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Protective efficacy of commercial inactivated Newcastle disease virus vaccines in chickens against a recent Korean epizootic strain

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Despite the intensive vaccination policy that has been put in place to control Newcastle disease virus (NDV), the recent emergence of NDV genotype VII strains in Korea has led to significant economic losses in the poultry industry. We assessed the ability of inactivated, oil-emulsion vaccines derived from La Sota or Ulster 2C NDV strains to protect chickens from challenge with Kr-005/00, which is a recently isolated Korean epizootic genotype VII strain. Six-week-old SPF chickens were vaccinated once and challenged three weeks later via the eye drop/intranasal route. All vaccinated birds were fully protected from disease, regardless of the vaccine strains used. All vaccinated and challenged groups showed significant sero-conversion 14 days after challenge. However, some vaccinated birds, despite being protected from disease, shed the challenge virus from their oro-pharynx and cloaca, albeit at significantly lower titers than the unvaccinated challenged control birds. The virological, serological, and epidemiological significance of our observations with regard to NDV disease eradication is discussed.

Keywords: Newcastle disease, protection, vaccination, virus shedding

Introduction

Newcastle disease (ND) is caused by the ND virus (NDV) and it is one of the most important diseases that affects birds, in particular chickens. The epizootic nature of the disease has caused severe economic losses in the poultry industry worldwide since the 1920s. NDV is a member of the avian paramyxovirus type 1 viruses that belong to the *Avulavirus* genus of the *Paramyxoviridae* family [17].

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NDV strains can be classified into three pathotypes (lentogenic, mesogenic and velogenic) on the basis of the severity of disease they cause in chickens. Severity is determined by *in vivo* pathogenicity test parameters, including the mean death time in specific pathogen-free (SPF) chicken embryos and the intra-cerebral pathogenicity index in day-old SPF chickens [1]. A lethal infection by a velogenic NDV pathotype results in high mortality (up to 100%) in chickens and a severe drop in the egg productivity of laying hens. Molecular biologically, multiple basic amino acids at the C-terminus of the F2 protein and phenylalanine at residue 117, which is the N-terminus of the F1 protein, have been demonstrated in the virulent virus [18].

Within the single NDV serotype, eight genotypes (designated I to VIII) have been determined by restriction site mapping and sequence analysis of the fusion (F) gene [2,6,21]. Since the 1920s, at least four genotypes (III, V, VI and VII) have been responsible for epizootic episodes in Korea [10,11]. A genotype III virus was involved in the first epizootic episode, which took place before the 1960s [11]. Genotypes V and VI viruses were most common during the second epizootic (before 1984) and third epizootic episodes (the late-1980s to mid-1990s) [10,11]. A genotype VII virus first emerged in 1984 and then reemerged in 1995. This genotype was prevalent during the most recent "epizootic episode", which has been ongoing since the 2000s [11].

To contain the spread of NDV, chickens are routinely vaccinated with NDV vaccines derived from known strains such as La Sota or Ulster 2C. Many researchers have recently reported that the viruses isolated in Korea, China, Japan and Taiwan belong to the VIId sub-genotype of genotype VII [4,8,11,13,14,16,20-22]. Some of these isolates, including the Korean isolate Kr-005/00, were obtained from chickens that had been vaccinated several times with current NDV vaccines [4,22]. This suggests that these VIId isolates are antigenically distinct from the

currently available NDV vaccine strains. Alternatively, it is possible that the vaccines are not sufficiently immunogenic to prevent the spread of NDV.

In this paper, we assessed the ability of two commercial oil-emulsion NDV vaccines based on the La Sota or Ulster 2C strains to protect chickens from the recently isolated Korean epizootic sub-genotype VIId isolate, Kr-005/00. For comparison, we also challenged the vaccinated chickens with Kr-KJW/49, which is the standard genotype III challenge strain used in Korea [11].

