombinant NDV by inserting the HN protein C-terminal 39 amino acids derived from the enterotropic PHY-LMV42 strain into the HN gene of the respirotropic LaSota strain using reverse genetics techniques to assess the role of the HN C-terminal extension in virus tissue tropism. The pathogenicity of the recombinant virus, rLS-HN-ex, and its parental LaSota strain was assessed by conducting the standard mean death time (MDT) and intracerebral pathogenicity index (ICPI) assays. Growth ability of the recombinant and its parental viruses in tracheal and intestinal tissues was examined by virus isolation and titration. Analysis of the results allowed us to gain a better understanding of the role of HN protein extension in virus tissue tropism.

Materials and methods

Cell line, virus and RNA preparation

HEp-2 and DF-1 cell lines were grown in Dulbecco's Modified Eagle Medium (DMEM, Invitrogen, Carlsbad, CA) supplemented with 10 % fetal bovine serum (FBS, Invitrogen) and antibiotics (100 U/ml penicillin, 100 μg/ml streptomycin, 0.25 μg/ml amphotericin B, Thermo Scientific). The NDV LaSota strain was obtained from the pathogen repository bank at the Southeast Poultry Research Laboratory (SEPRL, USDA–ARS, Athens, GA, USA). The NDV PHY-LMV42 strain was obtained from CEVA Biomune (Lenexa, KS, USA). The previously rescued LaSota strain virus (rLS) was used as a control [17]. The modified vaccinia Ankara/T7 recombinant virus (MVA/T7) was used during virus rescue to provide the bacteriophage T7 RNA polymerase [18]. Viral RNA from the NDV-infected chicken embryo allantoic fluid was extracted using the TRIzol-LS reagent according to the manufacturer's instructions (Invitrogen).

Construction of the recombinant cDNA clone containing additional 39 amino acids at HN protein C-terminal

The infectious LaSota clone (pFLC-LaSota) previously generated [17] was used as backbone to construct a recombinant cDNA clone containing additional 39 amino acids at HN protein C-terminal as illustrated in Fig. 1b. Briefly, cDNA coding for HN extensions of 39 amino acids and a stop codon was generated by RT-PCR amplification from NDV PHY-LMV42 strain genomic RNA with a pair of specific primers (LS HN ex F and LS HN ex R, Table 1) using a Superscript™ III One Step RT-PCR system with Platinum Taq Hi-Fi kit (Invitrogen). Then the HN extension fragment was inserted immediately downstream of the HN gene ORF to replace the corresponding noncoding sequences in the pFLC-LaSota vector which was linearized by PCR with a pair of specific primers (LS HN ex Vet up and LS P-M Vet down, Table 1), using the In-Fusion® PCR Cloning Kit (Clontech). The resulting recombinant clone contained additional 39 amino acids at its HN protein

