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Characterization of a Recombinant Newcastle Disease Virus Vaccine Strain▽

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A recombinant La Sota strain (KBNP-C4152R2L) in which fusion (F) and hemagglutinin-neuraminidase (HN) genes were replaced with those of a contemporary genotype VIId virus, KBNP-4152, has been developed. To attenuate the virulence of the recombinant strain, the F cleavage motif was mutated from $_{112}$RRQKR$_{116}$ to $_{112}$GRQAR$_{116}$, and to reduce pathogenic instability, a codon which does not allow changes to basic amino acids by single point mutation was inserted at codon 115. In addition a six-nucleotide sequence was inserted into the intergenic region between matrix protein and F genes for attenuation without breaking the "rule-of-six." The HN protein length was increased from 571 to 577 as a marker. Serological tests revealed that the antigenicity of KBNP-C4152R2L was similar to that of KBNP-4152 but distinct from that of the La Sota strain. KBNP-C4152R2L was avirulent (intracerebral pathogenicity index, 0.0; mean death time, >168 h) and stable in pathogenicity through in vivo passages. The killed oil emulsion of and live KBNP-C4152R2L were completely protective against mortality and egg drop caused by virulent strains, and KBNP-C4152R2L was applicable to in ovo vaccination. Therefore, KBNP-C4152R2L is a promising vaccine strain and viral vector in terms of antigenicity, productivity, safety, and pathogenic stability.

Newcastle disease virus (NDV) is an enveloped, single-stranded negative-sense RNA virus which belongs to the *Avulavirus* genus of the family *Paramyxoviridae* (33). NDV strains have been classified into three pathotypes from low to high virulence: lentogen, mesogen, and velogen. These classifications are based on conventional in vivo pathogenicity indices: the mean death time (MDT) of chicken embryo and the intracerebral pathogenicity index (ICPI) (4).

The NDV genome is comprised of six genes: nucleoprotein (NP), phosphoprotein (P), matrix protein (M), fusion glycoprotein (F), hemagglutinin-neuraminidase (HN) glycoprotein, and large polymerase protein (L) (37). The F and HN glycoproteins are present on the virion envelope, enabling the virion to attach to and enter a target cell. The dibasic amino acids at the proteolytic cleavage site ($_{113}$R-X-K/R-R$_{116}$) in the F protein are relevant to systemic replication, and the HN protein determines the tropisms of NDV (10, 19, 39). The V protein which is expressed via RNA editing of the P gene and the structure of noncoding regions of the viral genome such as gene start, gene end, and intergenic region (IGR) also contribute to virulence and replication of *Paramyxovirus* (12, 23, 35, 48, 54).

On the basis of phylogenetic analysis of the F gene, NDVs are grouped into 10 genetic groups (I to X). The genetic groups VI and VII are subdivided into subgenotypes (VIa to VIh and VIIa to VIIe) (7, 17, 25, 27, 29, 30, 56, 58, 59). Genotypes II, VI, VII, VIII, IX, and X have been reported in China and Taiwan, but only genetic groups VIf, VIIa, and VIId have been reported in Korea (25, 27, 29, 56). Recently, VIId viruses have been found to be prevalent in the Far East (28).

Serologically, NDV is categorized into the serogroup avian paramyxovirus 1 (50), and it has been further classified into distinct antigenic subtypes by panels of monoclonal antibodies (MAbs) (2, 6, 50). F and HN proteins are protective antigens, and their epitopes have been mapped by using MAbs. Several conformational epitopes of F and HN proteins and one major linear epitope composed of 345 to 353 amino acid residues of HN protein have been defined (15, 21, 22, 41, 55). A single amino acid mutation (E347K) in the linear epitope of HN protein enables the virus to evade neutralization by a specific MAb (15, 36). The E347K mutation in field NDV strains is not rare, and in fact, an antigenic variant, KBNP-4152 in Korea, and some NDV strains registered in GenBank were identified as carrying the exact same or similar mutations in the linear epitope (8, 9, 24). In Korea, an intensive vaccination policy has been implemented and annual use of ND vaccine has increased, but ND outbreaks have still occurred periodically across the nation. Economic losses, mainly resulting from decreased egg production rates (egg drops), even on well-vaccinated farms have raised questions about the antigenic variation of NDV and the efficacy of conventional vaccines (8). Although conventional vaccine strains have been considered to be safe and stable, theoretically they can convert to virulent strains by single point mutation in the proteolytic cleavage site of the F protein. In reality, such conversions of vaccine strains to virulent pathogens by mutation of F protein have been reported (5, 11, 16).

