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Infectious Bronchitis Virus S2 Expressed from Recombinant Virus Confers Broad Protection Against Challenge

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SUMMARY. We developed a recombinant Newcastle disease virus (NDV) LaSota (rLS) expressing the infectious bronchitis virus (IBV) S2 gene (rLS/IBV.S2). The recombinant virus showed somewhat-reduced pathogenicity compared to the parental lentogenic LaSota strain but effectively elicited hemagglutination inhibition antibodies against NDV and protected chickens against lethal challenge with virulent NDV/CA02. IBV heterotypic protection was assessed using a prime-boost approach with a commercially available attenuated IBV Massachusetts (Mass)-type vaccine. Specific-pathogen-free chickens primed ocularly with rLS/IBV.S2 at 4 days of age and boosted with Mass at 18 days of age were completely protected against challenge at 41 days of age with a virulent Ark-type strain. In a second experiment, we compared protection conferred by priming with rLS/IBV.S2 and boosting with Mass (rLS/IBV.S2+Mass) versus priming and boosting with Mass (Mass+Mass). We also modified the timing of vaccination to prime at 1 day of age and boost at 12 days of age. Challenge with virulent Ark was performed at 21 days of age. Based on clinical signs, both vaccinated groups appeared equally protected against challenge compared to unvaccinated challenged chickens. Viral loads in lachrymal fluids of birds receiving rLS/IBV.S2+Mass showed a clear tendency of improved protection compared to Mass+Mass; however, the difference did not achieve statistical significance. A significant difference ($P < 0.05$) was determined between these groups regarding incidence of detection of challenge IBV RNA in the trachea; viral RNA was detected in 50% of rLS/IBV.S2+Mass-vaccinated chickens while chickens vaccinated with Mass+Mass and unvaccinated challenged controls showed 84 and 90% incidence of IBV RNA detection in the trachea, respectively. These results demonstrate that overexposing the IBV S2 to the chicken immune system by means of a vectored vaccine, followed by boost with whole virus, protects chickens against IBV showing dissimilar S1.

RESUMEN. El gene S2 del virus de la bronquitis infecciosa expresado en virus recombinantes confiere una amplia protección contra el desafío.

Se ha desarrollado un virus recombinante de la enfermedad de Newcastle (NDV) LaSota (rLS) que expresa el gene S2 del virus de la bronquitis infecciosa (IBV) (rLS/IBV.S2). El virus recombinante mostró patogenicidad ligeramente reducida en comparación con la cepa La Sota lentogénica que sirvió de origen, pero indujo efectivamente anticuerpos inhibidores de la hemaglutinación contra el virus de Newcastle y protegió a los pollos contra el desafío letal con la cepa virulenta NDV/CA02. Se evaluó la protección heterotípica contra el virus de bronquitis a través de un enfoque de primovacunación y de refuerzo con una vacuna Massachusetts (Mass) atenuada disponible en el mercado. Pollos libres de patógenos específicos primovacunados ocularmente con el virus rLS/IBV.S2 a los cuatro días de edad y revacunados con la cepa Massachusetts a los 18 días de edad estuvieron completamente protegidos contra la exposición a los 41 días de edad con una cepa virulenta cepa de tipo Ark. En un segundo experimento, se comparó la protección conferida por la primovacunación con rLS/IBV.S2 y la revacunación con Massachusetts (rLS/IBV.S2+Mass) en comparación con la primovacunación y refuerzo con Massachusetts (Mass+Mass). También se modificó el momento de la vacunación para primovacunar al primer día de edad y con refuerzo a los 12 días de edad. El desafío con una cepa Ark virulenta se realizó a los 21 días de edad. Con base en los signos clínicos, los dos grupos vacunados aparecieron igualmente protegidos contra el desafío en comparación con pollos desafiados y no vacunados. La carga viral en los fluidos lacrimales de las aves que recibieron el virus rLS/IBV.S2 +Mass mostraron una clara tendencia de mejor protección en comparación con el esquema Mass+Mass, sin embargo, la diferencia no alcanzó significancia estadística. Se determinó una diferencia significativa ($P < 0.05$) entre estos grupos con respecto a la incidencia de detección del ARN del virus de bronquitis de desafío en la tráquea; se detectó ARN viral en 50% de los pollos vacunados con rLS/IBV.S2 +Mass mientras que los pollos vacunados con el esquema Mass+Mass y los controles no vacunados y desafiados mostraron una incidencia 84% y 90% en la detección del ARN del virus de la bronquitis infecciosa en la tráquea, respectivamente. Estos resultados demuestran que la sobreexposición del sistema inmune del pollo al gene S2 del virus de bronquitis al mediante una vacuna recombinante, seguido por revacunación con virus completo, protege a los pollos contra virus de bronquitis infecciosa que muestran genes S1 diferentes.

Key words: IBV S2, genetic variation, NDV vector, IBV vaccine

Abbreviations: aa = amino acid; Ark = Arkansas; ArkDPI = Ark Delmarva Poultry Industry; EID_{50} = 50% embryo infectious dose; G = glycoprotein; IB = infectious bronchitis; IBV = IB virus; ICPI = intracerebral pathogenicity index; IN = intranasal; IO = intraocular; M = matrix gene; MAbs = monoclonal antibodies; Mass = Massachusetts; MDT = mean death time; MVA = modified vaccinia Ankara; ND = Newcastle disease; NDV = ND virus; P = phosphoprotein gene; PBS = phosphate-buffered saline; rLS = recombinant LaSota virus; rLS/IBV.S2 = rLS expressing the IBV S2 gene; RT-PCR = reverse transcriptase PCR; S = spike protein; SEPRL = Southeast Poultry Research Laboratory; SPF = specific-pathogen-free; $TCID_{50}$ = 50% tissue infectious dose; USDA = United States Department of Agriculture

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Table 1. Analysis of 18 United States IBV complete S2 sequences available in GenBank.

