Newcastle disease virus vectored infectious laryngotracheitis vaccines protect commercial broiler chickens in the presence of maternally derived antibodies

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ABSTRACT

Newcastle disease virus (NDV) recombinants expressing the infectious laryngotracheitis virus (ILTV) glycoproteins B and D have previously been demonstrated to confer complete clinical protection against virulent ILTV and NDV challenges in naive chickens. We extended this study to assess whether maternally derived antibody (MDA) against NDV and ILTV would interfere with protection in vaccinated broiler chickens. Chickens with a mean NDV MDA hemagglutination inhibition (HI) titer of 6.4 ($\log_2$) and detectable ILTV neutralization (VN) antibodies at hatch were vaccinated with rLS/ILTV-gB or rLS/ILTV-gD at 1 or 10 day of age (DOA) or on both days. Groups of birds vaccinated with the commercial ILT vaccines (FP-LT and CEO) or sham inoculated were also included in this study. All vaccinated birds were challenged with virulent ILTV strain at 21 DOA. By that time, NDV HI titers declined to 2.6 ($\log_2$) in unvaccinated birds, whereas the HI titers in NDV vectored vaccine groups increased to 3.5-6.3 ($\log_2$). At standard dosages, both vaccine candidates conferred significant clinical protection; however, the protection elicited by the rLS/ILTV-gD was superior to that of rLS/ILTV-gB. Recombinant rLS/ILTV-gD reduced ILTV shedding from tracheal and ocular tissues by approximately 3 $\log_{10}$ TCID₅₀. Notably, there was no improvement in protection after booster vaccination at 10 DOA. Overall results indicate that the presence of maternal antibodies to NDV and ILTV did not significantly interfere with the ability of the NDV LaSota strain-vectored ILTV gB and gD vaccine candidates to elicit protective immunity against infectious laryngotracheitis.

Keywords:

NDV; ILTV; Recombinant vaccine; Maternal antibody; Interference; Protection

1. Introduction

Infectious laryngotracheitis (ILT) is an acute upper respiratory tract disease of chickens caused by the alphaherpesvirus infectious laryngotracheitis virus (ILTV) or gallid herpesvirus type 1 (GaHV-1). Despite the extensive use of attenuated, and more recently recombinant vaccines, ILT continues to affect poultry on a worldwide basis [1]. Historically, the commonly used live ILT vaccines were first attenuated in the 1950s by either multiple passages in embryonated eggs (chicken embryo origin [CEO]) or in tissue culture (tissue culture origin [TCO]) [2, 3]. Although these vaccines protect against clinical disease, they have residual virulence, which is exacerbated by continued infections of naïve birds from productively infected animals and latent carriers [4-6]. In high-density poultry rearing facilities there is a continuous reservoir of ILT viruses, both virulent and vaccinal. It has been reported that CEO vaccinal “revertants” can become the dominant field strains in poultry populations and cause outbreaks [7, 8]. Although the recombinant vaccines, turkey herpesvirus (HVT) and fowlpox virus (FPV) expressing ILTV antigens are safe [9-14], they only induce partial protection when compared with that induced by live-attenuated vaccines [15]. Thus, a significant need exists to revise the ILT control strategies particularly regarding the development of next generation vaccines that are safe, protective and incapable of vaccinal reversion.

Recently, we developed Newcastle disease virus (NDV) recombinants expressing the ILTV glycoproteins B and D as bivalent vaccines [16]. Vaccination of one-day-old specific pathogen free (SPF) and 3-week-old commercial boiler chickens conferred complete clinical protection against virulent ILTV and NDV challenges [16]. However, there was a general concern that the maternally derived antibodies (MDA) may interfere with the immunoresponses of the chicks to the corresponding vaccines [17-19]. It has been reported that the NDV MDA confers protection to young chicks against ND, but it can also interfere with NDV vaccine efficacy at 1 day of age (DOA) [17, 19]. This interference is dependent on the titer of maternal antibodies and the strain of the NDV vaccine administered [20, 21]. The LaSota vaccine strain has been demonstrated to overcome MDA interference better than the Hitchner, B1 or V4 strains [21]. Higher levels of circulating maternal antibodies to the LaSota strain were required to depress the immunoresponse than that required to do the same for the other three strains [21]. In contrast, maternal antibodies to ILTV do not confer protection against disease nor interfere with the immune response following vaccination [22].

