Vaccine xxx (2014) xxx–xxx

Contents lists available at ScienceDirect

Vaccine

journal homepage: www.elsevier.com/locate/vaccine

Novel in-ovo chimeric recombinant Newcastle disease vaccine protects against both Newcastle disease and infectious bursal disease

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ARTICLE INFO

Article history:
Received 25 September 2013
Received in revised form 1 January 2014
Accepted 14 January 2014
Available online xxx

Keywords:
Recombinant chimeric Newcastle disease virus
Newcastle disease
Infectious bursal disease
In-ovo vaccination

ABSTRACT

Development of a safe and efficient in-ovo vaccine against Newcastle disease (NDV) and very virulent infectious bursal disease virus (vvIBDV) is of great importance. In this study, a chimeric NDV LaSota virus with the L gene of Clone-30 (rLaC30L) was used to generate a recombinant chimeric virus expressing the VP2 protein of vvIBDV (rLaC30L-VP2). The safety and efficacy of rLaC30L-VP2 in-ovo vaccination was then evaluated in 18-day-old special pathogen free (SPF) chicken embryos and commercial broiler embryos for prevention of NDV and vvIBDV. Hatchability and global survival rate of the hatched birds was not affected by in-ovo rLaC30L-VP2 vaccination. However, rLaC30L-VP2 in-ovo vaccination induced significant anti-IBDV and anti-NDV antibodies in SPF birds and commercial broilers, and 100% of vaccinated chickens were protected against a lethal NDV challenge. In-ovo rLaC30L-VP2 vaccination also provided resistance against vvIBDV challenge in a significant amount of animals. These results suggest that rLaC30L-VP2 is a safe and efficient bivalent live in-ovo vaccine against NDV and vvIBDV.

1. Introduction

Newcastle disease virus (NDV) and infectious bursal disease virus (IBDV) cause highly contagious and deadly disease that result in severe economic losses in the poultry industry [1,2]. Thus, vaccination has become an essential mean of preventing ND and IBD outbreaks. Current vaccines include live vaccines and inactivated vaccines, and each has limitations. Live vaccines conflict with maternal antibodies remained and inactivated vaccines are expensive [3].

In-ovo vaccination strategy is a safe, effective and convenient method for large-scale delivery of vaccines in the poultry industry [4–6], and has been used to protect chickens from Marek's diseases since 1982 [7]. Current NDV live vaccines, which are usually used after chicks hatch, cause embryonic lethality are not safe for in-ovo vaccination [5]. Recently, a complex of IBDV live vaccine and anti-IBDV antibodies has been developed for in-ovo vaccination [4], but the rate of IBDV live vaccine and anti-IBDV antibodies in the complex is crucial for in-ovo inoculation safety. Thus, developing a safe and effective in-ovo vaccine against NDV and IBDV is still needed.

The NDV genome encodes at least six proteins, of which fusion protein [8,9], hemagglutinin–neuraminidase protein [10,11], and large polymerase protein (L) [12] play a considerable role in NDV virulence. In this study, we replaced the L gene of the LaSota NDV with that of a lentogenic NDV strain, Clone-30 to create a chimeric NDV virus. We then constructed a new recombinant virus expressing the major host-protective antigen VP2 protein of IBDV using this chimeric NDV vector. Our aims were to develop a safe in-ovo vaccination delivery method using SPF embryos, and to test the efficacy of this vaccination in the preventing ND and IBD in NDV antibody-positive chickens.