Since the first isolation of NDV from cDNA in 1999, various recombinant NDV vaccine strains with low virulence have been developed (14, 20, 34, 35, 43, 44, 46). However, NDV vaccine strains with all the desirable characteristics including virulence low enough to allow for in ovo vaccination, antigenic

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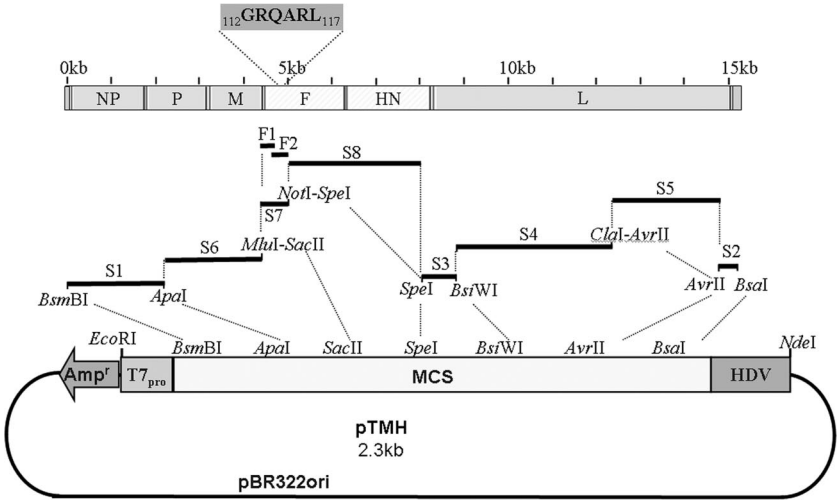

FIG. 1. Schematic strategy of cloning of the full-length NDV genome. Six gene fragments (S1 to S6) spanning the genome of La Sota, except for the F and HN genes, were generated from viral RNA by RT-PCR. After cloning and sequencing of the fragments they were ligated in order from S1 to S6 into the pTMH vector. To generate attenuated and pathologically stable recombinant NDV, a gene fragment (F2, nucleotides 214 to 429 of KBNP-4152) was modified to encode $_{112}$GRQARL$_{117}$ instead of $_{112}$RRQKRF$_{117}$ by DA-PCR. Codon 115 was mutated to CAA (A), which does not allow changes to basic amino acids by any single point mutation. A gene fragment (F1) was amplified from genomic RNA of KBNP-4152, and it was ligated to F2 by splicing-of-extension PCR to generate the S7 fragment. The S7 fragment was ligated between the S6 and S3 fragments, and then S8 was ligated between S7 and S3. The ligation of the full-length NDV cDNA between the T7 promoter ($T7_{pro}$) and the hepatitis delta virus (HDV) ribozyme by BsmBI and BsaI guaranteed the authentic 3′ and 5′ termini without additional nucleotides. Abbreviations: F, fusion protein; Amp^r, ampicillin resistance gene; MCS, multicloning site.

contemporaneity, and genetic stability are still elusive. In the present study a chimeric recombinant La Sota strain (KBNP-C4152R2L) in which F and HN genes were replaced with those of a current antigenic variant, KBNP-4152, was developed and its biological traits, pathogenicity, genetic stability, and immunogenicity were investigated.

MATERIALS AND METHODS

Virus, cells, and antiserum. KBNP-4152 and SNU5074 were isolated from a chicken and a stork, respectively, in Korea as previously reported (9). The strains are antigenic variants that possessed amino acid changes in (E347K) and near (M354K) the linear epitope of HN (8, 9). The KJW strain (AY6304009), which is a classic velogenic strain (genotype III) and a standard challenge strain for vaccine efficacy tests in Korea, has been maintained in our laboratory. The La Sota strain was obtained from the ATCC (ATCC VR-699). Avinew is the most commonly used commercial live vaccine (Merial Ltd.) in Korea, and a commercial vaccine product was directly used in the present study. All the NDV strains were propagated and tested as previously reported (9). The recombinant vaccinia virus (13), which expresses T7 RNA polymerase, was propagated with Vero cells. Chicken embryo fibroblasts (CEF) were cultured in 199 and F10 media (Gibco-BRL, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS; Gibco-BRL). HEp-2 and Vero cells were grown in Dulbecco modified Eagle medium (Gibco-BRL) supplemented with 10% FBS and incubated at 37°C with humidified 5% CO_2. The chicken polyclonal antisera against a lentogenic vaccine strain (La Sota), KBNP-4152, and the recombinant NDV strain (KBNP-C4152R2L) which was recovered in the present study were produced as previously described (8).

Reverse transcription-PCR (RT-PCR) and sequence analysis. La Sota and KBNP-4152 strains in allantoic fluid were pelleted by ultracentrifugation, and the RNA was purified with a Viral Gene Spin kit (iNtRON Biotechnology, Korea) according to the manufacturer's recommendations. Whole viral genome was divided into eight fragments (S1 to S8) (Fig. 1), and primer sets for amplification and cloning of the fragments were designed. The RNA of the La Sota strain was used for amplification of S1 to S6, and that of the KBNP-4152 strain was used for S7 and S8. cDNA was synthesized as previously described (48), and all PCRs were carried out as previously described except that they used high-fidelity *Pfu* DNA polymerase (iNtRON Biotechnology, Korea) (25). The amplified DNA fragments were cloned by using an XL-TOPO TA cloning kit (Invitrogen) after purification, and the nucleotide sequences were determined as previously described (25).