To evaluate whether maternal antibodies interfere with protection induced by the LaSota strain-vectored ILTV recombinants, rLS/ILTV-gB and rLS/ILTV-gD, we carried out vaccination/challenge experiments with one-day-old commercial broiler chickens that had a mean NDV MDA HI titer of 6.4 ($\log_2$) and detectable ILTV virus neutralization (VN) antibodies. The immunoresponses following vaccination and protection indices (e.g. clinical signs, body weight gain and virus shedding after ILTV challenge) suggest significant protection against ILTV challenge even in the presence of maternally derived antibodies specific for NDV and ILTV.

2. Materials and methods

2.1. Vaccines, viruses and cells

The generation of NDV LaSota strain-vectored ILTV vaccine candidates, rLS/ILTV-gD and rLS/ILTV-gB, were previously described [16] and propagated in 9-day-old SPF chicken embryos at passage level 3. The titers of these vaccine stocks were determined by the 50% egg infective dose (EID₅₀) assay in 9-day-old SPF chicken embryos [23]. The commercial ILTV vaccines, Vectormune (FP-LT) and Trachivax (CEO), were purchased from Ceva Biomune (Lenexa, KS) and Merck Animal Health (Summit, NJ), respectively. The ILTV strain (63140/C/08/BR) was obtained from the pathogen repository bank at the Poultry Diagnostic and Research Center (PDRC, University of Georgia, Athens, GA) and used as the challenge virus [11, 24]. A recombinant ILTV expressing the green fluorescent protein (ILTV/GFP) was used to infect LMH cell line (ATCC[®] CRL-2117[™]) in order to measure neutralizing maternal antibody titers against ILTV [25]. LMH cells were cultured in Dulbecco's Modified Eagle Medium (DMEM, Life Technologies, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS, Life Technologies, Carlsbad, CA) and antibiotics (100U/ml Penicillin, 100µg/ml Streptomycin, 0.25µg/ml Amphotericin B, Thermo Scientific, Suwanee, GA) at 37°C in 5% CO₂ atmospheres. The titers of the ILTV challenge virus and the ILTV/GFP virus stocks were determined by the 50% tissue infectious dose (TCID₅₀) assay in primary chicken kidney (CK) cells in a 96-well format [11, 23].

2.2 Animals

One-day-old commercial broiler chickens were obtained from Fieldale Farms (Baldwin, GA). The breeders of these commercial broiler chickens were vaccinated with ILT vaccine (TCO) once and with NDV vaccines (B₁B₁ and LaSota strains) multiple times before layering eggs. Newly hatched birds were transferred to the BLS-2E animal facilities at the Southeast Poultry Research Laboratory (SEPRL). Birds were housed in Horsfal isolators (Federal Designs, Inc., Comer, GA) with *ad libitum* access to feed and water. At the termination of the experiments all birds were humanely euthanized in accordance to an SEPRL's Institutional Animal Care and Use Committee approved animal use protocol.

2.3 Animal experimental design

One hundred and fifty one-day-old commercial broiler chickens were randomly divided into ten groups of 15 birds. Each bird in groups 1 and 2 was inoculated with 100 µl of phosphate buffered saline (PBS) via intranasal (IN) and intraocular (IO) routes and served as unvaccinated controls. Birds in groups 3, 4, and 5 were vaccinated with 100 µl of rLS/ILTV-gD (1.0×10^7 EID₅₀/ml) per bird via IN/IO routes at 1 (group 3) or 10 (group 4) DOA or both days (group 5). Birds in groups 6, 7, and 8 were vaccinated with 100 µl of rLS/ILTV-gB (1.0×10^7 EID₅₀/ml) per bird via IN/IO routes at 1 (group 6) or 10 (group 7) DOA or both days (group 8). Birds in groups 9 and 10 were vaccinated with one dose of commercial vaccines via the subcutaneous (Vectormune FP-LT at 1 DOA) or eye drop (CEO at 10 DOA) routes as instructed by the manufacturers (Ceva and Merck, respectively). At 21 DOA, the birds in groups 2-10 were challenged with the virulent ILTV (strain 63140/C/08/BR) at a dose of 10^4 TCID₅₀/per bird via intra-tracheal route. Birds in group 1 were untreated and served as non-challenged controls for clinical sign observations and body weight gains. After challenge, clinical signs of ILT were observed and recorded daily for ten days as previously described [16, 26]. Briefly, breathing patterns, conjunctivitis, and the level of depression were scored on a scale of 0 to 3: normal (0), mild (1), moderate (2) and severe (3). A score of 9 was given to a bird that died. The total clinical sign scores per group per day were calculated. Blood samples were collected prior to both vaccination (1 DOA and 10 DOA) and challenge (21 DOA) for determining MDA titers and immunoresponses. Body weights were measured prior to challenge and at 10 days post challenge (DPC). Tracheal and ocular swabs were collected from each bird at 4, 7 and 10 days post-challenge (DPC) and stored in Brain Heart Infusion (BHI) medium (Becton, Dickinson and Company, Sparks, MD) at -80°C until further needed.