	Identity scores (%)																	
	Cal 95-9437	Cal 99	Cal 557 2003	CAV 56b	CAV 1013	ArkD-PI	M41	DE072	Conn 46	GA98	FL-18288	Md27	Iowa	Holte	Gray	JMK	GA-V92	Ark99
Cal 95-9437		**98.4**	**94.4**	**96.8**	**94.9**	**91.4**	**89.8**	**75.7**	**91.2**	**76.2**	**90.9**	**90.9**	**91.4**	**91**	**91**	**91.4**	**92**	**92**
Cal99	99		**95.5**	**98.1**	**95.2**	**91.7**	**90.1**	**76.3**	**91.5**	**76.8**	**91.2**	**91**	**91.7**	**91.5**	**91.4**	**91.7**	**92.2**	**92.3**
Cal557 2003	97.6	98.2		**95.2**	**93.3**	**89.8**	**88.8**	**75.7**	**90.4**	**76.2**	**90.1**	**89.4**	**90.6**	**91**	**89.8**	**89.8**	**90.1**	**90.6**
CAV 56b	98.2	98.9	97.8		**94.2**	**90.9**	**89.1**	**76.6**	**90.7**	**77.1**	**90.4**	**90.1**	**90.7**	**90.6**	**90.4**	**90.9**	**91**	**91.4**
CAV 1013	97.3	97.9	97.4	97.4		**92.6**	**89.8**	**76.2**	**92.2**	**76.6**	**91.8**	**92**	**90.9**	**91.7**	**92.5**	**92.6**	**93.6**	**93.9**
ArkDPI	95.5	96.2	95.7	95.7	96.8		**94.6**	**75.7**	**95.8**	**75.5**	**95.5**	**97.8**	**94.1**	**94.9**	**98.9**	**99.7**	**93.6**	**93.9**
M41	94.9	95.5	94.9	94.7	95.4	97.4		**74.4**	**94.4**	**74.4**	**94.2**	**93.6**	**94.2**	**94.4**	**94.1**	**94.2**	**90.1**	**90.4**
DE072	87	87.7	87.5	88	87.4	86.7	86.2		**76.6**	**98.1**	**76.3**	**75.4**	**74.9**	**75.8**	**75.8**	**75.7**	**76.2**	**76.3**
Conn 46	95.8	96.5	96.3	96	96.8	97.9	97.3	87.4		**76.5**	**99.7**	**95.7**	**95.4**	**94.1**	**95.7**	**95.8**	**92.6**	**93**
GA98	87.5	88.2	88	88.5	87.8	86.6	86.1	98.4	87.2		**76.2**	**75.4**	**74.7**	**75.7**	**75.7**	**75.5**	**76.6**	**76.8**
FL18288	95.7	96.3	96.2	95.8	96.6	97.8	97.1	87.2	99.8	87		**95.4**	**95**	**93.8**	**95.4**	**95.5**	**92.3**	**92.6**
Md27	95.5	96.2	95.8	95.5	97	98.7	96.8	86.9	97.6	86.9	97.4		**93.4**	**94.1**	**97.4**	**97.8**	**93.6**	**94.1**
Iowa	95.5	96.2	95.7	95.4	95.7	96.8	97	86.2	97.4	86.1	97.3	96.5		**94.9**	**93.9**	**94.1**	**89.8**	**90.1**
Holte	95.8	96.5	96	95.7	96.3	97.8	97.6	86.6	97.8	86.4	97.6	97.4	97.8		**94.6**	**94.9**	**90.9**	**91.2**
Gray	95.2	95.8	95.5	95.4	96.5	99.4	96.8	86.6	97.6	86.4	97.4	98.6	96.5	97.4		**99.2**	**93.4**	**93.8**
JMK	95.5	96.2	95.7	95.7	96.8	99.7	97.1	86.7	97.9	86.6	97.8	98.7	96.8	97.8	99.7		**93.6**	**93.9**
GAV92	95.2	95.8	95.8	95.4	96.8	97.1	95.7	86.2	96.5	86.7	96.3	97.1	95.4	95.8	96.8	97.1		**98.9**
Ark99	95.2	95.8	95.8	95.2	96.6	97	95.5	86.1	96.3	86.6	96.2	97.1	95.2	95.7	96.8	97	99.4	
	Similarity scores (%)																	

Infectious bronchitis (IB) is one of the most prevalent avian diseases in the world's poultry industry. Effective control of IBV is particularly difficult due to genotype-phenotype diversity and continuing evolution of the causal coronavirus (10). The S1 subunit of the spike protein (S) polypeptide of IBV is responsible for viral attachment to cells and is of distinct importance for host protective immune responses (4,14). The extensive variation exhibited by S1 among IBV populations (16,17) facilitates this virus' immunologic escape and evolutionary success. Based on S1 sequences and serology, IBV isolates commonly isolated from outbreaks of disease during the last decade in the United States belong to the types Arkansas (Ark), Connecticut, DE072, Massachusetts (Mass), Georgia variants, and California variants as well as a few variant profiles that do not fully match reference viruses (11,27). IBV types commonly isolated in other regions of the world have been listed in a recent review (10). Control of the disease has been largely based on a multiplicity of type-specific IBV attenuated vaccines. In general, these vaccines work effectively against homologous challenge. However, accumulating evidence indicates that attenuated IBV vaccines may also be contributing to the emergence and circulation of vaccine-like viruses in the poultry industry (24,26).