Construction of plasmids. The transcription vector of the full-length NDV genome was constructed as follows. The replicon and ampicillin resistance genes of pBR322 were isolated from pBR322 by digestion with EcoRI and NdeI. They were then ligated to a T7 promoter-multicloning site fragment (T7-MCS) that was synthesized by dual asymmetry-PCR (DA-PCR) (Fig. 1) as previously described (57). The ribozyme sequence of hepatitis delta virus and T7 terminator sequence were amplified by PCR from TV vector. They were then ligated to pBR322 replicon-amp-T7-MCS at the BsaI and NdeI sites located just after T7-MCS, and the resulting plasmid was designated pTMH (Fig. 1). Gene fragments (S1 to S6) spanning the genome of La Sota, except for the F and HN genes, were generated from viral RNA by RT-PCR. Each fragment was cloned into the XL-TOPO TA cloning kit and sequenced, and the fragments were ligated in order from S1 to S6 into the pTMH vector according to the shared restriction enzyme sites (Fig. 1). S7 containing a part of attenuated F of KBNP-4152 was ligated between the S6 and S3 fragments, and then S8 containing the remaining part of F and most of the HN genes of KBNP-4152 was ligated between the S7 and S3 fragments (Fig. 1). The ligation of the full-length NDV cDNA between the T7 promoter and the hepatitis delta virus ribozyme by BsmBI and BsaI guaranteed the authentic 3′ and 5′ termini without additional nucleotides (Fig. 1). The resulting full-length NDV genome transcription vector was designated pTMH-NDV. The plasmids expressing NP, P, and L genes of La Sota from the T7 promoter were generated by cloning full amplicons of NP, P, and L genes into the plasmid pcDNA3.1 Topo (Invitrogen) (pcDNA-NP, pcDNA-P, and pcDNA-L, respectively), and the cloned genes were confirmed by sequencing.

Mutagenesis of F and HN genes. To generate attenuated and pathologically stable recombinant NDV, a gene fragment (F2) containing the cleavage site of the F gene (positions 214 to 429; numbered from A of the start codon of the F gene) was synthesized by DA-PCR. The primers used for DA-PCR were designed based on the nucleotide sequence of KBNP-4152, and the cleavage site sequence was modified to encode $_{112}$GRQARL$_{117}$ instead of $_{112}$RRQKRF$_{117}$. Codon 115 was mutated to CAA (A), which does not allow changes to basic amino acids by any single point mutation. A gene fragment (F1) containing an artificially added six-nucleotide sequence to reduce pathogenicity of NDV without breaking the "rule-of-six" (45) and covering the IGR between M and F and the N-terminal region of F (1 to 213) was amplified from genomic RNA of KBNP-4152. F1 was ligated to F2 by splicing-of-extension PCR to generate the S7 fragment (57). To extend the length of HN from 571 (KBNP-4152) to 577 (La

Sota), the S8 fragment covering partial F and HN (1 to 569) of KBNP-4152 was amplified and ligated to fragment S3 containing positions 569 to 577 of HN and partial L of La Sota (Fig. 1). The ligation resulted in an amino acid change from R to G at residue 570 (R570G) of HN.

Recovery of recombinant NDV. HEp-2 cells grown to 80% confluence in six-well plates were washed twice with Opti-MEM (Invitrogen, CA) just before transfection and infected with 0.3 focus-forming unit/cell of recombinant vaccinia virus expressing T7 RNA polymerase for 1 h. Uninfected viruses were then removed. The transfection mixture was prepared by adding 2 μg of pTMH-NDV, 1 μg of pcDNA-NP, 1 μg of pcDNA-P, 0.1 μg of pcDNA-L, and Lipofectamine 2000 (Invitrogen) to 0.2 ml of Opti-MEM/well, and transfection was conducted per the manufacturer's instructions. After 12 h the medium was replaced with 2 ml of Opti-MEM containing 20 μg/ml of acetylated trypsin (Sigma-Aldrich Co., MI) and 40 μg/ml of cytosine arabinoside to inhibit the vaccinia virus. On the fourth day after transfection, the supernatant was harvested and inoculated into fresh HEp-2 cells, and after a 4-day incubation, virus was propagated in 10-day-old embryonated chicken eggs (ECEs).

Characterization of a recombinant NDV. The MDT was determined for each virus as previously described (4). According to the MDT, the NDV strains were classified as velogenic (<60 h), mesogenic (60 to 90 h), and lentogenic (>90 h). The ICPI was determined as previously described (4). Bacterial contamination of inoculated viruses was excluded by bacterial culture on brain heart infusion agar (Difco, MI). The 50% egg infection dose (EID_{50}) was determined to compare the productivities of NDV strains, and the hemagglutination-elution assay was carried out as previously described (53). CEF in each well were infected with 200 50% tissue culture infective doses ($TCID_{50}$) of each virus and incubated for 3 days with medium containing 0.5% FBS and 20 μg/ml trypsin, and cytopathic effects were examined. The antigenicities of the NDV strains were compared with cross-hemagglutination inhibition (HI) and virus neutralization (VN) tests as previously described (3, 9).

Distribution of the recombinant virus in internal organs. Twenty-one-day-old specific-pathogen-free (SPF) chicks were inoculated intraocularly with 10^7 EID_{50}/chick, and five chicks were sacrificed 3, 5, 7, and 14 days postinoculation (DPI). Tracheas, cecal tonsils, spleens, livers, and kidneys were sampled, and the EID_{50}/ml of pooled samples of each tissue was measured.

Pathogenic stability of the recombinant virus. Twenty-one-day-old SPF chicks were divided into two groups and inoculated with KBNP-C4152R2L and La Sota via the intracerebral route. Chicks were sacrificed at 10 DPI, and homogenized cerebral samples were prepared. Five 10-day-old ECEs were inoculated with each sample (KBNP-C4152R2L-CB1 and La Sota-CB1). KBNP-C4152R2L was passaged 10 times through 10-day-old ECEs (KBNP-C4152R2L-E10), and then five 1-day-old SPF chicks were inoculated intraocularly with 10^7 EID_{50}/chick. On DPI 5 chicks were sacrificed, trachea samples were homogenized, and three 10-day-old ECEs were inoculated with the mixture. The same procedure was repeated five times (KBNP-C4152R2L-E15-C5).