2.4. Quantitation of ILTV load in tracheal and ocular swabs.

The ILTV viral load in each tracheal and ocular swab sample was quantified using a quantitative real-time PCR assay (qPCR) with the host $\alpha 2$ -collagen gene as an internal control [16, 27]. DNAs were isolated from the swab samples using a DNeasy 96 Blood & tissue kit (Qiagen). The amount of ILTV DNA was measured by the qPCR assay on an Applied Biosystems® 7500 Fast Real-Time PCR System (Life Technologies, Carlsbad, CA) with specific primers and probes as described previously [16]. A threshold value of 0.05 was used for consistent evaluation of different 96-well plates. The amount of challenge infectious laryngotracheitis virus shed in tracheal and ocular samples was calculated by two methods: standard curve and the relative $2^{-\Delta\Delta CT}$ method. A series of 10-fold dilutions of titrated ILTV challenge virus stock was subjected to the DNA isolation and the qPCR assay in order to establish a standard curve correlating ILTV titers with copy numbers of viral DNA. Samples with any recorded threshold cycle number (C_t) value were considered positive and used to approximate ILTV titers. Based on the qPCR C_t values of the samples, the ILTV titers were converted by referencing the standard curve of ILTV titers/qPCR C_t values. The mean titers of ILTV from each of the treatment groups were expressed in Log_{10} plus standard deviation (SD). The second method, relative $2^{-\Delta\Delta CT}$ was used to calculate the fold reduction in virus shedding between ILTV vaccines (rLS/ILTV-gB, rLS/ILTV-gD, CEO, and FP-LT) relative to PBS. ΔC_T values were calculated from C_T values measured for the gC amplicon minus those of the endogenous control ($\alpha 2$ -collagen).

3.5. Serology

NDV antibody titers were determined using the standard hemagglutination inhibition (HI) test [23], whereas the ILTV antibody titers were measured using a virus neutralization (VN) assay. The VN test was adapted from that published by Zuckermann et al. [28] with modifications. Briefly, the ILTV/GFP virus stock was serially diluted 10-fold. Fifty microliters of the diluted virus were incubated with an equal volume of 50-fold diluted serum at 37° C for 1 hour. After incubation, the virus/serum mix was used to inoculate monolayers of LMH cells in a 96-well plate. The inoculated cells were incubated at 37° C in 5% CO_2 atmospheres for 5 days. The ILTV titers were calculated by counting the green fluorescent formation units (FFU) in the infected cells and the VN titers were calculated by comparison of FFUs in cells containing the

serum samples with those containing SPF chicken serum. The mean VN titers from each of the treatment groups were expressed in Log₁₀ plus standard deviation (SD).

3.6. Statistical analysis

The percentage of body-weight gains in each of the vaccinated/challenged bird groups was compared with that of the control (unvaccinated/non-challenged) group using the student t-Test with a 5% level of significance (Microsoft Excel). The ILTV titers shed in tracheal and ocular swabs from vaccinated/challenged groups were compared with that from the unvaccinated/challenged group using the student t-Test with a 1% level of significance (Microsoft Excel).

3. Results

3.1. Detection of MDA and immunoresponses following vaccination

To determine the titers of NDV- and ILTV-specific maternally derived antibodies in commercial broiler chickens and immunoresponses following vaccination, serum samples collected at 1 DOA (before vaccination) and at 10 and 21 DOA (before challenge) were subjected to the HI (for NDV antibodies) and VN (for ILTV antibodies) tests. These results (Table 1) showed that unvaccinated birds at 1 DOA possessed a mean NDV HI titer of 6.4 (log₂) and a mean ILTV VN titer of 0.51 (log₁₀). The NDV HI titer decreased to 3.64 (log₂) at 10 DOA and 2.59 (log₂) at 21 DOA. In rLS/ILTV-gD and rLS/ILTV-gB vaccinates higher NDV HI titers [3.50 to 6.28 (log₂)] were induced against the NDV vector at 21 DOA. However, vaccination at 10 DOA induced only marginally higher HI titers than those detected in birds vaccinated at 1 DOA. Furthermore, the booster vaccination at 10 DOA did not enhance NDV HI antibody response.