Unlike S1, the S2 portion of S is highly conserved among different IBV strains (15). For example, Souza *et al.* (22) produced monoclonal antibodies (MAbs) against the S2 protein of IBV strain M41. Using ELISA, these antibodies recognized the M41, Ark-99, and Connecticut strains as well as numerous Brazilian indigenous strains (22). Our own analysis of 251 complete S2 sequences available in GenBank from all over the world indicates that the percent amino acid sequence identity among them varies between 74.4 and 99.7% (examples of U.S. isolates in Table 1). The N-terminal portion of S2 has been shown to contain immunodominant regions and different neutralization epitopes recognized by polyclonal and MAbs. Thus, S2 has been suggested to be suitable for vaccine development (18). An accurate mapping of the epitopes of four MAbs that recognize the immunodominant region in the S2 subunit of IBV was reported by Kusters *et al.* (15). MAbs directed against the S2 protein defined two epitope clusters. MAbs directed against cluster S2-G moderately to strongly neutralized IBV at titers higher than 2 $\log_{10}$ (14). From a teleologic perspective, exposing conserved regions to the immune system would be detrimental to the success of this virus family. Thus, probably due to protein folding or other mechanisms, S2 remains largely unexposed to the immune system during natural infection.

Analogous to IBV, the globular head of avian influenza virus HA is subject to continuing genetic evolution while the structure of the stem region (HA2 domain) is more conserved (6,29). Recent results have shown that prime and boost vaccination elicits antibodies to epitopes in the HA2 stem region which broadly neutralize heterosubtypic H1N1 influenza viruses (5,30). Specifically, vaccination with plasmid DNA encoding H1N1 influenza HA, and boosting with seasonal vaccine or replication-defective adenovirus 5 vector encoding HA, stimulated the production of broadly neutralizing influenza antibodies. This prime-boost combination increased the neutralization of diverse H1N1 strains dating from 1934 to 2007, as compared to either component alone, and conferred protection against divergent H1N1 viruses in mice and ferrets. These antibodies were directed to the conserved stem region of the HA and were also elicited in nonhuman primates (30).

Based on the information on antigenic sites and analogous evidence described above, we hypothesized that overexposing the IBV S2 to the chicken immune system by means of a vectored vaccine, followed by boosting with whole virus, would protect the host against IBV showing dissimilar S1.

In this study we developed recombinant Newcastle disease (ND) virus (NDV) LaSota vector (rLS) expressing the IBV S2 gene. ND is a relevant infectious disease of poultry, and virulent NDV outbreaks require reporting to the World Organisation for Animal Health (O.I.E.) by member nations (31). The use of the NDV LaSota as a live vaccine vector has several advantages including increased safety, routine use in poultry as a live vaccine throughout the world, stability (recombination events are rare), easy mass-administration via the drinking water or by aerosol, and induction of strong local and systemic immune responses (2,3,7,9). rLS viruses expressing the glycoprotein (G) of avian metapneumovirus subgroup C have been previously generated and evaluated *in vitro* and *in vivo* as bivalent vaccine candidates (8,32).

Table 2. Biological assessments of the NDV/IBV-S2 recombinant virus.

Virus	MDT[A] (hr)	ICPI[B]	HA[C]	EID_{50}[D]	$TCID_{50}$[E]
LaSota	110	0.15	1,024	6.8×10^8	3.5×10^7
rLS/IBV-S2	122	0	4,096	1.76×10^9	1.58×10^8

[A]MDT = mean death time in embryonated eggs.
[B]ICPI = intracerebral pathogenicity index in 1-day-old chickens.
[C]HA = hemagglutination titer.
[D]EID_{50} = 50% embryo infectious dose in embryonated eggs.
[E]$TCID_{50}$ = 50% tissue infectious dose on DF-1 cells.

For boosting, we decided to use a commercially available attenuated Mass-type vaccine, as this type of vaccine has been registered and is used worldwide. For challenge purposes we used a wild virulent Ark strain which shows only 77.6% S1 amino acid (aa) sequence identity with the S1 of the Mass vaccine strain.

MATERIALS AND METHODS

Chickens. White leghorn chickens hatched from specific-pathogen-free (SPF) fertile eggs (Sunrise Farms, Catskill, NY) were used in all experiments. Experiments to evaluate protection against NDV virulent challenge were performed in biosafety level 3+ facilities at the United States Department of Agriculture's (USDA) Southeast Poultry Research Laboratory (SEPRL). Protection against IBV challenge was assessed in biosafety level 2 facilities at Auburn University College of Veterinary Medicine, Auburn, AL. Experimental procedures and animal care were performed in compliance with all applicable federal and institutional animal use guidelines.