Safety and efficacy of KBNP-C4152R2L as an in ovo vaccine. The 10^6 $TCID_{50}$/0.1 ml of KBNP-C4152R2L or La Sota virus or phosphate-buffered saline (PBS) (control) was injected into the 18-day-old embryos from a commercial broiler breeder, and the hatching rates and health conditions of the chicks were observed until virus challenge. Serum samples were collected at 2, 17, and 34 days, and they were challenged with 1×10^6 $TCID_{50}$ of KBNP-4152 at 35 days. Clinical signs and mortality were observed for 12 days after the challenge.

Protection of egg drops by killed oil emulsion recombinant vaccine. To prepare killed oil emulsion vaccines of La Sota and KBNP-C4152R2L, they were inactivated with 0.3% formaldehyde, and the inactivated allantoic fluid was emulsified with ISA70 oil (30:70, volume/volume ratio). Fifty-eight 115-day-old commercial layer chickens (Hy-Line brown) which had been vaccinated two and three times with commercial oil emulsion and live vaccines, respectively, were divided into two groups. Then, one group (40 chickens) was vaccinated with the KBNP-C4152R2L oil vaccine and the other group (18 chickens) was vaccinated with the La Sota oil vaccine. The two groups were kept in the same room. On DPI 21 serum samples were collected from each group and VN titers for three different virus strains, KJW, KBNP-4152, and SNU5074, were measured. The clinical signs of infection and egg production rates for 1 week before challenge were recorded as control. On DPI 22 all chickens were challenged with 10^5 EID_{50} of SNU5074, and clinical signs of infection and weekly egg production rates were observed for 5 weeks.

Cross-protection of the live KBNP-C4152R2L and the killed KBNP-C4152R2L oil emulsion vaccines against a different genotype (III) virus, KJW. For the live vaccine efficacy test 74 1-day-old SPF chicks were assigned to five groups: a control group and four vaccine groups which were subdivided into four groups according to the vaccine titers from 10^5 to 10^8 EID_{50} (see Table 3). The chicks were inoculated with live KBNP-C4152R2L vaccine via the intraocular route, and serum samples were collected on DPI 21 for the HI test. On DPI 22 all chickens were challenged with 2×10^5 $TCID_{50}$ of a genotype III virus, KJW, via the intranasal route. Challenged chickens were observed for clinical signs and mortality for 14 days.

For the killed oil emulsion vaccine efficacy test 49 7-week-old SPF chickens (Nam-Deog Sanitek Co., Korea) were assigned to three experimental groups: the control group, the recombinant vaccine group, and the La Sota vaccine group (see Table 4). Vaccinated chickens were inoculated with 0.5 ml of each vaccine via the subcutaneous route, and serum samples were collected on DPI 7 and 21 for the HI test. On DPI 22 all chickens were challenged with 2×10^5 $TCID_{50}$ of a heterologous genotype III virus, KJW, via the intranasal route. Challenged chickens were observed for clinical signs of infection and mortality for 28 days.

Statistical analysis. The survival rates between La Sota and KBNP-C4152R2L vaccine groups or between the KBNP-C4152R2L vaccine group and the control group were evaluated via chi-square and Fisher's exact tests, and the average numbers of eggs produced from conventional vaccine and KBNP-C4152R2L vaccine groups at each week postchallenge were compared via Student's *t* test (95% confidence interval), using SPSS for Windows version 12.0.

Nucleotide sequence accession number. The nucleotide sequence of the cloned full-length NDV cDNA was determined and deposited in GenBank (EU140955).

RESULTS

Construction of full-length NDV cDNA. All the nucleotide sequences of the full-length viral cDNA including the molecular markers such as the cleavage site of the F gene encoding $_{112}$GRQARL$_{117}$, R570G of the HN protein, and the MluI site between S6 and S7 were conserved.

Generation of recombinant virus. Ten-day-old ECEs were inoculated with the hemagglutination-positive allantoic fluid to propagate the recombinant NDV strain. The recombinant NDV strain induced syncytia only after treatment with acetylated trypsin, just as La Sota did. The genetic markers which were introduced into the genome of recombinant virus were confirmed by sequencing, and the recombinant NDV strain was designated KBNP-C4152R2L.

Characterization of KBNP-C4152R2L. The pathogenicity of KBNP-C4152R2L was assessed by MDT and ICPI and compared with those of La Sota and KBNP-4152. KBNP-4152 and La Sota were confirmed as velogenic (MDT = 48; ICPI = 1.92) and lentogenic (MDT = 114; ICPI = 0.25) strains, respectively. KBNP-C4152R2L was lentogenic and less virulent than La Sota on the basis of both MDT (>168 h) and ICPI (0). The EID_{50} of KBNP-C4152R2L was determined to be $10^{10.1}$/ml, and it was similar to that of La Sota.