The VN tests revealed that the commercial broilers had detectable levels (0.51 log₁₀) of maternally derived neutralizing antibodies to ILTV at 1 DOA, which decreased to undetectable levels at 21 DOA (Table 1). Blood samples from birds in the rLS/ILTV-gD (1 DOA) group and those in the CEO (10 DOA) group had markedly low levels of VN antibodies to ILTV at 21 days [0.23 (log₁₀) and 0.61 (log₁₀), respectively] in comparison to the VN titer [>4.30 (log₁₀)] of the commercial anti-ILTV serum.

3.2. Clinical protection against virulent ILTV challenge

To evaluate efficacy of the NDV-vectored ILTV vaccines in the commercial broiler chickens with maternal antibodies to NDV and ILTV, the vaccinated chickens were challenged with virulent ILTV at 21 DOA. As shown in Table 2 and Fig. 1, chickens in the unvaccinated (PBS/Chal) group exhibited typical clinical signs of the disease from 3 DPC onward with signs of depression, conjunctivitis or respiratory distress, which were scored 1-3 depending on the severity. Between 5-6 DPC, the infected birds showed severe clinical signs with 13.3% mortality, and gradually decreased in severity thereafter. In contrast, the chickens vaccinated with rLS/ILTV-gD at 1 DOA (rLS/ILTV-gD D1) showed no signs of disease during the entire 10 days period. Only two chickens (13.3%) from the rLS/ILTV-gD D10 and the rLS/ILTV-gD D1+10 groups showed some clinical signs of the disease (scored as mild). Similarly, 2-4 chickens (13.3%-26.6%) from the rLS/ILTV-gB vaccine groups displayed mild clinical signs. Clearly, all birds vaccinated with the recombinant ILTV vaccines were protected against the lethal infection. One bird in the rLS/ILTV-gB D10 vaccinated group died accidentally at 4 DPC as it was trapped in the feeder. In the commercial CEO vaccinated group 1 chicken showed mild conjunctivitis, during 4-8 DPC. Whereas clinical signs were observed in 4 birds (36%) from the group vaccinated with the other commercial vaccine (FP-LT) and one bird died on 4 DPC within this group. As expected all birds in the non-challenge (PBS/Nchal) group remained healthy during the entire observation period.

3.3. Body-weight gain after ILTV challenge

In broiler chickens it has been reported that ILTV infection causes a decrease in body-weight gains [1]. To investigate whether vaccination prevents body weight loss following challenge, the body weights of the broiler chickens were measured prior to challenge (21 DOA) and at the termination of the experiment at 31 DOA. As shown in Table 3, the rates of body-weight gains of all NDV/ILTV vaccines immunized birds except those in the rLS/ILTV-gB D1+10 group, CEO vaccinated birds, and PBS/non-challenged control birds were similar among them, but significantly higher than that in the PBS/Chal control group ($P<0.05$). The rates of body-weight gain in the rLS/ILTV-gB D1+10 group (74.57%) and the FP-LT vaccine group (89.99%) were higher than that in the PBS/Chal group (56.64%). However, statistically the difference was not significant ($P>0.05$). These data indicated that the NDV-vectored ILTV vaccines administered

at 1 DOA prevented immunized birds from a decrease of body-weight gains following ILTV challenge, suggesting that the MDA did not significantly interfere with vaccinal protection.