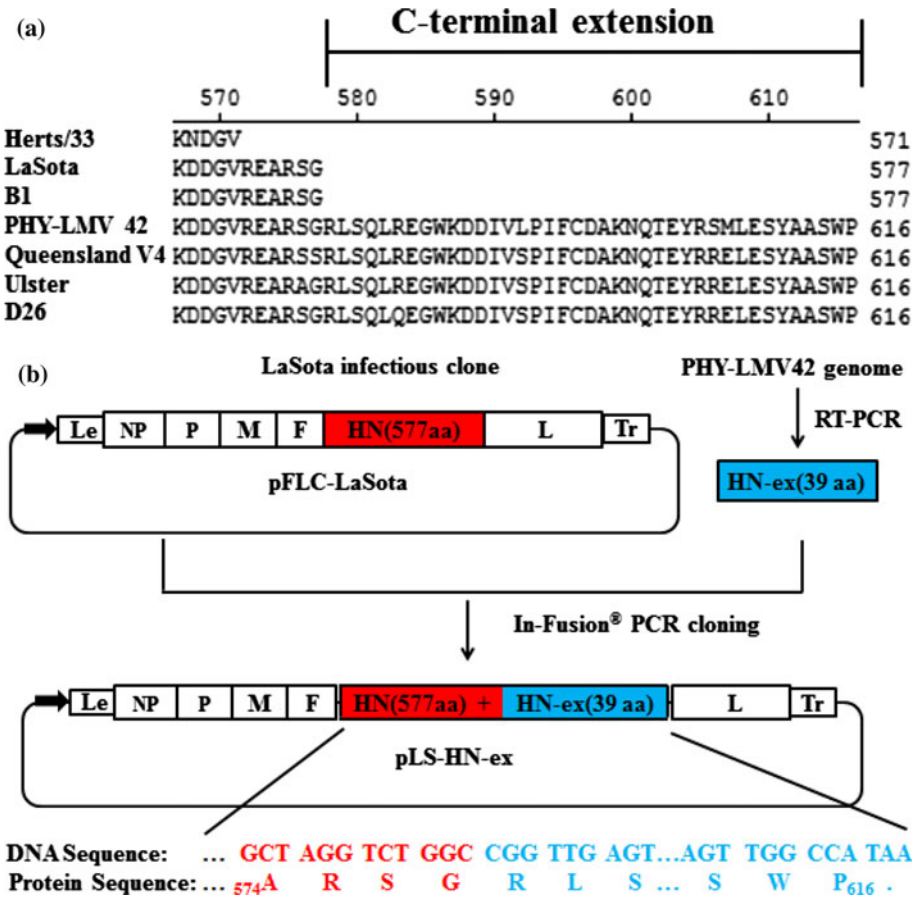

Fig. 1 Amino acid alignment of HN C-terminus of NDV strains and schematic representation of pLS-HN-ex construction. **a** Amino acid alignment of HN C-terminus of NDV strains. NDV strains demonstrated three different genotypes according to the different location of the termination codon. The HN proteins of 571 amino acids were only detected in velogenic strains, such as Herts/33. In contrast, the longest HN protein, 616 amino acids in length, has only been found in lentogenic NDV with enterotropic tissue tropism, such as PHY-LMV42, Queensland V4, Ulster, and D26 strain. The respirotropic NDV vaccine strains, such as B1 and LaSota, contain the HN gene with 577 amino acids in length. **b** Schematic representation of pLS-HN-ex construction. cDNA coding for the PHY-LMV42 strain HN C-terminal 39 amino acids (HN-ex), generated from virus genomic RNA, was inserted immediately downstream of the HN gene ORF of the pFLC-LaSota vector by using the In-Fusion® PCR cloning kit (Invitrogen). The recombinant clone, pLS-HN-ex, contained the HN gene with 616 amino acids (additional 39 amino acids) in length

C-terminal, designated as pLS-HN-ex, was amplified in Stbl2 cells at 30 °C for 24 h and purified using a QIAprep Spin Miniprep kit (Qiagen).

Virus rescue and propagation

The procedures of transfecting the full-length cDNA clone and supporting plasmids into HEp-2 cells were described previously [19]. In brief, the HEp-2 cells were grown overnight in a six-well plate to ~80 % confluency and infected with MVA/T7 which provided the T7 polymerase at a multiplicity of infection (MOI) of 3. Subsequently, the cells were cotransfected with 2 μg of pLS-HN-ex, 1 μg of pTM-NP, 0.5 μg of pTM-P, and 0.1 μg of pTM-L by using 6 μl Lipofectamine™ 2000 (Invitrogen). After incubation for 6 h at 37 °C, the cells were washed once with PBS buffer and maintained in DMEM medium containing 2 % FBS. At 72 h post-infection, the rescued virus, rLS-HN-ex, was harvested by freeze–thawing the infected cells three times. The rescued virus was amplified by inoculating 100 μl of the cell lysate into the allantoic cavity of 9-day-old specific pathogen free (SPF) chicken embryos and incubating the embryos at 37 °C. After 4 days of incubation, the allantoic fluid (AF) was harvested and used for detection of rescued virus by the hemagglutination (HA) test. The AF was terminally diluted during subsequent passages to remove any possible MVA contamination. The rescued virus that had been positively confirmed by the HI test was amplified in SPF chicken embryos three times and the AF was harvested, aliquoted, and stored at −80 °C as a stock.