The neuraminidase activities of NDV strains were tested by the elution rate test. According to the test, La Sota was classified as a slow eluter but KBNP-C4152R2L was classified as a rapid eluter together with KBNP-4152. The antigenicity of KBNP-C4152R2L was compared with those of La Sota and KBNP-4152 with the HI test. The mean HI titers of anti-La Sota antiserum were 7.13, 4.13, and 4.50 for La Sota, KBNP-4152, and KBNP-C4152R2L, respectively. Further the mean HI titers of anti-KBNP-4152 antiserum were 9.00, 11.00, and 11.00 for La Sota, KBNP-4152, and KBNP-C4152R2L, respectively. Anti-La Sota and anti-KBNP-4152 antisera inhibited hemagglutination of both KBNP-4152 and KBNP-C4152R2L about eight times less and about four times more effectively than they did that of La Sota, respectively. Therefore, KBNP-C4152R2L is antigenically similar to KBNP-4152 but clearly different from La Sota.

TABLE 1. Distribution and reisolation of KBNP-C4152R2L from internal organs

Organ[a]	3 DPI		5 DPI		7 DPI		14 DPI	
	RR[b] (%)	Titer[c]	RR (%)	Titer	RR (%)	Titer	RR (%)	Titer
Tra	80	$10^{4.5}$	60	$10^{3.5}$	40	$10^{2.2}$	0	NT[d]
CT	60	$10^{2.2}$	0	NT	0	NT	0	NT
Sp	0	NT	0	NT	0	NT	0	NT
Liv	0	NT	0	NT	0	NT	0	NT
Kid	0	NT	0	NT	0	NT	0	NT

[a] Tra, trachea; CT, cecal tonsil; Sp, spleen; Liv, liver; Kid, kidney.
[b] RR, reisolation rate.
[c] EID_{50}/ml.
[d] NT, not tested.

Distribution of KBNP-C4152R2L in internal organs. To understand tissue tropism of KBNP-C4152R2L and duration time of virus shedding, the virus was inoculated into 21-day-old SPF chickens via the intraocular route, and virus isolation was tried on DPI 3, 5, 7, and 14. KBNP-C4152R2L was isolated from cecal tonsils and tracheas but not from spleens, livers, and kidneys in inoculated chickens and did not cause any lesions such as exudates, congestion, and hemorrhages in the examined tissues. KBNP-C4152R2L was isolated from 40 to 80% of the trachea samples between 3 and 7 DPI but not on DPI 14. KBNP-C4152R2L was also present in the cecal tonsils, but it disappeared on DPI 5 (Table 1). Therefore, it can be concluded that KBNP-C4152R2L caused only local infection without systemic spread.

Pathogenic stability of KBNP-C4152R2L. The pathogenicities of passaged viruses KBNP-C4152R2L-E10, KBNP-C4152R2L-E15-C5, KBNP-C4152R2L-CB1, and La Sota-CB1 were assessed by ICPI. KBNP-C4152R2L-E10 and KBNP-C4152R2L-E15-C5 were classified as avirulent strains on the basis of the ICPI values, 0.000 and 0.038, respectively. Interestingly, the virulence of La Sota-CB1 increased steeply (0.963) and it was classified as a mesogen. The high ICPI value of La Sota-CB1 was unexpected, but any bacterial contamination was excluded by bacterial culture. We repeated the same experiment, and the ICPI was 0.7. We analyzed the nucleotide sequence of the cleavage site of F protein by sequencing the amplicon, but we could not find any mutation. KBNP-C4152R2L-CB1 was not recovered from the specimens of inoculated chicks, and so its ICPI could not be determined.

Safety and efficacy of KBNP-C4152R2L as an in ovo vaccine. In ovo vaccination is convenient and effective because of the automated, uniform inoculation of vaccine into individual ECEs. But to date most vaccine strains are not applicable to in ovo vaccination because of virulence for the embryo. To test the applicability of KBNP-C4152R2L to in ovo vaccination, 18-day-old commercial ECEs were inoculated with KBNP-C4152R2L, La Sota, and PBS, and the antibody titer and survival rate of each group were compared with those of the other groups. The La Sota inoculation group showed a significantly lower survival rate until 17 days than did KBNP-C4152R2L and PBS inoculation groups (31.2% versus 88.2%; $P < 0.05$) (Table 2). KBNP-C4152R2L induced constant and slightly increasing HI titers from DPI 17 to 34 against KBNP-4152 and KBNP-C4152R2L, respectively. This was in contrast to the gradual decrease of HI titer in the control group (Table 2). The survival rate of the KBNP-C4152R2L group after challenge with KBNP-4152 was significantly higher than that of the control group (100% versus 13.3%; $P < 0.05$) (Table 2).

