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RESEARCH ARTICLE

Efficacy of a Newcastle Disease Vaccine Developed from Korean Strain NDRL0901 in Protecting against Recently Emerged Newcastle Disease Viruses in Southeast Asia

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ABSTRACT

New Newcastle disease viruses (NDVs) have been circulating among poultry flocks in several Southeast (SE) Asian countries since the mid-2000s. This study analyzes whether the NDV vaccine strain, NDRL0901, protects against genotype VIIh of NDV. The efficacy of the NDRL0901 vaccine was tested in week-old and three-week-old specific pathogen free (SPF) chickens. Week-old chickens that had been vaccinated had geometric mean hemagglutination inhibition (HI) titers of $2^{4.1}$ to $2^{4.7}$ two weeks after vaccination. The vaccinated birds were completely protected against potentially fatal clinical disease from both the MY3519/2010 and VN5/2007 NDV strains, which were administered via eye drops. No virus was detected in the vaccinated birds, regardless of the challenge virus. When three-week-old SPF chickens were vaccinated, the birds had geometric mean HI titers of $2^{6.3}$ two weeks after vaccination. The NDRL0901 vaccine provided complete clinical protection against fatal challenges from the NDV isolate, KN5/2011, which was administered via the intramuscular route. No virus shedding was detected in the vaccinated birds after administration of the challenge virus. Our results indicate that locally developed NDV vaccines using NDRL0901 vaccine strain could provide complete protection against the emergent NDV genotype VII in SE Asia.

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INTRODUCTION

One of the most prevalent of poultry diseases, Newcastle disease (ND) can cause substantial economic loss and remains a major threat to the poultry industry around the world. The causative agent of the disease is the ND virus (NDV), which belongs to the *Avulavirus* genus from the *Paramyxoviridae* family (Mayo, 2002; Amarasinghe *et al*., 2017). Based on the severity of clinical manifestation in susceptible chickens (OIE, 2012), NDV isolates are categorized into at least four pathotypes including asymptomatic, lentogenic (the least virulent), mesogenic (moderately virulent), and velogenic (highly virulent) pathotypes. Velogenic isolates are further divided into viscerotropic velogenic (Asiatic velogenic) and neurotropic velogenic pathotypes. These pathotypes have usually been determined by *in vivo* pathogenicity tests, such as the intra-cerebral pathogenicity index (ICPI) of inoculated day-old chickens and the mean death time (MDT) of inoculated embryonated chicken eggs. Using nucleotide sequence information from the fusion protein gene, NDV isolates are classified into two phylogenetic groups: Class I (single genotype) and Class II (more than sixteen genotypes) (Miller *et al.*, 2010; Diel *et al*., 2012). Class I viruses and most Class II genotype I and II viruses are of low virulence for chickens, whereas most Class II viruses (with the exception of genotypes I and II) are velogenic viruses (Miller *et al.*, 2010; Jabeen *et al*., 2015).

ND is still endemic in many Asian countries. Southeast (SE) Asia, in particular, is considered to be a major epidemic center for the disease. Recently, NDV isolates that are genetically different from known NDVs have been reported in SE Asia (Liu *et al*., 2008; Ke *et al*., 2010; Choi *et al*., 2013). These NDV isolates have been classified as a new subtype of Class II genotype VII (VIIh) (Miller *et al*., 2015). They are regarded as being almost a new genotype (Choi *et al*., 2014). These VIIh viruses were first detected in Malaysia (2004), Indonesia (2009), Cambodia (2011) and Vietnam (2011) (Tan *et al*., 2010; Xiao *et al*., 2012a; Choi *et al*., 2013). This suggests that velogenic VIIh viruses

have spread widely across countries in SE Asia since the mid-2000s and are predicted to become one of the major VII subtypes with a wide distribution in the region (Berhanu *et al*., 2010; Rui *et al*., 2010; Choi *et al*., 2014).

The objective of this study was to investigate whether NDV vaccines developed from locally isolated apathogenic strain of NDV can protect birds against the velogenic NDVs that are spreading rapidly in SE Asia. The protection efficacy test was carried out using a commercial NDV vaccine strain. The test analyzed the viral shedding from vaccinated chickens in addition to the standard observation of morbidity and mortality after challenge.