Table 1 Primer sequences used in the study

Primer	Primer sequence[c]	Primer name
1[a]	5′-gaagctaggtctggcCGGTTGAGTCAACTG-3′	LS HN ex F
2[a]	5′-atgagcactggctgaTTATGGCCAACTGGC-3′	LS HN ex R
3[b]	5′-gccagacctagcttcTCTAA-3′	LS HN ex Vet up
4[b]	5′-tcagccagtgctcatGCGAT-3′	LS HN ex Vet down

[a] Primers 1 and 2 were used to RT-PCR amplify the HN extension region from NDV PHY-LMV42 strain genomic RNA

[b] Primers 3 and 4 were used to amplify or linearize the vector

[c] Nucleotides shown in lower case letters represent homology sequences with a vector backbone, which were used to facilitate the RE independent cloning using the In-Fusion® PCR cloning kit (Clontech)

Sequence analysis

The full-length plasmid and purified RT-PCR product generated during the FLC construction or from the rescued recombinant virus were sequenced directly with M13 universal primers or specific primers using the Applied Biosystems-PRISM fluorescent big dye sequencing kit and the ABI 3730 DNA sequencer (ABI, Foster City, CA). Nucleotide sequence editing, assembling, and comparison analysis were carried out using the DNASTAR program (Madison, WI).

Virus titration and pathogenicity assay

Analyses of the virus stock titers were performed by conducting the standard HA test in a 96-well microplate, the 50 % tissue infectious dose ($TCID_{50}$) assay on DF-1 cells, and the 50 % egg infective dose (EID_{50}) assay in 9-day-old SPF chicken embryos [20]. Virus titers in cell cultures and eggs were calculated by the Reed and Muench method [21]. The pathogenicity of the recombinant viruses, rLS-HN-ex and rLS, were assessed by performing the standard mean death time (MDT) and intracerebral pathogenicity index (ICPI) tests [20]. The MDT was statistically analyzed using the unpaired, two tailed Student's *t* test with a 1 % level of significance (Microsoft Excel, Redmond, WA).

Detection of recombinant virus rLS-HN-ex in chicken trachea and intestine

A group of forty 1-day-old chickens were randomly divided into three groups of 10 birds each and housed in Horsfal isolators (Federal Designs, Inc., Comer, GA) with ad libitum access to feed and water in the SEPRL BLS-2E animal facility. Birds in groups 1, 2, and 3 were inoculated with 100 µl of 1.0×10^7 EID_{50}/ml of rLS, rLS-HN-ex, and NDV PHY-LMV42 strain, respectively, with 50 µl intranasal (IN) and 50 µl intraocular (IO). Three birds from each group were killed at 2, 4, and 6 days post-inoculation (DPI) and tissue samples (trachea and intestine) were collected and homogenized for virus titration. The virus titers were determined in 9-day-old SPF chicken embryos and expressed as EID_{50}. The virus titers were statistically analyzed using the unpaired, two tailed Student's *t* test with a 1 % level of significance (Microsoft Excel, Redmond, WA).

Results

Generation of the rLS-HN-ex virus

A cDNA fragment of 120 nucleotides (nts) coding for the PHY-LMV42 strain HN C-terminal 39 amino acids and a stop codon was successfully amplified, and inserted into the LaSota full-length clone at the HN protein C-terminal through RT-PCR and In-Fusion PCR cloning (Fig. 1b). The total length of the full-length cDNA clone was 15,186 nts and divisible by 6, which abides by the "Rule of Six" [22]. The plasmid pLS-HN-ex was cotransfected into HEp-2 cells with three supporting plasmids expressing the NP, P, and L proteins. At 72 h post-infection, the supernatants were harvested and inoculated into SPF chicken embryonated eggs. After 4 days of incubation, the rescued recombinant virus rLS-HN-ex was harvested and confirmed by HA and HI test. Sequencing analysis of the RT-PCR products of the viral genome verified the HN C-terminal extension in the LaSota genome (Fig. 2), and confirmed the nucleotide sequence fidelity (data not shown).