Materials and Methods

Chickens

Six-week-old, SPF chickens from White Leghorn parents (Lohmann, Germany) were used in this study. Before the chickens were introduced into the laboratory experimental facilities of the National Veterinary Research and Quarantine Service of Korea, they were tested for the major viral diseases that affect chickens, including NDV, infectious bursal disease, and infectious bronchitis. The SPF birds were maintained in air-filtered bio-security isolation units (ThreeShine, Korea) with feed and water *ad libitum* for the duration of the study. All experiments were performed at animal facilities which followed the ethical guidelines of animal welfare committee recommended by the National Veterinary Research and Quarantine Service.

Viruses

The two highly virulent NDV strains Kr-005/00 and Kr-KJW/49 were used for challenge in this study. The sub-genotype VIId Kr-005/00 strain was isolated from a laying chicken that had been routinely vaccinated with live and inactivated vaccines during the recent epizootic episode in Korea in 2000 [11]. The genotype III Kr-KJW/49 strain was originally isolated in 1949 from a dead chicken that had been raised free in a backyard in Korea. All viruses were propagated using SPF chicken eggs, titrated as described elsewhere [9], and stored at -70°C until use.

Animal experiments

Seventy-three SPF chickens were divided into nine groups (Table 1). Two locally-produced, inactivated, oil-emulsion vaccines that were derived from La Sota or Ulster 2C were used in this study. Fifty-eight birds were immunized via the intramuscular route with a single dose of the La Sota vaccine (n = 29) or the Ulster 2C vaccine (n = 29), according to the manufacturer's recommendations. Fifteen birds were not vaccinated and served as a control group. Three weeks post-vaccination (pv), birds in each vaccine group were challenged with Kr-005/00 ($10^{4.5}$ EID_{50}/100 µl) (n = 12 per vaccine group) or Kr-KJW/49 ($10^{4.8}$ EID_{50}/100 µl) (n = 12 per vaccine group) via the eye drop (50 µl) and intranasal (50 µl) routes (ED/IN route). Unvaccinated birds were also challenged with Kr-005/00 (n = 5) or Kr-KJW/49 (n = 5) as described above. Fifteen birds, including five unvaccinated, five La Sota-vaccinated, and five Ulster 2C-vaccinated birds, were sham challenged with the same volume (100 µl) of PBS *via* the ED/IN route. All birds were monitored daily for overt clinical signs (depression, respiratory signs, diarrhea *etc*) and mortality. Serum samples were taken by wing bleeds on day 0 pv, 0 post-challenge (pc) and 14 pc. Oro-pharyngeal and cloacal swabs were collected from birds on day 0, 1, 3, 5, 7, 10 and 14 pc.

Virus isolation

Swab samples collected for virus isolation were placed in 3 ml transport medium (Micro Test M4RT; Remel, USA) and stored at -70°C until use. For viral isolation, the samples were clarified by centrifugation at 3,000 × g for 10 min. A 100-µl aliquot of the supernatant was serially diluted 10-fold and inoculated onto monolayers of primary chicken embryo fibroblast cells in 96-well tissue culture microtitre culture plates (Nunc, Denmark). The cells were incubated for five d at 37°C and observed daily for cytopathic effects, which are characterized by scattered focal areas of cell rounding and syncytia formation.

Serology

Serum titers to NDV were determined by hemagglutination inhibition (HI) tests in U-bottom microtiter plates as described elsewhere [1]. Two-fold dilutions of test sera in PBS (pH 7.4) were mixed with the same volume of La Sota antigen (4 HA units). The mixture (50 µl per well) was then incubated at room temperature for 30 min. Fifty µl of 0.5% chicken RBC in PBS was then added. The HI endpoint was determined as the last dilution that showed complete inhibition of HA activity. All tests were repeated twice. HI titers are expressed as reciprocal $\log_2$ in this study.