Protection from egg drops by a killed oil emulsion vaccine of the recombinant NDV. The field NDV has been suspected to cause egg drops even in highly vaccinated egg layers in Korea, but to date direct evidence has never been presented. Therefore, experimental demonstration of egg drops in highly vaccinated

TABLE 2. Results of safety and efficacy tests for KBNP-C4152R2L as an in ovo vaccine

Group	Dose (log_{10} EID_{50}/egg)	Survival rate after vaccination[a]	Test virus	Mean HI titer (log_2) at chick age (days):			Survival rate after challenge[b]
				2	17	34	
Control	0	88.2 (15/17)	La Sota	6.2 ± 1.5	3.3 ± 1.0	0.5 ± 0.7	13.3*[d] (2/15)
			KBNP-C4152R2L	4.4 ± 1.6	1.2 ± 1.2	0.9 ± 1.0	
			KBNP-4152	5.2 ± 1.5	2.6 ± 1.1	0.7 ± 1.0	
KBNP-C4152R2L	5.1	88.2* (15/17)	La Sota	NT[c]	4.9 ± 1.4	4.0 ± 1.6	100* (15/15)
			KBNP-C4152R2L	NT	5.1 ± 1.6	6.6 ± 1.5	
			KBNP-4152	NT	5.2 ± 1.4	5.5 ± 1.6	
La Sota	5.0	31.2* (5/16)	La Sota	NT	6.0 ± 1.2	4.0 ± 1.4	
			KBNP-C4152R2L	NT	4.6 ± 1.1	4.8 ± 1.8	
			KBNP-4152	NT	5.0 ± 1.0	3.8 ± 0.8	

[a] Survival rate from hatching to 17 days of age after in ovo vaccination with vaccine viruses (10^6 $TCID_{50}$/0.1 ml) and PBS (0.1 ml), shown as percent (number surviving/total number vaccinated).
[b] Survival rate after challenge with KBNP-4152 (1×10^6 $TCID_{50}$/chicken at 35 days of age via intranasal route), shown as percent (number surviving/total number vaccinated).
[c] NT, not tested.
[d] *, significant difference ($P < 0.05$).

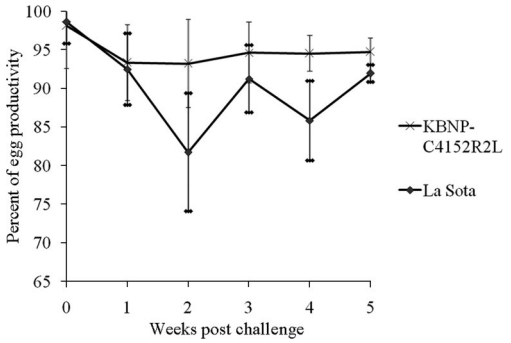

FIG. 2. Comparison of efficacies of the killed KBNP-C4152R2L and La Sota oil emulsion vaccines against egg drops in highly vaccinated commercial layers. Fifty-eight 115-day-old commercial layer chickens (Hy-Line brown) which had been highly vaccinated with commercial vaccines were divided into two groups, KBNP-C4152R2L and La Sota oil vaccine groups. All chickens were challenged with 10^5 EID_{50} of SNU5074, and clinical signs of infection and weekly egg production rates were observed for 5 weeks. The chickens of the two groups were kept in the same room.

layers after challenge with the genotype VIId virus is very important. Highly vaccinated commercial layers were vaccinated with killed oil emulsion vaccines of KBNP-C4152R2L and La Sota, and their vaccine efficacies were evaluated in terms of antibody titers and protection against egg drops caused by a contemporary NDV strain in Korea. The recombinant vaccine group showed higher mean VN antibody titers (log_2) against all virus strains than did the La Sota vaccine group (11.9 ± 0.9 versus 9.4 ± 1.0 for KJW, 13.7 ± 1.0 versus 10.6 ± 1.3 for KBNP-4152, and 13.3 ± 1.1 versus 10.2 ± 1.0 for SNU5074), and the antigenic difference between genotypes III and VIId was not high but was apparent. The egg production rates of each group were compared for 6 weeks: 1 week before challenge and 5 weeks after challenge. The recombinant vaccine group showed no difference in egg production rates during the observation period, but the La Sota vaccine group showed significant egg drops in the second and fourth weeks, 81.7% versus 93.2% and 87.3% versus 94.5%, respectively ($P < 0.05$) (Fig. 2).

Cross-protection of KBNP-C4152R2L live and killed oil emulsion vaccines against a different genotype (III) virus, KJW. To evaluate cross-protection efficacies of the recombinant vaccines, a heterologous genotype III virus, KJW, was used to challenge SPF chickens which had been vaccinated with KBNP-C4152R2L live and killed oil emulsion vaccines. The recombinant live vaccines were highly protective against KJW, resulting in zero mortality even at low titers of vaccine virus, while in the control group there was 100% mortality (Table 3). The lowest dose, 10^5 EID_{50}, of vaccine was completely protective, and the serum-neutralizing titers were as high as those from chicks immunized with up to 1,000-fold more virus. The KBNP-C4152R2L killed oil emulsion vaccine generated higher HI titers than did the live vaccines on DPI 21 and protected chickens completely. This was in sharp contrast to the survival rates of the La Sota and control groups, 89% and 10%, respectively (Table 4).