Biological characterization of the rLS-HN-ex virus

To evaluate the influence of the HN extension on NDV replication and pathogenicity, the rescued virus was examined in vitro and in vivo by conducting virus titration, MDT, and ICPI assays according to the standard procedures [20]. As shown in Table 2, the recombinant virus appeared to be slightly attenuated in embryonated eggs and day-old chickens with a longer MDT (142.8 h) and a lower ICPI (0.0) than the parental LaSota strain. The HA titers, EID_{50}, and $TCID_{50}$ of the recombinant virus measured in either embryonated eggs or in DF-1 cells were approximately one log lower than that of the parental rLS virus (Table 2), indicating that the HN C-terminal extension marginally affected the growth ability of the virus.

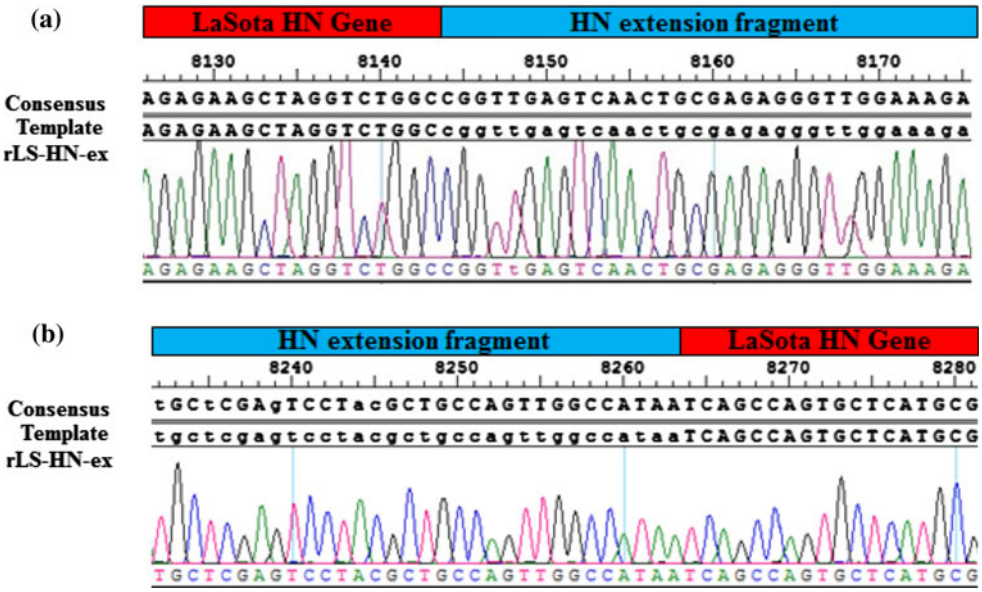

Fig. 2 Sequencing analysis of the recombinant virus rLS-HN-ex at the insertion region. **a** The junction of the LaSota HN gene and the 5′ end of the HN extension insert derived from the HN PHY-LMV42 strain. **b** The junction of the 3′ end of the HN extension and the downstream noncoding region of the LaSota HN gene. To validate the rescued virus, RT-PCR products of the viral genome were sequenced with M13 universal primers or specific primers using the Applied Biosystems-PRISM fluorescent big dye sequencing kit and the ABI 3730 DNA sequencer (ABI, Foster City, CA)