Statistical analysis

A three-way ANOVA and paired t-test were performed to evaluate the influence of multiple variables on the serological data. The vaccine strain (La Sota or Ulster 2C), the challenge virus (Kr-005/00 or Kr-KJW/49), and the test time (0 day pc or 14 day pc) served as independent variables. Variation among these three variables was analyzed by using a three-way ANOVA. Variation in the antibody titers at 0 day pc and 14 day pc within each factor category was analyzed by using paired t-test. Variation between or within groups was considered to be significant at $p < 0.05$.

Results

Protection of vaccinated SPF chickens against challenge

No overt clinical signs of ND were observed in any birds

prior to challenge. Protection from virulent NDV challenge was determined by the absence of clinical signs and death during the 14 day pc period (Table 1). None of the birds that had been vaccinated with a La Sota- or Ulster 2C-derived vaccine showed clinical signs after challenge with Kr-005/00 or Kr-KJW/49. In contrast, 100% of the unvaccinated birds that had been challenged with Kr-005/00 or Kr-KJW/49 died within 5 day pc. All dead birds displayed severe depression between day 2-4 pc. All unchallenged birds in each of the three groups remained normal during the course of the experiment.

Serological response of SPF chickens to NDV

Sera were taken from chickens on day 0 pv, 0 pc, and 14 pc and the anti-NDV antibody titers were determined by the HI test. None of the sham control birds had detectable HI antibodies to NDV during the course of the study. The serologic results were analyzed by using a three-way ANOVA based on three independent variables (Table 2). Prior to challenge (0 day pc), the Ulster 2C vaccine group had a significantly higher geometric mean HI antibody titer compared to that of the La Sota vaccine group ($p < 0.05$). On day 14 after being challenged with Kr-005/00 or Kr-KJW/49, both the La Sota- and Ulster 2C-vaccinated groups showed significant increases in HI antibody titers (paired *t*-test, $p < 0.05$). These rises in HI titers between 0 and 14 day pc were not observed in either non-challenged control vaccine group (paired *t*-test, La Sota group $p = 0.62$, Ulster 2C group $p = 0.74$). The geometric mean HI titers on day 0 and 14 pc differed significantly between challenge and non-challenge control groups but not between the challenge groups.

Table 1. Protection of vaccinated SPF chickens with inactivated oil-emulsion vaccines against challenge with virulent Newcastle disease virus

Group (Vaccine Strain)	HI titer (log_2) prior to challenge	Challenge*			Protection†	
		Strain	Route	No. birds	Clinical signs	Mortality
La Sota	5.2 ± 1.4	Kr-005/00	ED/IN	12	0/12	0% (0/12)
	4.3 ± 1.9	Kr-KJW/49	ED/IN	12	0/12	0% (0/12)
	5.4 ± 1.3	PBS	ED/IN	5	0/5	0% (0/5)
Ulster 2C	7.5 ± 1.1	Kr-005/00	ED/IN	12	0/12	0% (0/12)
	6.6 ± 1.3	Kr-KJW/49	ED/IN	12	0/12	0% (0/12)
	5.8 ± 2.6	PBS	ED/IN	5	0/5	0% (0/5)
Control	0	Kr-005/00	ED/IN	5	5/5	100% (5/5)
	0	Kr-KJW/49	ED/IN	5	5/5	100% (5/5)
	0	PBS	ED/IN	5	0/5	0 % (0/5)

*Six-week-old SPF birds were vaccinated with a commercial inactivated oil-emulsion vaccine and challenged three weeks later with $10^{5.0}$ EID_{50} of virulent virus *via* the eye drop and intranasal (ED/IN) route. †Protection of chickens against challenge was observed over the two weeks following the challenge.