Construction of a recombinant LaSota cDNA clone containing the S2 gene of IBV. The previously generated full-length LaSota cDNA clone was used as a backbone (8). The complete S2 gene sequence of the virulent Ark Delmarva Poultry Industry (ArkDPI) isolate (S2 amino acid sequence GenBank accession no. AAF82269) was optimized to the chicken codons and synthesized. This S2 sequence shows ≥98.7% aa identity with the S2 of all Ark virus sequences available in GenBank and 94% identity with the S2 of Mass strains. The codon-optimized IBV S2 gene flanked by the NDV gene start (GS) and gene end (GE) sequences was inserted into the rLS vector between the phosphoprotein (P) and matrix (M) genes as an additional transcription unit using the In-Fusion® PCR cloning kit (Clontech, Mountain View, CA). The resulting recombinant clone, designated as pLS/IBV.S2, was amplified in Stbl2 *Escherichia coli* cells and purified using a QIAprep Spin MiniPrep kit (Qiagen, Valencia, CA). The sequence fidelity of the recombinant clone was confirmed by nucleotide sequencing with the Applied Biosystems PRISM® fluorescent BigDye® sequencing kit and the ABI 3730 DNA Sequencer (Applied Biosystems, Foster City, CA). The total length of this clone obeys the rule of six, which is critical for efficient replication of the virus genome.

Virus rescue and propagation. Rescue of the rLS expressing the IBV S2 gene (rLS/IBV.S2) virus was performed by transfecting the full-length cDNA clone and supporting plasmids into modified vaccinia Ankara (MVA)/T7-infected HEp-2 cells using Lipofectamine™ 2000 (Invitrogen, Grand Island, NY) according to the manufacturer's instructions. At 6-hr post transfection, cells were washed with phosphate-buffered saline (PBS) and maintained in Dulbecco's modified eagle's medium (DMEM) medium containing 2% fetal bovine serum (FBS) and antibiotics. At 72 hr post transfection, the transfected-infected cells were harvested by freeze-thawing three times. The rescued virus was amplified by inoculating 100 μl of the transfected-infected cell lysate into the allantoic cavity of 9-day-old SPF chicken embryos. After 4 days of incubation, the allantoic fluid was harvested and used for detection of rescued virus by HA test (1). The HA-positive allantoic fluid was terminally diluted during subsequent passages to remove any possible MVA contamination. The rescued virus, designated as rLS/IBV.S2, was amplified in SPF chicken embryos three times and the allantoic fluid harvested and stored at −80 C as a stock.

Confirmation of the rescued rLS/IBV.S2. To confirm the sequence fidelity of the rescued virus, the S2 gene insertion region of rLS/IBV-S2 was examined by reverse transcriptase PCR (RT-PCR) amplification with a pair of specific primers and followed by sequence analysis. The complete genome of the rescued rLS/IBV.S2 was sequenced to rule out any undesired mutation in the recombinant virus. Confirmation of S2 protein expression in cell cultures was performed by immunofluorescence assay as previously described (8) using a hyperimmune serum.

Biological assessment of rLS/IBV.S2. *rLS/IBV.S2 virus titration and pathogenicity assays.* Analysis of the recombinant rLS/IBV.S2 viral stock titers was completed using the standard hemagglutination assay (HA) in 96-well microplates, the 50% tissue infectious dose ($TCID_{50}$) assay on DF-1 cells, and the 50% embryo infective dose (EID_{50}) assay in 9-day-old specific-pathogen-free (SPF) chicken embryos and compared to the parental LaSota virus (1). Pathogenicity of the recombinant virus was assessed by performing the standard mean death time (MDT) and intracerebral pathogenicity index (ICPI) tests (1) and then compared to the parental LaSota virus originally obtained from the American type culture collection (ATCC).

rLS/IBV.S2 immunization and challenge experiment. Thirty, 1-day-old chickens were divided into three groups of 10 birds each and housed in Horsfall-type isolators (Federal Designs, Inc., Comer, GA) with *ad libitum* access to feed and water in the SEPRL biosafety level 3+ animal facility. Each bird in group 1 (negative control) was inoculated with 100 μl PBS via the intranasal (IN) and intraocular (IO) routes. Birds in group 2 were vaccinated with 100 μl of rLS/IBV.S2 (1.0×10^7 EID_{50}/ml), and birds in groups 3 were vaccinated with 100 μl of the parental LaSota virus (1.0×10^7 EID_{50}/ml) per bird via IN-IO routes. At 14 days postvaccination, blood samples were collected from each bird to detect serum antibody responses against NDV by a hemagglutination inhibition test (1,23). Immediately after blood collection, all birds were challenged with the velogenic NDV/CA02 virus with a dose of 10^5 EID_{50}/bird via IN-IO routes as previously described (13). Mortality of the NDV/CA02-challenged birds was monitored and recorded daily for 2 wk.

Evaluation of IBV heterotypic protection. *IBV protection experiment 1.* We established five chicken groups (n = 12–14 each group). Chickens in group 1 were primed ocularly with $10^{6.0}$ EID_{50}/bird of rLS/IBV.S2 at 4 days of age and boosted ocularly with a commercially available attenuated IBV Mass-type vaccine (Fort Dodge Animal Health, Fort Dodge, IA) at 18 days of age using the dose recommended by the manufacturer. Control groups included chickens vaccinated on the same days with the empty vector (rLS/E) followed by Mass (group 2), groups 3 and 4 vaccinated with rLS/E only, and group 5 of unvaccinated controls. Chicken groups 1 through 3 were challenged at 41 days of age via the ocular and nasal routes with 10^5 EID_{50}/bird of an Ark-type IBV virulent strain previously described (26). Groups 4 and 5 were not challenged. Protection was evaluated 5 days after challenge by disease signs and viral load determined by quantitation of viral RNA in the lachrymal fluids of individual chickens by quantitative reverse transcription PCR (qRT-PCR). Detection of virus at 5 days post-challenge is consistent with the O.I.E. guidelines for IBV vaccines (31). Signs were assessed as previously described (27). In brief, respiratory rales (nasal, tracheal, or both) were assessed without knowledge of treatment group by listening to each individual bird of each group.