MATERIALS AND METHODS

Viruses: NDRL0901, a commercial live NDV vaccine strain derived from the wild duck NDV isolate (KR/duck/13/07, genotype I) (Lee *et al*., 2009; Kim *et al*., 2016) was used for immunization. Three genotype VII viruses from Vietnam (VN5/2007, VIId and KN5/2011, VIIh) and Malaysia (MY3519/2010, VIIh) were used for the challenge (Choi *et al*., 2014). All viruses used were propagated using SPF-embryonated chicken eggs (ECEs) (Valo Biomedia, IA, USA). The infective titer was expressed as 50% egg infective dose (EID_{50}) according to the Reed and Muench formula (Reed and Muench, 1938).

Animal experiments: All experiments were approved by our institutional Animal Experiment and Ethics Committee. The SPF chickens were hatched from SPF ECEs and raised in air-filtered bio-security isolation units. Protection efficacy tests of vaccines against virulent NDVs from SE Asia were performed in two ways, as described below.

Experiment I: The SPF chicks (n=34) was randomly divided into five groups (Table 1). At one week old, Groups I to III were immunized with the NDRL0901 strain (10^6 EID_{50} per dose) via eye drops, whereas Groups IV and V were mock infected with phosphate buffered saline (PBS) via the same route. Fourteen days later, Group I (n=9), Group II (n=8), Group IV (n=4) and Group V (n=5) were challenged with the MY3519/2010 (10^6 EID_{50} per dose) or the VN5/2007 (10^6 EID_{50} per dose) strains via the intranasal route. Group III (n=8) was raised without any challenge. Clinical signs were monitored daily for 14 days post challenge (dpc). Blood samples were collected from all birds at 0, 14 and 21 days post vaccination (dpv). Cloacal swabs were taken on days four and seven after the challenge for re-isolation of shedding viruses.

Experiment II: Twenty-four SPF chicks were randomly divided into two groups of twelve chicks each. When three weeks old, Group A (n=12) was immunized with NDV strain NDRL0901 (10^6 EID_{50} per dose) via eye drops, whereas Group B (n=12) was inoculated with PBS via the same route. On 14 dpv, all birds were challenged with the NDV isolate KN5/2011 (10^6 EID_{50} per dose) by the intra-muscular (i.m.) route. Clinical signs were monitored daily for two weeks after the challenge. All the birds were bled at 0 and 14 dpv for serological testing. Cloacal swabs were taken on 4 and 7 dpc for re-isolation of shedding viruses.

Serological testing: Serological testing was carried out using microtiter hemagglutination inhibition (HI) using four HA units of NDV antigen and 1% (vol/vol) chicken RBC in V-bottomed 96-well plates as described elsewhere (OIE, 2012). The HI antibody titer was determined as the reciprocal $\log_2$ of the highest dilution showing complete inhibition.

Virus re-isolation: Re-isolation and titration of the shedding NDVs from swab samples was conducted in primary chicken embryo fibroblast (CEF) cells in 96-well flat-bottomed tissue culture plates as described elsewhere (Choi *et al*., 2013). Titers (50% tissue culture infective dose, $TCID_{50}$) of shedding viruses were calculated according to the Reed and Muench formula.

Statistical analysis: Comparison of serological data was carried out using the statistical software program SAS. Variables were compared using the Student t-test (within a group) or one-way analysis of variance (ANOVA) (among groups). In one-way ANOVA, post hoc, multiple comparisons were performed using Duncan (D) multiple comparison tests. Variation between or within groups was considered to be significant at $P<0.05$.