DISCUSSION

Since the first recombinant NDV was generated by reverse genetics, various types of chimeric recombinant NDV strains have been established. But to date, none of the recombinant NDV strains had been applied to vaccines to replace conventional vaccines in the field. A good ND vaccine should be antigenically contemporary, productive, avirulent, and stable in pathogenicity. Therefore, the goal of the present study was to generate a highly practical, tailor-made recombinant vaccine strain that satisfies all prerequisites for a good ND vaccine. In the Far East the genotype VIId viruses have become prevalent, and among them, antigenic variants of mutated HN linear epitopes have been increasing in Korea (8, 28, 31). Therefore, KBNP-4152 was selected as the representative of contemporary field NDVs, and F and HN genes were used for the recombinant NDV (9). The pathogenicity of NDV is a multigenic trait. The multibasic amino acids at the cleavage site of F protein can be a very important virulence determinant in the attenuation of a velogenic genotype VIId virus, ZJ1, but high virulence could not be achieved in a lentogenic virus by the multibasic amino acids (18, 44). HN protein is a multifunc-

TABLE 3. Efficacy of live KBNP-C4152R2L vaccine against a different genotype (III) virus, KJW

Group	Vaccine titer (EID_{50})	Test virus	HI titer at 21 DPI (log_2)	Survival rate[a]
Control	0	La Sota	0	0/13 (0)
		KBNP-C4152R2L	0	
KBNP-C4152R2L	10^5	La Sota	4.6 ± 0.9	11/11 (100)
		KBNP-C4152R2L	5.3 ± 0.5	
	10^6	La Sota	5.3 ± 1.1	12/12 (100)
		KBNP-C4152R2L	5.7 ± 0.6	
	10^7	La Sota	4.7 ± 1.3	13/13 (100)
		KBNP-C4152R2L	5.2 ± 0.7	
	10^8	La Sota	5.1 ± 1.2	13/13 (100)
		KBNP-C4152R2L	5.3 ± 1.0	
Avinew	1 dose	La Sota	7.3 ± 1.2	12/12 (100)
		KBNP-C4152R2L	6.2 ± 1.3	

[a] Survival rate after challenge with KJW (2×10^5 $TCID_{50}$/chicken on DPI 22 via the intranasal route), shown as number surviving/total number challenged (percent).

TABLE 4. Efficacy of killed KBNP-C4152R2L oil emulsion vaccine against a different genotype (III) virus, KJW

Group	Vaccine titer (EID_{50})	Test virus	HI titer (log_2) at DPI:		Survival rate[a]
			7	21	
Control	0	La Sota	0	0	1/10 (10)
		KBNP-C4152R2L	0	0	
KBNP-C4152R2L	$10^{9.1}$	La Sota	NT[b]	7.1 ± 1.1	15/15 (100)
		KBNP-C4152R2L	0.2 ± 0.4	7.5 ± 1.2	
La Sota	$10^{9.2}$	La Sota	0.2 ± 0.6	6.9 ± 1.5	8/9 (89)
		KBNP-C4152R2L	0	4.6 ± 1.5	

[a] Survival rate after challenge with KJW (2×10^5 $TCID_{50}$/chicken on DPI 22 via the intranasal route), shown as number surviving/total number challenged (percent).
[b] NT, not tested.

tional protein with roles such as receptor recognition, neuraminidase activity on sialic acid-containing receptors, and fusion promotion, and it plays an important role in the tropism and virulence of NDV (19).

The pathogenicity of NDV is determined not by the length of HN protein, 571 (velogen), 577 (mesogen/lentogen), and 616 (lentogen) amino acids, but by the balanced high receptor binding and neuraminidase activities of HN protein (19, 26, 49). V protein is expressed via RNA editing of the P gene to function as an alpha interferon antagonist by targeting STAT1 for degradation, and it is responsible for virulence and efficient replication (36, 55).

The nucleotide sequence of the gene start, the length of the uridine tract of the gene end, and the length of the IGR of the paramyxoviruses affect the efficiency of transcription termination and initiation. This ultimately affects the gene expression and growth rates, as well as the titer and virulence of the virus (12, 23, 47). The lengths of the IGRs between NP and P, P and M, and M and F are one or two nucleotides, but those of the IGRs between F and HN and between HN and L are 31 and 47 nucleotides, respectively. The increased length of the IGR, therefore, may be associated with a gradual reduction in the expression from the NP to L genes (12, 47).

Reverse genetics studies revealed that recombinant NDVs which carried additional nucleotides or foreign genes were more attenuated than their lentogenic parent NDVs. A 12-nucleotide insertion at the upstream noncoding region of NP caused a slight decrease in ICPI of a recombinant NDV from 1.28 to 1.18 compared to that of its parent virus (44). Also, insertions of foreign genes such as H5 and H7 of avian influenza viruses between P and M decreased the pathogenicity of recombinant NDVs from lentogenicity to avirulence. However, insertion of H7 dramatically reduced pathogenicity of a recombinant NDV from mesogenicity to lentogenicity (14, 43). Studies of viral growth kinetics revealed that most recombinant NDVs carrying foreign genes replicated slowly and produced a lower titer than did their parent or control recombinant NDVs (14, 20, 43). Therefore, the length of intergenic sequence can be a novel alternative target of NDV attenuation. KBNP-4152 was a virulent strain, but KBNP-C4152R2L became still less virulent than La Sota after changes at the cleavage site of F; change in the length and an amino acid substitution, R570G, of HN protein; and the insertion of a six-nucleotide sequence at the IGR between M and F. The amino acid sequence of the cleavage site of F protein is almost the same as that in La Sota, and the HN protein, nearly unchanged, shows activity (rapid elution of chicken erythrocytes) similar to that of its velogenic parent. Therefore, the lack of virulence of KBNP-C4152R2L may be due to the intended mutations at the cleavage site of F and the six-nucleotide insertion at the IGR between M and F, but it should be demonstrated by further studies. The tissue tropism study of KBNP-C4152R2L revealed its restricted replication in the respiratory and alimentary tracts and supported the finding of its avirulence. Considering the high productivity of KBNP-C4152R2L, the six-nucleotide insertion may be appropriate for additional attenuation of a lentogenic to an avirulent virus without hampering its productivity.