Growth of the rLS-HN-ex virus in day-old chicken trachea and intestine

To investigate if the HN protein C-terminal extension affects the virus tissue tropism, the virus replication was examined in day-old chicken trachea and intestine by virus titration. As shown in Fig. 3a, all three viruses, rLS, rLS-HN-ex, and PHY-LMV42 strain, were able to grow in the tracheal tissues with a titer of approximately 3–5 $\log_{10}$ EID_{50}/g from 2 to 6 days post-infection. It appeared that the rLS and rLS-HN-ex replicated consistently in the tracheas tissues during the first 6 days of infection, although the titer of rLS-HN-ex was slightly lower than that of rLS; whereas the replication of the PHY-LMV42 strain decreased slightly as the infection progressed. Nevertheless, there were no statistically significant difference in virus titers among these three viruses in trachea ($p > 0.05$). In contrast, replication of the recombinant virus rLS-HN-ex and its parental virus rLS in the gastrointestinal tract tissues could not be detected on any of three testing days post-inoculation (Fig. 3b). As expected, the enterotropic NDV PHY-LMV42 strain was able to replicate in the intestine with a relatively higher titer (5.16–6.07 $\log_{10}$ EID_{50}/g) (Fig. 3b) than that (2.99–4.8 $\log_{10}$ EID_{50}/g) of the virus in trachea (Fig. 3a). These results indicated that the HN C-terminal extension did not alter the recombinant virus tissue tropism, and the rLS-HN-ex virus still exhibited a similar respirotropic tissue preference as its parental LaSota virus.

Table 2 Biological assessments of the NDV recombinant virus

Virus	MDT (h)[a]	ICPI[b]	HA[c]	EID_{50}^{d}	$TCID_{50}^{e}$
rLS	122	0.2	1,024	2.37×10^9	1.76×10^9
rLS-HN-ex	143	0	1,024	3.87×10^8	1.76×10^8

[a] Mean death time assay in embryonating eggs

[b] Intracerebral pathogenicity index assay in day-old chickens

[c] Hemagglutination assay

[d] The 50 % egg infective dose assay in embryonated eggs

[e] The 50 % tissue infectious dose assay on DF-1 cells

Discussion

In the present study, we have successfully constructed and rescued a NDV respirotropic strain LaSota-based recombinant virus, rLS-HN-ex, possessing a HN protein C-terminal extension of 39 amino acids derived from the enterotropic NDV PHY-LMV42 strain using reverse genetics techniques. The rescued recombinant virus was evaluated in vitro and in vivo for its biological properties to investigate the role of the HN C-terminal extension of NDV in virus virulence and tissue tropism. The MDT and ICPI results indicated that the HN C-terminal extension slightly attenuated virus replication in embryonated chicken eggs and day-old chickens compared to its parental virus rLS. The exact mechanism by which this recombinant virus was marginally attenuated is not clear, but it is likely that its HN protein with the C-terminal extension would require a step of cleavage process to be biologically active as found in some of asymptomatic NDV strains that contain the HN C-terminal extension [11–13]. This step of cleavage process might slow down the virus replication and partially contribute to the marginal virulence attenuation.

The HN protein has been shown to play an important role in tissue tropism independent of the amino acid sequence of the F protein [23, 24]. In the meanwhile,