Table 3. Serum antibody response against NDV after vaccination and survival of vaccinated chickens after challenge with a lethal dose of NDV/CA02.

Vaccination	Antibody response		Survivors
	Seropositive birds	HI titer[A]	
PBS	0/10	0	0/10
rLS/IBV.S2	10/10	3.6 ± 1.6	10/10
rLaSota	10/10	4.8 ± 0.4	10/10

[A]Hemagglutination inhibition (HI) titer was expressed as mean $\log_2 \pm$ SD.

Severity scores (1 = normal; 2 = respiratory rales detected at the examiner's ear; 3 = respiratory rales detected at a distance [without approaching the bird to the examiner's ear]) were recorded for each bird. Data were compared among groups by ANOVA and a Tukey multiple comparisons *post hoc* test.

Evaluation of viral load by qRT-PCR. Viral RNA was extracted from individual tear samples obtained as described (25) from each chicken of all groups using the Qiagen QIAmp viral RNA mini kit (Qiagen) following the manufacturer's protocol. A fluorescence resonance energy transfer (FRET) qRT-PCR detecting the IBV N gene was performed as previously described (28). Melting point analysis confirmed the specificity of the positive reactions.

IBV protection experiment 2. In experiment 2 we compared the protection conferred by priming with rLS/IBV.S2 and boosting with Mass (rLS/IBV.S2+Mass) to protection conferred by priming and boosting with Mass (Mass+Mass). We also modified the timing of vaccination to prime at 1 day of age and boost at 12 days of age. Challenge with virulent Ark was performed at 21 days of age. The rationale for this modification was that the incidence of outbreaks of IBV in broilers often rises after 20 days of age (27). Dosage and evaluation were performed as described above, except that in addition we performed viral RNA detection in the trachea of each individual chicken by our previously described, more-sensitive conventional RT-PCR assay (27).

RESULTS

Molecular characterization of rLS/IBV.S2. The RT-PCR product generated from the rescued rLS/IBV.S2 virus was about 2.0 kb larger than that from the parental LaSota virus (not shown). Sequence analysis of the RT-PCR product confirmed that the synthetic IBV S2 gene had been inserted into the LaSota genome between the P and M genes (not shown). The complete genome sequence of the rescued rLS/IBV.S2 confirmed absence of undesired mutations. Finally, we confirmed S2 protein expression in cell cultures by immunofluorescence assay using a hyperimmune serum (not shown).

rLS/IBV.S2 biological properties. As seen in Table 2, both the MDT and ICPI demonstrated a slight reduction of virulence of rLS/IBV.S2 compared to the parental NDV LaSota. MDT increased from 110 to 122 hr and ICPI decreased from 0.15 to 0.0 hr. In addition, the titers determined by HA, EID_{50}, and $TCID_{50}$ were higher for the rLS/IBV.S2 virus compared to the parental LaSota. Furthermore, as seen in Table 3, rLS/IBV.S2 induced specific hemagglutination inhibition antibodies in vaccinated chickens, and these chickens were protected against challenge with a lethal dose of highly virulent NDV/CA02.