All of the lentogenic and avirulent vaccine strains had a codon, GGA, for G at residue 115 of the F protein cleavage site ($_{112}$GRQGR$_{116}$). However, this codon can be changed by a single point mutation to codon CGA or AGA for basic amino acid K or R. Therefore, the single point mutation in codon 115 of lentogenic NDV strains can result in conversion to a mesogen due to generation of the motif R-X-K/R-R, if the L at codon 117 also mutates to F. All of the reported lentogenic recombinant NDVs were generated from B1, La Sota, or a subclone of La Sota (clone 30). Therefore, single point mutation can also cause generation of the virulent cleavage site motif (40, 44, 48). In reality, single intracerebral passage in 1-day-old chicks was enough to generate mutation G115R or L117F (11). The appearance of virulent strains after successive passages of avirulent field NDV strains through chickens has already been reported to be caused by mutations in the cleavage site of F (53, 60). KBNP-C4152R2L was highly stable in terms of pathogenicity in various passages through ECEs and tracheas, and the intracerebral passage did not change its virulence, in contrast to La Sota. The repeated experiment supported the apparent increase of ICPI of La Sota, but we could not observe any mutations in the cleavage site of F protein by direct sequencing of the amplicon. The pathogenicity of NDV is a multigenic trait, and further studies of mutations in HN protein may be required (19). Therefore, KBNP-C4152R2L may be an even safer recombinant vaccine virus and viral vector than any other recombinant NDVs reported to date.

In ovo vaccination has been used for turkey herpesvirus and infectious bursal disease virus to prevent Marek's disease and infectious bursal disease, respectively (51, 52). It has proven to be effective and convenient because of the uniform inoculation of vaccine into individual ECEs by using automated machines. Most conventional lentogenic ND vaccine strains could not be

employed for in ovo vaccination in their current form due to embryonic lethality; therefore, efforts to develop safe in ovo vaccine strains have been made. A B1 strain treated with an alkylating reagent was reported to be safe and immunogenic for 18-day-old chicken embryos (1). Additionally, a recombinant ND vaccine strain with a defect in expression of the V protein was reported as an in ovo vaccine candidate (34). But the use of a chemical reagent is not preferable, and the V protein-defective recombinant virus could not induce an antibody response sufficient to overcome maternal antibodies (1, 34). Recently, several escape mutants of La Sota were evaluated as in ovo vaccine candidates, but their embryonic and neonatal lethality rates were still high (32). On the other hand, KBNP-C4152R2L displayed several outstanding properties as an embryo vaccine candidate. Administration of KBNP-C4152R2L to 18-day-old ECEs of commercial broiler breeders did not lead to any decrease in hatchability or abnormal health conditions in chicks up to 34 days. Hatched chicks showed high levels of antibodies with no deterioration for 34 days after hatching despite a high level of maternal antibodies and were completely protected from mortality after lethal challenge with KBNP-4152. These results support the idea that complete protection might be possible in broiler chickens until the end of their feeding period in Korea by a single in ovo vaccine.

NDV has generally been regarded as comprising a single serogroup; however, its antigenic variations have been detected in several MAb studies (6, 42). Although commercial vaccines have been considered to be protective against mortality caused by virulent NDV strains, the existence of antigenic variations has been suspected (9, 42, 59). Recently, the mutations in and around the linear epitope of HN protein, E347K and M354K, were reported in a genotype VIId virus, KBNP-4152. According to the results of HI and VN tests, KBNP-4152 was found to be antigenically distinct from La Sota (9). The presence of identical or somewhat different amino acid changes of the linear epitope in the GenBank database was reported (9, 24). The newly emerging genotype VIId viruses in Korea during the initial period of 2000 and 2001 possessed no mutations at residue 347, just like all of the commercial vaccine strains. However, since the first appearance of the E347K mutant in 2002, the E347K-M354K mutant has become predominant in 2005, and the accumulations of amino acid changes in and around the linear epitope may be the result of antigenic selections (8).

Conventional vaccines can protect against mortality caused by field NDV strains, but protection against egg drop resulting from contemporary field NDV strains has never been demonstrated. In Korea, egg drop protection by conventional vaccinations has been assumed and some layer and breeder farms have chosen a vaccine program employing multiple regular vaccinations with oil emulsions and live vaccines. The challenge study reported herein revealed that even layer chickens hyperimmunized with conventional vaccines could not be completely protected by KBNP-4152. These results support the conclusion that the vaccine homologous with the challenge virus is more protective than the heterologous vaccines (38). Therefore, to achieve complete protection against mortality and egg drop, a contemporary homologous virus should be selected as the vaccine strain. The efficacy of live and killed KBNP-C4152R2L against the different genotype (III) virus, KJW, was demonstrated by challenge experiments. The higher serum neutralization titers of serum samples (against KJW) from the KBNP-C4152R2L-vaccinated group than of serum samples from the La Sota-vaccinated group demonstrated better cross-protection of KBNP-C4152R2L against the heterologous virulent genotype III virus than conventional vaccines. Therefore, KBNP-C4152R2L is a promising practical vaccine strain and a viral vector in terms of antigenicity, productivity, safety, and pathogenic stability.

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