3.4. Reduction in shedding of the challenge ILTV in vaccinated birds

Reduction in shedding of the challenge virus from tracheal and ocular swabs was another important criterion in the evaluation of the vaccine candidates. The virus load in trachea and eyes was inferred by measuring the amount of ILTV DNA in the tracheal lumen and tears of chickens using real time qPCR. The relative quantitation of challenge virus was measured using two methods: standard curve and relative $2^{-\Delta\Delta CT}$. For the first method, Ct values were converted to ILTV titers using a standard curve of ILTV titers/qPCR Ct values established in this study. For the second method, the fold reduction in virus load was measured relative to the Ct value of samples from sham vaccinates receiving PBS. As shown in Fig. 2, approximately 1-3 log₁₀ reductions of ILTV shedding in the tracheal lumen and tears of all vaccinated chickens were quantified when compared with those samples from the PBS/challenge birds at 4 and 7 DPC. Overall the fold reduction in virus load was more pronounced in tracheal samples than in ocular samples. The greatest reduction in virus load was in tracheal samples collected at day 7 from birds vaccinated with rLS/ILTV-gD at day 10 (123,601-fold reduction: $P < 0.0001$). Strikingly this was a magnitude higher than the reduction observed in tracheal samples from CEO vaccinates. This difference continued with samples collected at 10 DPC although less dramatic. At 10 DPC the ILTV shedding titers from the PBS/challenge group decreased to comparable levels as that noted in the vaccinated groups, especially with the ocular samples, indicating infected birds shed virus for at least 10 days. At this time, three logs of virus could still be detected in tracheal swabs. Statistical analysis verified that ILTV shedding titers from birds in the rLS/ILTV-gD vaccinated groups were significant lower ($p < 0.01$) than those from the PBS/challenge group in all swab samples, except those from tear samples at 10 DPC. Not surprisingly, ILTV shedding titers from birds in the rLS/ILTV-gB vaccinated groups were also significantly lower ($p > 0.01$) than that from the PBS/challenge group at 4 and 7 DPC, but not at 10DPC. This is likely due to the low levels of virus shed at this late time point. Using the standard curve method, the average titers of virus shedding in the trachea of bird vaccinated with rLS/ILTV-gD were nearly identical to that noted in birds vaccinated with the CEO vaccine, the gold standard of ILTV vaccine. However, this was dependent on the day post challenge and

when the samples were taken. At early sampling times (day 4) CEO outperformed rLS/ILTV-gD, but at days 7 and 10 this was reversed and a greater reduction in virus load was noted in vaccinates receiving rLS/ILTV-gD at 10 days old. Unfortunately, 1-day-old birds were only vaccinated with the recombinant vaccine candidates and not CEO, so a comparison of CEO D1 vs gB D1 or gD D1 could not be made. It is worthy to note (i) vaccination at D1 with rLS/ILTV-gD resulted in a greater reduction in virus load with lower titers than that observed in samples from birds vaccinated with either the rLS/ILTV-gB candidate or a vectored fowlpox recombinant expression glycoprotein B and the phosphoprotein UL32 (FP-LT) and (ii) boost vaccination at 10 DOA did not enhance the reduction of the virus shedding nor did it augment protection when compared with the single dose vaccination at either 1 or 10 DOA.

4. Discussion

In this study, we evaluated the protective efficacies of two NDV LaSota strain-vectored ILTV vaccine candidates rLS/ILTV-gB or rLS/ILTV-gD in commercial maternal antibody positive broiler chickens. The results showed that both bivalent vaccine candidates conferred clinical protection, maintained body-weight gains, and significantly reduced ILTV load following challenge. The data suggest that the presence of maternally derived antibodies capable of neutralizing ILTV and high titers of NDV MDA did little to interfere with their abilities to elicit protective immunity against ILTV.

Previously we have demonstrated that the rLS/ILTV-gB outperformed rLS/ILTV-gD in decreasing the amount of virus shed in both ocular and tracheal samples from SPF birds vaccinated at 1 day of age [16]. In commercial broilers vaccinated at day 21 with rLS/ILTV-gB, virus load was only reduced 17 fold in trachea samples. In the current study a fold reduction as high as 9000 (gB D10) could be demonstrated. However, this was dependent on the day the bird received the vaccine and the day the samples for virus load analysis were taken. Higher fold reductions in virus load (>120,000) could be demonstrated in tracheal samples from birds receiving the rLS/ILTV-gD vaccine. Greater fold reductions in virus load were consistently noted in samples taken from the lumen of the trachea, the primary site of ILTV replication, than those noted in tear samples. Overall in the presence of maternally derived antibodies, the rLS/ILTV-gD outperformed the rLS/ILTV-gB vaccine candidate. This was surprising in light of data from our previous studies, but in line with results from protection studies involving NDV