Table 2. Serologic responses before and after challenge in vaccinated SPF chickens with an inactivated oil-emulsion vaccine

Group (Vaccine Strain)	Challenge strain					
	Kr-005/00 (VIId)		Kr-KJW/49 (III)		Sham-challenge	
	0 dpc	14 dpc	0 dpc	14 dpc	0 dpc	14 dpc
Vaccine						
La Sota	5.2 ± 1.4* (n = 12)^{a,p}	9.3 ± 1.5^{b,q}	4.3 ± 1.9 (n = 12)^{a,p}	8.0 ± 2.4^{b,q}	5.4 ± 1.3 (n = 5)^{a,p}	5.6 ± 1.8^{a,p}
Ulster 2C	7.5 ± 1.1 (n = 12)^{a,q}	10.0 ± 1.0^{b,q}	6.6 ± 1.3 (n = 12)^{a,q}	8.7 ± 1.8^{b,q}	5.8 ± 2.6 (n = 5)^{a,p}	6.0 ± 2.4^{a,p}
Control	0 (n = 5)^{c,r}	NT	0 (n = 5)^{c,r}	NT	0 (n = 5)^{c,r}	0

*The data are geometric mean HI antibody titers (log_2) ± SD. $^{a, b, c}$Values with different superscripts within row differ significantly ($p < 0.05$). $^{p, q, r}$Values with different superscripts within column differ significantly ($p < 0.05$). dpc: day post challanege.

Virus shedding in vaccinated chickens after fatal challenge

Oro-pharyngeal and cloacal swabs were collected from the birds on day 0, 1, 3, 5, 7, 10, and 14 pc and subjected to virus isolation tests. The results of these tests are presented in Table 3 and Fig. 1. NDV was not isolated from any of the swabs taken from unchallenged birds. All unvaccinated, challenged birds were positive for viral shedding and their viral titers increased precipitously after challenge until the birds died. The maximal virus titers shed by the unvaccinated, Kr-005/00-challenged birds were approximately 10^5 $TCID_{50}$/ml on day 5 pc. The unvaccinated, Kr-KJW/49-challenged birds had maximal viral titers of $\geq 10^7$ $TCID_{50}$/ml, *i.e.*, at least hundred-fold higher compared to the unvaccinated, Kr-005/00-challenged birds.

In the vaccinated, Kr-005/00-challenged birds, NDV was isolated from 83% and 42% of the oropharyngeal swabs from the La Sota- and Ulster 2C-vaccinated birds, respectively, and from 25% of the cloacal swabs collected from both the La Sota- and Ulster 2C-vaccinated birds. In the vaccinated, Kr-KJW/49-challenged birds, NDV was isolated from 75% and 42% of the oropharyngeal swabs from the La Sota- and Ulster 2C-vaccinated birds, respectively, and from 33% of the cloacal swabs collected from both the La Sota- and Ulster 2C-vaccinated birds.

The patterns of virus shedding by each challenge group are presented in Fig. 1. The titer of viral load is represented as the highest titers measured in each group at sampling time. These data show that unvaccinated animals revealed skyrocketed rise prior to death in viral shedding from both swab sites. All vaccinated animals challenged with Kr-005/00 showed peak titer in the oro-pharynx and cloaca ($10^{3.0}$ $TCID_{50}$/ml) on day 3 and 5 pc, respectively. In contrast, vaccinated animals challenged with Kr-KJW/49

Table 3. Virus shedding from oro-pharyngeal and cloacal swabs of vaccinated chickens with an inactivated oil-emulsion vaccine after challenge

	Challenge strain					
	Kr-005/00		Kr-KJW/49		Sham-challenge	
	Oropharynx	Cloaca	Oropharynx	Cloaca	Oropharynx	Cloaca
Vaccine						
La Sota (n = 29)	10/12* (83%)^{a,p}	3/12 (25%)^{b,q}	9/12 (75%)^{a,p}	4/12 (33%)^{b,q}	0/5 (0%)^{c,r}	0/5 (0%)^{c,r}
Ulster 2C (n = 29)	5/12 (42%)^{b,q}	3/12 (25%)^{a,q}	5/12 (42%)^{b,q}	4/12 (33%)^{b,q}	0/5 (0%)^{c,r}	0/5 (0%)^{c,r}
Control (n = 15)	5/5 (100%)^{a,p}	5/5 (100%)^{a,r}	5/5 (100%)^{a,p}	5/5 (100%)^{a,p}	0/5 (0%)^{c,r}	0/5 (0%)^{c,r}

*The data are the number of birds that shed virus during the experiment / number of test birds. $^{a, b, c}$Values with different superscripts with row differ significantly ($p < 0.05$). $^{p, q, r}$Values with different superscripts with column differ significantly ($p < 0.05$). dpc: day post challanege.