2. Materials and methods

2.1. Viruses and cells

BHK-21 cells were grown in Eagle's minimum essential medium containing 10% fetal bovine serum. The NDV vector virus rL was rescued from the genomic cDNA of the NDV LaSota vaccine strain [13]. Recombinant NDV strains were grown and titrated in 9-day-old specific-pathogen-free (SPF) chicken egg embryos by inoculation of the allantoic cavity. The modified vaccinia strain Ankara expressing the T7 RNA polymerase (a generous gift from Dr. Bernard Moss, National Institutes of Health) [14] was grown and titrated in

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0264-410X/$ – see front matter © 2014 Published by Elsevier Ltd.
http://dx.doi.org/10.1016/j.vaccine.2014.01.020

primary chicken embryo fibroblasts. The very virulent IBDV strain GX (vvIBDV GX), was isolated from the bursa of a chicken that died from IBDV in the Guangxi Province of China [15]. Viral titration results were calculated according to Reed and Muench [16]. All viruses were stored at −70 °C before use.

2.2. Eggs and chickens

Embryonated eggs from SPF white Leghorn chickens or commercial broiler breeders (from a local chicken farm) were used. Eggs were incubated at 37.5 °C and a relative humidity of approximately 60%. All chicks were hatched in a contained environment and transferred to negative pressure isolators for experiments.

2.3. Construction of full-length chimeric and recombinant plasmids

The full-length antigenomic cDNA of NDV strain LaSota (pLa) was generated as previously described, and used to recover the recombinant virus rLaSota [13].

For this study, the published nucleotide sequences of NDV strains Clone-30 (GenBank accession number Y18898.1) and LaSota (GenBank accession number AY845400) were used to construct the chimeric viruses. To replace the L gene of pLa, two unique *Not* I and *Mlu* I restriction sites were created before the gene start motif and after the gene end motif of the L gene on full-length cDNAs of LaSota and Clone-30 (Fig. 1A). Briefly, the *Not* I-*Mlu* I fragment of the L gene was amplified from both Clone-30 cDNA and pLa, and subcloned into pBluescript (K/S) (Promega, Madison, WI). In the first step, fragment *Xba* I–*Not* I of pLa was PCR amplified using the primers *Xba*I-PF (5′-AATAC*TCTAGA*CCAGATGAG-3′) and *Not* I-PR (5′-ACCATGA*GCCGGC*TTGCCTTG-3′) and the *Not* I-*Mlu* I fragment of the L gene was amplified using the primers *Not* I-PF (5′-TACAAGGCAA*GCCGGC*TCATG-3′) and *Mlu* I-PR (5′-GTCCTAA*CGCGT*CAGGGTTC-3′). The *Mlu* I-*Sac* II fragment of the pLa was PCR amplified using the primers *Mlu* I-PF (5′-GAACCCTGACTCCTTAGGAC-3′) and *Sac* II-PR (5′-AGCCCCGCGGGGGCGCCCTCCCTT-3′) and subcloned between the *Xba* I and *Sac* II sites of pBluescript (K/S), resulting in pBS (*Xba* I–*Sac* II). The second *Not* I–*Mlu* I fragment of L gene was amplified from Clone-30 cDNA using primer C30*Not*I-PF (5′-CAAGGCAA*GCCGGC*TCATG-3′) and C30*Mlu*I-PR (5′-TCCTAA*CGCGT*CAGGGTTCAAC-3′), and was subcloned into the plasmid pBS (*Not* I–*Mlu* I) at the *Eco*RV site. *Not* I and *Mlu* I sites were introduced by mutating two nucleotides individually. *Not* I–*Mlu* I fragment of the L gene was excised from pBS (*Xba* I–*Sac* II) and replaced with other *Not* I–*Mlu* I fragment of L gene excised from pBS (*Not* I–*Mlu* I). The full-length antigenome cDNA of pLa was digested with *Xba* I and *Sac* II to replace the L gene. The full-length cDNA plasmid of rLaSota with the L gene of Clone-30 was designated pLaC30L.