1 recombinants expressing ILTV protein gB or gD chimeras [29]. We expected the gB construct to
2 be superior in protection since it has previously been shown to be an important target for both
3 humoral and cell-mediated responses [10, 14, 30] and should contain more epitopes just based on
4 its size (gB 882aa vs gD 434aa). Apparently, this is unimportant and even detrimental when
5 vaccinating birds with high titers of maternally derived antibodies. Since humoral immunity has
6 only a minor role in protection against herpesviruses, and reportedly protection can occur in the
7 absence of any detectable neutralizing antibodies [29], the protection afforded by the rLS/ILTV-
8 gD candidate is probably due to its capacity to elicit a stronger cell-mediated immune response
9 than the rLS/ILTV-gB candidate.

10 It is a common practice to vaccinate breeder chickens with NDV and other vaccines in many
11 countries around the world [31, 32]. Consequently, the antibodies induced by these vaccines in the
12 breeder chickens would be transmitted to offspring via eggs. During the first 1-14 days of a chick's
13 life, protection is conferred by the maternally derived antibodies. Further protection is achieved by
14 active vaccination. However, high levels of maternal antibodies can often interfere with the
15 induction of an effective immune response by vaccination at a young age. The broiler chickens used
16 in this study were obtained from a commercial chicken farm in Georgia, USA. The breeders of these
17 broiler chickens were vaccinated with ILT vaccine (TCO) once and with NDV vaccines (B₁B₁ and
18 LaSota strains) multiple times before layering eggs. Therefore, these broiler chickens had a high
19 level of NDV HI titers ($6.42 \pm 1.44 \log_2$) and a detectable level of ILTV VN titers ($0.51 \pm 0.10 \log_{10}$)
20 at 1 DOA. It has been reported that the degree of interference due to NDV MDA on vaccinal
21 efficacy is dependent on MDA titers and the genotype of the vaccine strain [20, 21], whereas ILTV
22 maternal antibodies do not appear to interfere with vaccinal efficacy [22]. Our data showed that
23 vaccination of the NDV MDA positive birds with the LaSota strain-based ILTV vaccines at 1 DOA
24 induced marginally lower ($1 \log_2$) HI titer than vaccination at 10 DOA, indicating that the higher
25 NDV titer (HI $6.42 \pm 1.44 \log_2$ at 1 DOA) had stronger interference with immunoresponses than the
26 lower MDA (HI $3.64 \pm 0.81 \log_2$ at 10 DOA). However, all vaccinated birds had much higher ($2-4$
27 $\log_2$) HI titers at 21 DOA than the unvaccinated controls. More importantly, the immunized
28 chickens were clinically protected against the virulent ILTV challenge with significant reductions of
29 the challenge virus shedding when compared with the unvaccinated control birds. Overall the data
30 suggest that the NDV maternal antibodies at the level of HI titers $6.42 \pm 1.44 \log_2$ and the presence
31 of ILTV MDA did not significantly interfere with protective immunoresponses to the NDV LaSota

1 strain-based ILTV vaccines. Our results are similar to the findings that relatively high levels of
2 circulating maternal antibodies against the LaSota strain are required to dampen the
3 immunoresponses following LaSota vaccination [21]. Such high levels are not needed to demonstrate
4 inference following homologous challenge with other NDV strains.

5 The mechanism of maternally derived antibody interference with immune response to
6 vaccination is not well understood. An often-suggested explanation for the lack of vaccination
7 success is that neutralization of vaccine virus by circulating maternal antibodies causes the
8 amount of viral antigen to fall below a certain (undefined) threshold and thereby interfere with
9 immune recognition [18]. The recombinant vaccine vector used in this study was based the
10 commonly used LaSota vaccine, which can robustly replicate in the upper respiratory tract.
11 Therefore, we assumed that although the circulating maternal antibodies to NDV could
12 neutralize the vector to some degree, the LaSota vector might still replicate sufficiently to
13 produce the above threshold amount of viral proteins, both vector- and foreign antigen-derived,
14 needed to elicit a protective immune response in chickens. In addition to the neutralization
15 theory, other proposed hypotheses for the maternal antibody interference with vaccination in
16 humans and other animals are in need of testing [18]. Therefore, further studies are required for
17 better understanding of the maternal antibody interference mechanism in chickens.