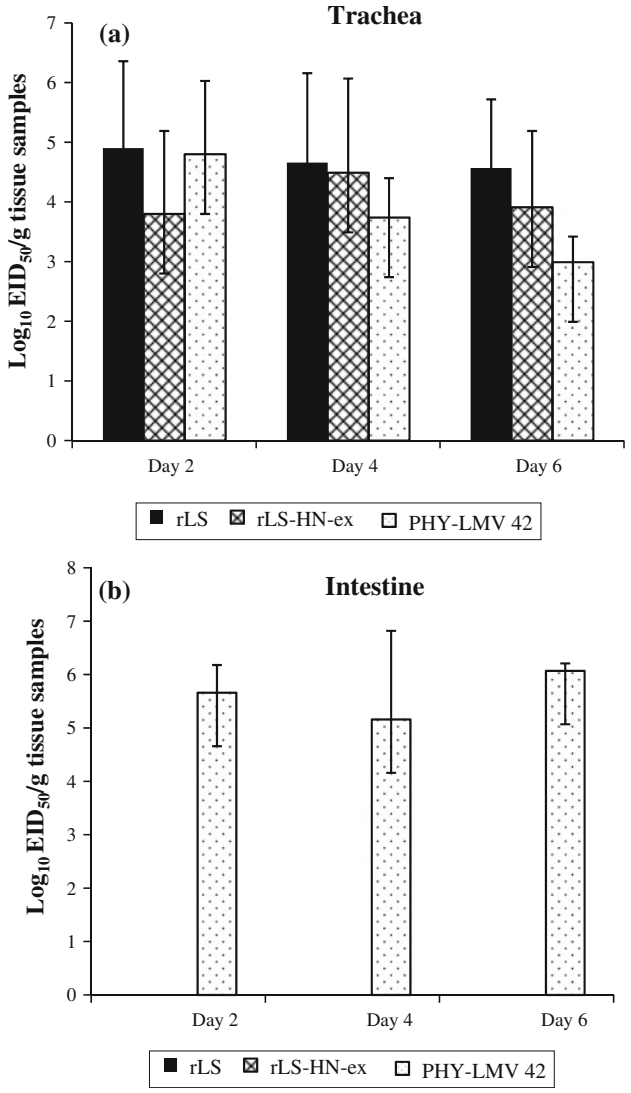

Fig. 3 Comparison of mean titers of NDV rLS, rLS-HN-ex, and PHY-LMV 42 viruses detected from trachea (**a**) and intestine (**b**). Chickens in each group were inoculated with rLS, rLS-HN-ex, or PHY-LMV42 strain, respectively, with 50 µl intranasal (IN) and 50 µl intraocular (IO). Three birds from each group were killed at 2, 4, and 6 days post-inoculation (DPI) and tissue samples (trachea and intestine) were collected and homogenized for virus titration. Virus titers were determined in eggs and expressed as EID_{50}/g for tissue samples

several studies on NDV pathogenesis have suggested that there is a correlation between the size of the HN protein and the tissue tropism. This is inferred from the fact that several NDV enterotropic vaccine strains (Queensland V4, D26/27, Ulster, and PHY-LMV42 strains) and Australian NDV isolates with HN extensions of 39 amino acids generally replicate in the intestine of the birds, while NDV associated with respiratory diseases usually have much smaller extensions [15, 16]. However, this assumption has so far neither been proved nor disproved. To test the hypothesis that HN protein C-terminal extension affects the virus tissue tropism, we examined the recombinant rLS-HN-ex virus replication in the tracheas and intestines of chickens infected with both viruses and a control. Our result showed that the recombinant virus rLS-HN-ex replicated in trachea, but not in the intestine. This result indicated that the HN protein C-terminal extension may not be the molecular basis of the NDV enterotropic characterization, nor the determinant of the virus tissue tropism.

Several reverse genetics studies have addressed the contribution of HN to tissue tropism by exchanging genes between strains. For example, de Leeuw et al. [25] exchanged the HN gene, globular head of HN protein and stem region of HN protein between the lentogenic LaSota virus and velogenic strain Herts, respectively, and showed that both the stem region and globular head of the HN protein are involved in determining virus tropism. Another study also confirmed this finding by exchanging the HN genes of a virulent Beaudette C strain and an avirulent LaSota strain [23]. The recombinant LaSota virus containing the HN protein of the virulent rBeaudette C strain exhibited a tissue preference similar to that of the virulent virus, and vice versa. The tissue tropism of the viruses was shown to be dependent on the origin of the HN protein. It is important to note that, the LaSota, Beaudette C, and Herts strains used in these studies all possess a smaller HN protein, rather than 616 amino acids. Thus, taking together with our result, it would be reasonable to conclude that the HN C-terminal extension is not the determinant of the virus enteric tropism. However, we can not rule out the possibility that the C-terminal extension may be still important, but require additional viral/host factors to alter the tissue tropism.

In summary, in the present study we successfully generated a NDV respirotropic strain LaSota-based recombinant virus, rLS-HN-ex, containing additional 39 amino acids at HN protein C-terminal. This recombinant virus was slightly attenuated and exhibited a similar tissue preference as rLS. All of these results suggested that the HN protein C-terminal extension is not the determinant of the virus enteric tropism.

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