Table 1: Protection efficacy of the NDRL0901vaccine following natural route challenge with SE Asia isolates of NDV

Group	Vaccination*	No.	Log_2 (Mean HI titer ± SD)			Challenge**	Survival rate	Virus shedding	
			0dpv	14dpv(0dpc)	21dpv(7dpc)			4dpc	7dpc
I	NDRL0901	9	$0.0\pm0.0^{a,p}$	$4.7\pm1.8^{b,q}$	$6.0\pm1.0^{c,r}$	MY3519/2010	100% (9/9)***	0/9(0.0±0.0)****	0/9(0.0±0.0)
II	NDRL0901	8	$0.0\pm0.0^{a,p}$	$4.1\pm1.0^{b,q}$	$5.5\pm0.5^{c,r}$	VN5/2007	100%(8/8)	0/8(0.0±0.0)	0/8(0.0±0.0)
III	NDRL0901	8	$0.0\pm0.0^{a,p}$	$4.6\pm1.2^{b,q}$	$6.3\pm1.0^{c,r}$	-	-	NT	NT
IV	-	4	$0.0\pm0.0^{a,p}$	$0.0\pm0.0^{a,p}$	$0.0\pm0.0^{a,p}$	MY3519/2010	0%(0/4)	4/4 (5.5±0.4)	NT
V	-	5	$0.0\pm0.0^{a,p}$	$0.0\pm0.0^{a,p}$	$0.0\pm0.0^{a,p}$	VN5/2007	0%(0/5)	5/5 (5.3±0.7)	NT

*Week-old SPF birds were vaccinated with NDRL0901 strain ($10^{6.0}$ EID_{50} per dose) via eye drops; **Vaccinated birds were challenged two weeks later with NDV isolates MY3519/2010 or VN5/2007 ($10^{6.0}$ EID_{50} per dose) via eye drops; *** Number of survived birds/ number of tested birds; ****Number of positive birds / number of tested birds (mean shedding virus titer ±SD (log_{10})); ^{abc} Values with different superscripts within row differ significantly ($P<0.05$); ^{pqr} Values with different superscripts within column differ significantly ($P<0.05$).

Table 2: Protection efficacy of the NDRL0901vaccine following intramuscular route challenge with SE Asia isolate of NDV

Group	Vaccination*	No.	Log_2(Mean HI titer ± SD)		Challenge**	Survival rate	Virus shedding	
			0dpv	14dpv			4 dpc	7 dpc
A	NDRL0901	12	$0.0\pm0.0^{a,p}$	$6.3\pm1.7^{b,q}$	KN5/2011	100% (12/12) ***	0/12(0.0±0.0)****	0/12(0.0±0.0)
B	-	12	$0.0\pm0.0^{a,p}$	$0.0\pm0.0^{a,p}$	KN5/2011	0% (0/12)	12/12(5.1±0.7)	NT

*Three-week-old SPF birds were vaccinated with NDRL0901 strain ($10^{6.0}$ EID_{50} per dose) via eye drops; **Vaccinated birds were challenged two weeks later with NDV isolate KN5/2011 ($10^{6.0}$ EID_{50} per dose) viai.m. route; ***Number of survived birds/ number of tested birds; ****Number of positive birds / number of tested birds (mean shedding virus titer ±SD (log_{10})); ^{ab} Values with different superscripts within row differ significantly ($P<0.05$); ^{pq} Values with different superscripts within column differ significantly ($P<0.05$).

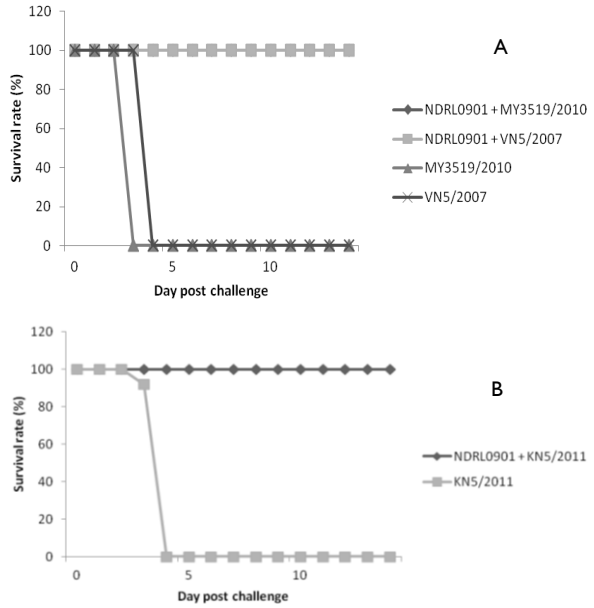

Fig. 1: Time course survival rate of vaccinated chickens after challenge with SE Asia isolates of NDV via (A) natural route or (B) intramuscular route.