18 In summary, we evaluated the protective efficacies of two NDV LaSota strain-vectored ILTV
19 vaccine candidates in commercial broiler chickens with maternally derived antibodies against
20 NDV and ILTV. The results showed that the NDV vectored ILTV gB and gD vaccine
21 candidates conferred protection against clinical signs after challenge with significant reductions
22 in ILTV shedding. The data suggest that the presence of maternal antibodies capable of
23 neutralizing ILTV and high titers of NDV MDA did not significantly interfere with the ability of
24 the two bivalent NDV/ILTV vaccines to elicit protective immunity against infectious
25 laryngotracheitis.

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Figure legend:

Fig. 1. Total clinical sign scores of broiler chickens recorded daily post-challenge. One-day-old commercial broiler chickens were vaccinated with rLS/ILTV-gB, rLS/ILTV-gD, commercial ILTV vaccines CEO and FP-LT, and PBS at one or 10 days of age or both days, and challenged with the virulent ILTV strain 63140/C/08/BR at 21 DPV. The birds were examined daily for clinical signs and the total scores from each group of birds were plotted.

Fig. 2. Infectious laryngotracheitis virus (ILTV) shed in tracheal and ocular samples post challenge. The intra-tracheal (A) and intra-ocular (B) swab samples were collected from ILTV-infected birds at 4, 7, and 10 days post challenge. Total DNA was isolated from the swab samples using a DNeasy 96 Blood & tissue kit (Qiagen). The amount of ILTV DNA was measured by a quantitative Real-time PCR (qPCR) assay on an Applied Biosystems® 7500 Fast Real-Time PCR System (Life Technologies, Carlsbad, CA). The relative $2^{-\Delta\Delta CT}$ method was used to calculate the fold reduction in virus shedding in the (A) trachea lumens and (B) tears between ITLV vaccines (rLS/ILTV-gB, rLS/ILTV-gD, FP-LT, and CEO) relative to challenge virus in sham (PBS) vaccinated birds. ΔC_T values were calculated from C_T values measured for the gC amplicon minus those of the control (collagen amplicon). Titers of shed challenge virus were also estimated using the relative standard curve method (C). A series of 10-fold dilutions of the ILTV challenge virus stock were subject to the DNA isolation and the qPCR assay to establish a standard curve of ILTV titers versa amounts of viral DNA. Based on the qPCR C_t values and the standard curve of ILTV titers/qPCR C_t values, the ILTV titers were converted and listed.

Table 1. MDA and immunoresponses following vaccination detected by the HI and VN tests

	Treatment groups	1 DOA		10 DOA	21 DOA	
		NDV HI ^a	ILTV VN ^b	NDV HI ^a	NDV HI ^a	ILTV VN ^b
1	PBS/NChal	6.42 ± 1.44	0.51 ± 0.10	3.64 ± 0.81	2.59 ± 0.87	0.00
2	PBC/Chal	6.42 ± 1.44	0.51 ± 0.10	3.64 ± 0.81	2.59 ± 0.87	0.00
3	rLS/ILTV-gD D1	N.D	N.D	N.D	5.13 ± 0.99	0.23 ± 0.15
4	rLS/ILTV-gD D10	N.D	N.D	N.D	6.28 ± 1.56	N.D
5	rLS/ILTV-gD D1+10	N.D	N.D	N.D	5.67 ± 1.50	N.D
6	rLS/ILTV-gB D1	N.D	N.D	N.D	3.62± 0.74	N.D
7	rLS/ILTV-gB D10	N.D	N.D	N.D	4.63± 1.34	N.D
8	rLS/ILTV-gB D1+10	N.D	N.D	N.D	3.50± 0.83	N.D
9	FP-LT D1	N.D	N.D	N.D	2.60± 1.07	N.D
10	CEO D10	N.D	N.D	N.D	3.11± 0.66	0.61 ± 0.15

^aNDV hemagglutination inhibition (HI) titer was expressed as log₂ of the mean ± standard deviation

^b ILTV virus neutralization (VN) titer was expressed as $\log_{10}$ of the mean $\pm$ standard deviation

N.D – not determined

Table 2. Observation of clinical signs in chickens at different days post-challenge

[illegible]

Table 3. Percentage body weight (% BW) gained from 21 to 31 days of age (DOA)