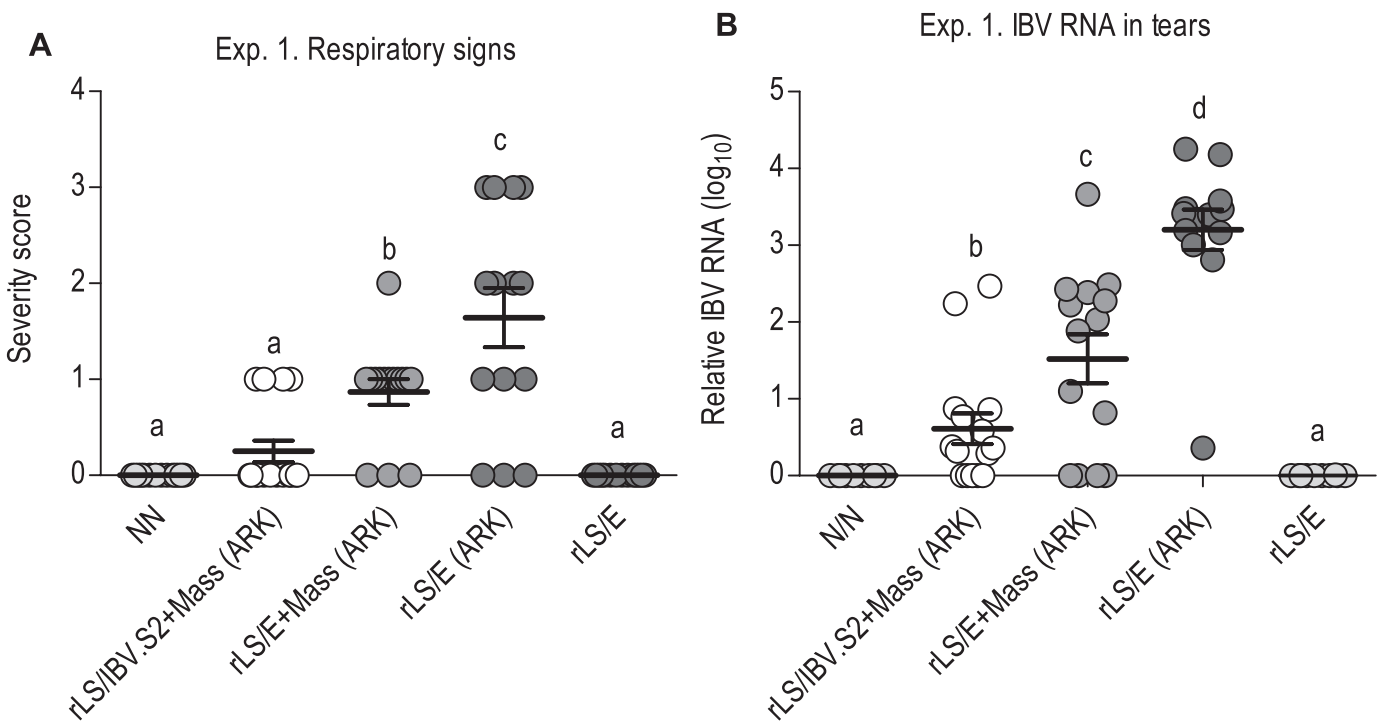

Fig. 1. Respiratory signs (A) and viral load (B) in vaccinated chickens 5 days after Ark virulent challenge at 41 days of age. Treatment groups (n = 12–14/group) include not vaccinated-not challenged (NN); rLS/IBV.S2+Mass = primed with rLS/IBV.S2 at 4 days, boost with attenuated Mass-type vaccine at 18 days of age; rLS/E+Mass = primed with empty vector, boost with Mass; rLS/E = primed empty vector; (ARK) = Ark challenged. Severity of respiratory signs scored blindly. Relative viral load determined by qRT-PCR in lachrymal fluids of individual chickens. Negative samples were assigned a value of 1 to allow calculation of $\log_{10}$ values. Lines = mean ± SEM. Different letters indicate significant differences ($P < 0.05$).