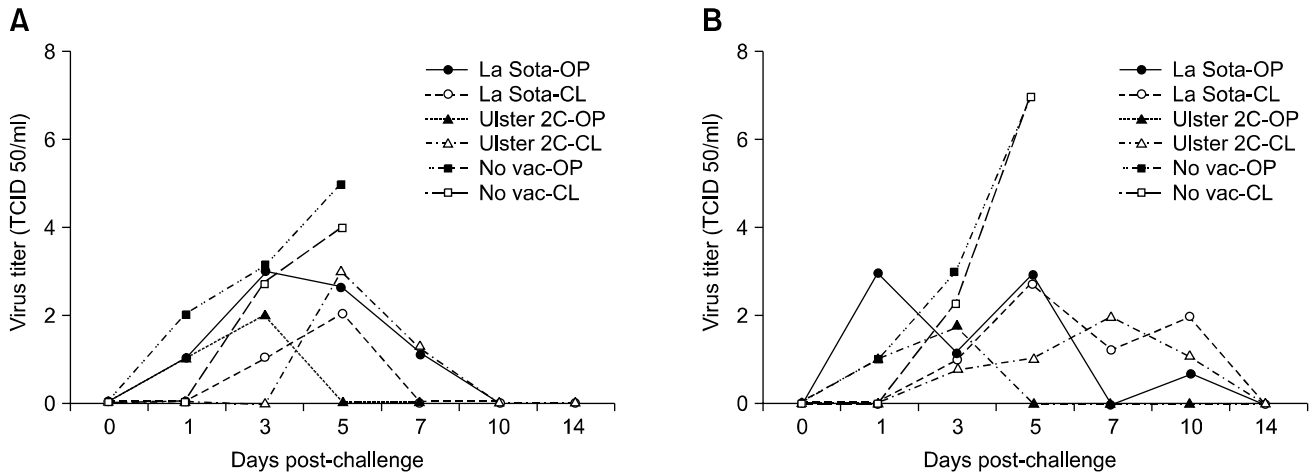

Fig. 1. Titers of Newcastle disease virus recovered from oro-pharyngeal and cloacal swabs after SPF chickens that had been vaccinated with La Sota- or Ulster 2C-derived vaccines were challenged with Kr-005/00 (A) or Kr-KJW/49 (B). OP; oropharyngeal swab, CL; cloacal swab.

showed prolonged duration of virus shedding. Briefly, the La Sota-vaccinated animals showed peak titer in the oro-pharynx ($10^{3.0}$ $TCID_{50}$/ml) until day 5 pc and in the cloaca ($\geq 10^{2.0}$ $TCID_{50}$/ml) up to day 10 pc. The Ulster 2C-vaccinated animals showed peak titer in the oro-pharynx and cloaca ($10^{2.0}$ $TCID_{50}$/ml) on day 3 and 7 pc, respectively. It indicates that virus shedding started from the oropharynx before cloacal shedding commenced.

Viral shedding titers from the La Sota-vaccinated birds (average $\log_2$ HI titers of 4.3 to 5.2) after challenge were lower than the Ulster-vaccinated birds (average $\log_2$ HI titers of 6.6 to 7.5) regardless of the challenge strain that was used. This seems to be related with the titers of HI antibodies that were induced by vaccination (Table 1). While all vaccinated birds (8 birds) that had an $\log_2$ HI titer of ≤ 4 at challenge exhibited virus shedding from their oropharynx or cloaca, NDV was not recovered from birds (11 birds) that had $\log_2$ HI titers exceeding 8 at challenge. This was true regardless of the vaccine strain that was used.