The cDNA of 1359 nucleotides representing the open reading frame (ORF) of the VP2 gene of the vvIBDV Gx strain was amplified using primers 5′-GACTGTTTAAACTTAGAAAAAATACGGGTAGAAGTGCC*ACC*ATGACGA ACCTGCAAGATC-3′ and 5′-GACTGTTTAAACTTACCTTAGGGCCCGGA TTATG-3′, including the gene end and gene start sequences (underlined) [17] and the optimal Kozak sequence (italic) [18]. The VP2 gene of Gx was introduced into the NDV genome in pLaC30L by introducing a *Pme* I site in the P-M intergenic region at position 3165 of the NDV genome. The resultant plasmid was designated pLaC30L-VP2.

2.4. Recovery of chimeric rNDVs

Chimeric recombinant NDVs (rNDVs) were recovered by co-transfection of pLaC30L and pLaC30L-VP2 into BHK-21 cells grown in a 6-well plate with support plasmids similar to a method described previously [13]. The rescued viruses were designated rLaC30L, rLaC30L-VP2.

2.5. Immunofluorescence of chimeric viruses

For immunofluorescence analysis, BHK-21 cells in 24-well plates were infected with rescued virus at a multiplicity of infection (MOI) of 0.1. After 24 h, cells were treated as described previously [13]. Primary antibodies were specific chicken polyclonal sera against IBDV or monoclonal antibodies against NDV. Secondary antibodies were FITC-conjugated rabbit-anti-chicken IgG (Sigma) and TRITC-conjugated goat-anti-mouse IgG (Sigma). DAPI (Sigma) was used to stain the cell nucleus.

2.6. Western blotting

BHK cells were infected with rescued virus at MOI of 0.5. After 24 h incubation at 37 °C, the medium was replaced with 2 ml Opti-MEM (Invitrogen Corp., Carlsbad, CA, USA) containing 2 μg of TPCK trypsin and the cells were incubated for another 12 h. VP2 expression was confirmed by using western blotting as described previously [13]. Cellular lysates were separated by using SDS–12%PAGE under denaturing conditions for Western blot analyses with chicken serum against NDV or VP2 or monoclonal antibody against β-actin (sigma). Chicken serum or mouse monoclonal antibody binding were detected with IRDye 700DX® conjugated goat-anti-chicken IgG (Rockland) or HRP-conjugated goat anti-mouse IgG (Sigma).

2.7. Growth characteristics of chimeric viruses in chicken embryos

To analyze the replication kinetics of chimeric viruses in chicken embryos, 10^3 EID_{50} of virus was inoculated into the allantoic cavities of 10-day-old SPF chicken embryos. Three embryos were chilled at 24-h intervals, allantoic fluid samples were harvested, and virus titers in samples were determined by EID_{50}.

2.8. Pathogenicity

The pathogenicity of rescued chimeric viruses was determined by the mean death time (MDT) test in 10-day-old SPF chicken embryos as described previously [13], the intracerebral pathogenicity index (ICPI) test in one-day-old SPF chicks as recommended in the OIE Manual [19], and the intravenous pathogenicity index (IVPI) test in 6-week-old SPF chickens [13].

2.9. In-ovo vaccination

Five groups of 50 18-day-old SPF chicken embryos were inoculated with graded doses of vaccines ($10^{5.5}$, $10^{4.5}$, $10^{3.5}$, $10^{2.5}$ EID_{50}) or PBS via the amniotic route. Briefly, embryos were candled for viability followed by disinfection of the egg shell. A small hole was punched through the large end with a drill. Vaccines were injected using a 21-gauge needle at the depth of one inch to make sure the large majority of the inoculum into the amniotic cavity. In addition, five groups of 50 NDV antibody-positive 18-day-old chicken embryos from a local commercial broiler chicken farm were inoculated in-ovo with graded doses of vaccine ($10^{6.5}$, $10^{5.5}$, $10^{4.5}$or $10^{3.5}$ EID_{50}) or PBS. Chicks were hatched in separate

Please cite this article in press as: Ge J, et al. Novel in-ovo chimeric recombinant Newcastle disease vaccine protects against both Newcastle disease and infectious bursal disease. Vaccine (2014), http://dx.doi.org/10.1016/j.vaccine.2014.01.020