	Treatment groups	Mean % BW ^A
1	PBS/NChal	88.79 (1.0000) ^a
2	PBS/Chal	56.64 (0.0002) ^b
3	rLS/ILTV-gD D1	95.72 (0.0275) ^c
4	rLS/ILTV-gD D10	86.11 (0.1821) ^a
5	rLS/ILTV-gD D1+10	92.80 (0.1510) ^a
6	rLS/ILTV-gB D1	83.62 (0.0994) ^a
7	rLS/ILTV-gB D10	90.33 (0.9330) ^a
8	rLS/ILTV-gB D1+10	74.57 (0.0041) ^b
9	FP-LT D1	89.99 (0.0291) ^b
10	CEO D10	95.44 (0.9288) ^a

^A (Mean BW post-challenge [31 DOA] - mean BW pre-challenge [21 DOA] / mean BW pre-challenge) x 100. The body-weight gain rate (%) in each of the challenged bird groups was compared with that of the control (PBS/NChal) group using the student t-Test with a 5% level of significance. The % BW with same lowercase letters were not statistically significant ($P > 0.05$).

Figure 1

Figure 2
A

Fold Reduction in Tracheal Virus Load

	T-Test							
	CEO D10	FP D1	gB D1	gB D1 & 10	gB D10	gD D1	gD D1 & 10	gD D10
Day 4	5.29E-07	1.01E-01	7.71E-03	3.67E-05	1.31E-03	3.47E-04	8.49E-04	3.27E-04
Day 7	2.61E-12	1.38E-03	5.50E-03	3.54E-03	4.30E-07	1.86E-07	4.03E-05	9.97E-22
Day 10	9.90E-06	5.61E-01	6.27E-01	1.12E-03	5.56E-05	2.04E-03	1.37E-01	7.63E-09

B

Fold Reduction in Ocular Virus Load

	T-Test							
	CEO D10	FP D1	gB D1	gB D1 & 10	gB D10	gD D1	gD D1 & 10	gD D10
Day 4	6.73E-07	1.75E-03	1.13E-02	2.69E-07	1.87E-08	6.43E-11	7.19E-10	4.20E-10
Day 7	5.12E-06	2.61E-01	4.91E-02	1.74E-01	9.79E-10	7.91E-06	7.96E-06	1.40E-12
Day 10	6.12E-01	1.98E-01	1.10E-01	5.42E-01	7.77E-05	9.36E-03	2.89E-01	2.61E-01

C

	Titers ($\text{Log}_{10} \text{TCID}_{50}/\text{ml}$) in Tracheal Swabs								
	PBS/CHAL	CEO D10	FP D1	gB D1	gB D1 & 10	gB D10	gD D1	gD D1 & 10	gD D10
Day 4	5.25 ± 0.39	1.42 ± 1.79	3.85 ± 1.89	3.00 ± 1.95	2.93 ± 1.59	3.39 ± 1.95	1.94 ± 2.40	3.13 ± 2.04	2.39 ± 2.39
Day 7	5.12 ± 0.42	0.77 ± 1.04	2.37 ± 1.68	3.21 ± 1.45	3.56 ± 1.58	2.13 ± 1.45	1.35 ± 1.64	2.07 ± 1.68	0.34 ± 0.56
Day 10	3.08 ± 0.82	1.20 ± 1.05	2.69 ± 1.41	3.29 ± 1.46	1.95 ± 0.76	1.12 ± 1.25	1.38 ± 1.51	1.39 ± 1.40	0.73 ± 0.70

	Titers ($\text{Log}_{10} \text{TCID}_{50}/\text{ml}$) in Ocular Swabs								
	PBS/CHAL	CEO D10	FP D1	gB D1	gB D1 & 10	gB D10	gD D1	gD D1 & 10	gD D10
Day 4	4.72 ± 0.93	1.82 ± 1.50	2.29 ± 1.82	2.80 ± 1.28	2.366 ± 1.08	1.67 ± 1.31	1.05 ± 0.77	1.05 ± 1.30	1.19 ± 1.22
Day 7	3.78 ± 0.60	2.03 ± 1.05	3.18 ± 1.11	2.94 ± 0.91	3.08 ± 1.47	1.48 ± 0.78	0.80 ± 1.57	1.39 ± 1.40	0.52 ± 0.79
Day 10	2.24 ± 0.53	1.91 ± 1.16	2.83 ± 1.11	1.39 ± 1.20	2.13 ± 1.04	1.14 ± 0.79	0.58 ± 1.30	2.40 ± 1.51	1.62 ± 1.09