Discussion

Analysis of the NDV strains that were recently isolated from vaccinated flocks in China, Taiwan and Korea has led several researchers to suggest that the antigenic variant genotype VII may be responsible for the continuing outbreaks of ND in Asia. Here, we assessed whether a genotype VII isolate can overcome the immunity of chickens induced by the currently available ND vaccines. We found that these vaccines provide chickens with full protection from disease caused by the genotype VII virus, as no mortality or disease symptoms were observed in any of the vaccinated chickens. The vaccines were also protective against Kr-KJW/49, which is a previously prevalent NDV strain (genotype III). Our results support the data reported by Lui *et al.* [14] that demonstrated that chickens vaccinated with the La Sota strain were fully protected from challenge with heterologous NDV strains of different genotypes.

Although none of the chickens vaccinated once with a La Sota- or Ulster 2C-derived vaccine became sick or died upon challenge in our study, most shed the challenge virus from their oropharynx and cloaca (although at considerably lower levels than those shed by unvaccinated chickens). Similar findings were also reported by Kapcynsky and King [7]. We also found that some of infected birds commenced viral shedding from their cloaca after virus shedding from their oropharynxes. This indicates systemic dissemination in the some of infected birds (especially with low $\log_2$ HI titers ≤ 4), despite vaccination. These observations suggest that vaccinated chickens may still act as reservoirs and be a source of NDV spread within and between farms, especially via their faeces or faeces-contaminated materials (i.e., eggs, vehicles and people).

In this study, the pattern of viral load in chickens infected with Kr-KJW/49 was significantly different from that in chickens infected with the Kr-Kr005/00. In the unvaccinated controls, Kr-KJW/49-challenged birds had viral loads 100 times higher than the Kr-005/00-challenged birds, despite similar titers of challenge virus. Vaccinated birds challenged with Kr-KJW/49 had a prolonged duration of virus shedding compared to vaccinated birds challenged with Kr-005/00, suggesting re-infection in chickens with low HI titers. Thus, birds with lower vaccine-induced immunity may continue to be susceptible to NDV infection. This may also explain why virulent NDV has been isolated from birds despite numerous vaccinations and how NDV field viruses can be circulated within farms in which chickens were vaccinated routinely [4,22]. It should be noted that young broiler chickens, having a short life span, are usually vaccinated with live attenuated vaccine *via* various routes (e.g. drinking water, sprays, and eye drops). Kapcynski and King [7] found significantly lower titers of virus shedding in chickens receiving live vaccines compared to chickens receiving inactivated NDV vaccines, presumably because of the development of stronger mucosal immunity. However, it should be noted that live vaccines are predominantly used for young birds of several weeks old or less in most of countries. Maternal antibodies against NDV can interfere with the immune response induced by live vaccine, resulting in lower and uneven immunization, depending on the level of maternal antibodies against NDV. This suggests that some young chickens may susceptible to NDV infection and act as reservoirs of NDV spreading.

In conclusion, it appears that poor vaccinal immune response is responsible for viral spreading rather than viral antigenic variation. We found that to protect chickens from NDV and prevent them from acting as viral reservoirs, it is important to maintain solid and potent immunogenicity by efficient vaccination program. In the field, routine vaccinations are not inducing sufficiently high levels of immunity to control the disease. This is due, presumably, to other factors such as maternal antibody interference in young chickens, immunosuppression by other agents, poor handling or administration of vaccines or inappropriate vaccination schedules. Thus, routine vaccinations should be supported by high bio-security measures. In this regard, the all-in/all-out program can be recommended as one of the most effective measures to control the disease. The DIVA (differentiation between vaccinated and infected animals) strategy that uses marker vaccines, including subunit, recombinant, and DNA vaccines [3,5,12,15,18,19], may be a tool ideal for removing the source of NDV in endemic areas, but it is still not applied to NDV eradication in endemic countries such as Korea. Alternatively, it is considerable to highlight more strengthened eradication

program and financial support and to offer incentives to encourage the farmers to report the disease earlier.

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