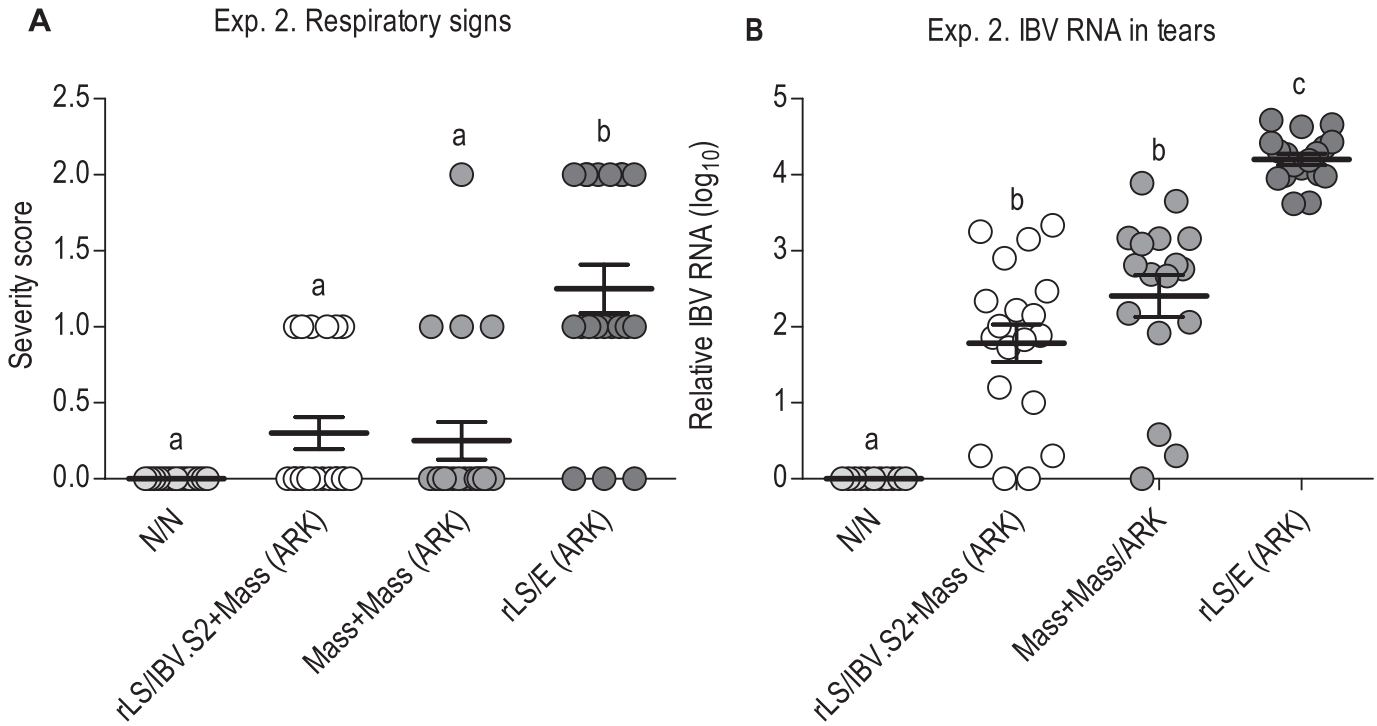

Fig. 2. Respiratory signs (A) and viral load (B) in vaccinated chickens 5 days after Ark virulent challenge at 23 days of age. Treatment groups (n = 17–19/group) include unvaccinated-not challenged (NN); rLS/IBV.S2+Mass (ARK) primed with rLS/IBV.S2 at 1 days, boost with attenuated Mass at 12 days of age, and Ark challenged; primed with Mass+Mass (ARK), boost with Mass, and Ark challenged; primed with rLS/E (ARK) empty vector and Ark challenged. Severity of respiratory signs scored blindly. Relative viral load determined by qRT-PCR in lachrymal fluids of individual chickens. Negative samples were assigned a value of 1 to allow calculation of log $_{10}$ values. Lines = mean ± SEM. Different letters indicate significant differences ($P < 0.05$).

IBV protection experiment 1. Based on clinical signs severity scores, chickens primed with rLS/IBV.S2 and boosted with an attenuated Mass-type vaccine (group 1) were protected against challenge with a wild virulent Ark-type strain (Fig. 1A). In contrast, positive control chickens (group 3) vaccinated only with the empty vector rLS/E showed significantly ($P < 0.05$) higher severity of clinical signs. Indeed, in several birds in the positive control group severe respiratory rales could be readily detected by the ear of the examiner without approaching the individual birds. Chickens of group 2 vaccinated with rLS/E+Mass showed significantly higher ($P < 0.05$) severity of respiratory signs than did rLS/IBV.S2+Mass-vaccinated chickens but significantly less-severe signs than the positive controls (Fig. 1A). This result indicates that Mass-type IBV vaccination does confer partial protection against Ark-type IBV challenge. However, only by priming with rLS/IBV.S2 did the protection become complete, i.e., respiratory sign scores were not significantly different from unchallenged control birds.

The clinical findings were consistently corroborated by the concentration of viral RNA detected in the lachrymal fluids of chickens after challenge. As seen in Fig. 1B, rLS/IBV.S2+Mass-vaccinated chickens showed lower IBV RNA than all other challenged groups. The reduction in viral load was significant ($P < 0.05$) compared to chickens vaccinated with rLS/E+Mass as well as to positive controls vaccinated with the empty vector only. Consistent with the clinical findings was the fact that chickens vaccinated with the empty vector +Mass also showed reduction of viral load. Unvaccinated-not challenged and rLS/E-not challenged controls (groups 4 and 5) showed neither apparent signs of disease nor detectable viral RNA in the tear fluids.

IBV protection experiment 2. As seen in Fig. 2A, we were not able to detect statistically significant clinical differences between rLS/IBV.S2+Mass- and Mass+Mass-vaccinated chickens. Both groups seemed to be equally protected when compared with the positive controls (rLS/E-only challenged). Results of viral load in lachrymal fluids (Fig. 2B) of birds receiving rLS/IBV.S2+Mass showed a clear tendency of improved protection compared to Mass+Mass but the difference between these groups did not achieve statistical significance. Both groups also exhibited a significant reduction of IBV RNA in the tears when compared to the positive controls. A significant difference ($P < 0.05$) was determined among the groups regarding incidence of IBV RNA detection in the trachea (Fig. 3). As seen in this figure, viral RNA was detected in 50% (10/20) of rLS/IBV.S2+Mass-vaccinated chickens. In contrast, chickens vaccinated with Mass+Mass and unvaccinated positive controls showed 84% (16/19) and 90% (18/20) incidence of IBV RNA in the trachea, respectively.

DISCUSSION

We successfully developed rLS expressing the S2 gene of IBV. The recombinant virus essentially maintained the biologic properties of the lentogenic parental LaSota strain, as it induced hemagglutination inhibition antibodies and protected chickens against lethal challenge with a virulent NDV strain. Interestingly, the recombinant virus showed a slight reduction in pathogenicity compared to the lentogenic parental strain, as determined by both MDT and ICPI. Also, no signs of adverse reactions became apparent in young SPF chickens after vaccination. Thus, the reduced pathogenicity displayed by the recombinant construct may provide an advantage over the parental strain as a vaccine candidate. Use of NDV-vectored vaccines has the advantage of providing protection against NDV, a

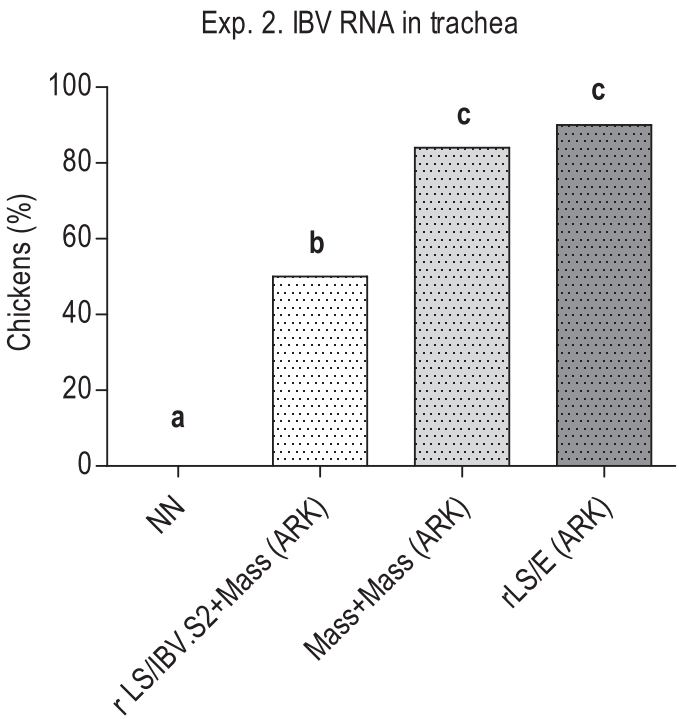

Fig. 3. Percent chickens with detectable IBV RNA in the trachea as determined by conventional RT-PCR. Groups and treatment described in Fig. 3. Different letters indicate significant differences ($P < 0.05$; Fisher exact test).

continuous risk for the world's poultry industry. However, use of NDV-vectored transgenes might not be suitable in areas of high NDV infective pressure, as preexisting immunity against the vector due to high levels of maternal immunity would likely prevent this vaccine from inducing strong protection against the expressed transgene. Use of this type of vaccine would theoretically prove successful in regions with low NDV infective pressure such as the United States or some European countries in which an NDV vaccine should readily break through low levels of maternal immunity.

We replicated rLS/IBV.S2 in SPF embryonated eggs before each animal experiment. Based on sequencing of the S2 transgene after three passages no changes were detected (data not shown). Thus the rLS/IBV.S2 is stable, like the previously produced recombinant LaSota viruses (8,32). Finally, replication of rLS/IBV.S2, both in egg and cell cultures, demonstrated high virus yields which would be essential for vaccine mass production.

Both experiments 1 and 2 demonstrated that rLS/IBV.S2 used in a prime-boost scheme with a Mass attenuated vaccine confers protection against Ark virulent challenge. Indeed, these experiments consistently showed a significant difference between vaccinated and unvaccinated positive controls, both in terms of clinical signs and viral load. The protective effect of vaccines depends largely on antigenic similarity between the antigenic characteristics of the immunogen and those of the challenge strain. Even though IBV S2 proteins are conserved among IBV, phylogenetically distant strains do show differences among their sequences (see Table 1). In the current experiments the S2 sequence used showed ≥98.7% aa identity with the S2 of all Ark virus sequences available in GenBank and 94% identity with the S2 of the Mass vaccine strain used. The results indicate that boost with Mass was able to elicit an S2 memory response, thus explaining the protective effect against Ark virulent challenge.

As mentioned in the introductory paragraph, S2 likely remains largely unexposed to the immune system during natural infection. The mechanisms by which the stem portion of the HA of AIV (30), as well as those by which S2 provides increased broad protection as reported herein, require further investigation. From a speculative perspective, overexposure of S2 likely induces an increase in B and T cells bearing receptors interacting with epitopes on the S2. Those few epitopes of S2 that become exposed during virus replication, as this likely happens during the booster vaccination with an attenuated IBV vaccine, may not only increase the number of memory cells but also increases the affinity and avidity toward those few epitopes exposed during challenge. Ultimately, the immune response against S2 may have achieved optimal effectiveness to enough antigenic sites that protection is conferred.

The question remains about protection against IBV strains showing increased S2 divergence; e.g., Georgia and Delaware IBV strains. From a speculative perspective, protection against phylogenetically distant strains might require using distinct S2 transgenes to achieve effective protection. Experiments toward evaluation of protective properties of the current construct against distant IBV strains are currently being performed.

In the first experiment birds were primed at 4 days of age, then 14 days separated priming from boosting, and challenge was performed at 41 days. In the second experiment priming was performed at 1 day of age, boost was 11 days after priming, and challenge was performed at 21 days of age. Thus, in the second experiment the conditions to allow vaccination to work most effectively were considerably weakened. Still, rLS/IBV.S2-vaccinated chickens were effectively protected against Ark challenge compared to unvaccinated challenged controls. However, the difference in protection compared to the Mass+Mass regime was not significant. Indeed, both vaccination strategies protected well against respiratory signs. However, a clear tendency for increased reduction of viral load in lachrymal fluids and a significant reduction ($P < 0.05$) in incidence of viral RNA detection in the trachea indicated improved protection by rLS/IBV.S2+Mass over Mass+Mass vaccination. The protective effect shown by Mass+Mass vaccination against Ark challenge was not expected but might be explained by the fact that experimental chickens were maintained under the best health and environmental conditions, allowing them to perform to their optimal immunologic potential.

Irrespective of the possible protective benefits of using combinations of different attenuated IBV strains to protect chickens against IBV, the use of recombinant vaccines provides superior advantages including, for example, 1) elimination of the risk of recombination between live attenuated IBV vaccines, wild IBV strains, or both; 2) avoidance of introduction of exotic live IBV strains (this is a serious problem, triggered mainly by vaccine companies of which many examples exist, the most recent being introduction of IBV 4/91 into Chile); and 3) eliminating the risk of emergence of vaccine-like strains resulting from point mutations as reported, for example, for attenuated ArkDPI derived vaccines (11,12,19,20).

This study provides initial evidence supporting the hypothesis that priming with S2 expressed from a recombinant virus provides protection against IBV challenge when compared to nonvaccinated controls. Proof of principle was obtained using a recombinant NDV virus. This particular vector may not be the most suitable for many regions in the world. However, it is theoretically plausible that any vector effectively expressing transgenes in chickens should work as effectively.

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