RESULTS

Protection against velogenic NDVs from SE Asia following exposure via natural routes: We assessed whether NDV vaccine developed from local strain can protect chickens against velogenic NDV from SE Asia following exposure via natural routes (Table 1). The vaccinated birds showed no clinical signs of disease and there were no deaths regardless of which challenge virus was administered (Table 1). However, when the non-vaccinated groups (Groups IV and V) were challenged with the MY3519/2010 and the VN5/2007, respectively, there was a 100% mortality rate within 4 dpc (Fig. 1). Titers of serum antibody for NDV were determined by the HI test (Table 1). In all three vaccination groups (Groups I, II and III), no birds had detectable NDV antibodies before vaccination. NDV antibodies were detected 14 dpv in all the vaccination groups (Groups I, II and III). These vaccination groups had geometric mean HI titers (log_2) of 4.1 to 4.7. The geometric mean HI titers of Groups I and II were significantly increased seven days after the challenge when compared with pre-challenge antibody titers. However, the difference in the HI titers between the vaccinated groups (Groups I and II) that were challenged and the vaccinated group that was not challenged (Group III) was not statistically significant ($P<0.05$). Viruses shed from the vaccinated birds that were challenged were examined by virus re-isolation from cloacal samples collected 4dpc and/or 7dpc. Both the non-vaccinated groups that were challenged (Groups IV and V) shed challenge viruses through cloaca before death. Titers of shedding viruses ranged from$10^{5.3}$ to $10^{5.5}$ $TCID_{50}$/0.1ml. There were no significant differences in titers of shedding viruses between the groups ($P<0.05$). However, challenge viruses were not detected in every vaccinated bird that was challenged, regardless of challenge virus. Our results indicate that the NDRL0901 vaccine strain can fully protect chickens against infection by velogenic NDVs from SE Asia when vaccinated birds are exposed to challenge viruses via natural infection route.

Protection efficacy against velogenic NDV following challenge via injection route: Protection efficacy tests of the NDV vaccine against velogenic NDV from SE Asia were further tested in three-week-old SPF chickens. None of the vaccinated birds showed any clinical signs of ND after vaccination with NDRL0901 at this age. In the serological test, vaccinated birds showed sero-conversion to NDV at 14 dpv. The geometric mean HI titers (log_2) of the group were 6.3±1.7 (Table 2). All birds from the non-vaccinated group had no detectable NDV antibodies over 14 days. On 14 dpv, all birds from both the vaccinated and non-vaccinated groups were challenged with velogenic NDV KN5/2011 (VIIh) isolate via the i.m. route. For the non-vaccinated birds, this resulted in 100% mortality within four days after the challenge. The vaccinated birds, on the other hand, displayed no clinical signs of disease, nor were there any deaths during the two weeks after the challenge (Fig. 1).

Shedding viruses from vaccinated birds that had been challenged were examined by virus re-isolation from cloacal swab samples 4 and/or 7dpc, respectively. Vaccinated birds that had been challenged with NDRL0901 did not shed challenge virus in cloacal swabs until 7 dpc. However, the non-vaccinated group shed challenge virus through cloaca with the infective titer of $10^{5.1\pm0.7}$ $TCID_{50}$/0.1ml on 4 dpc, before death. Our results indicate that the NDRL0901 vaccine can fully protect chickens against velogenic genotype VIIh NDV (KN5/2011) isolate when administered by the i.m. route.

DISCUSSION

ND in the commercial poultry industry has been controlled by appropriate prophylactic vaccination on farm for several decades. Most live or killed NDV vaccines administered on farm have been produced based on the least virulent NDVs belonging to genotypes I or II in Class II (Dortmans *et al*., 2012). These vaccine viruses are genetically divergent from virulent viruses of the same class that appear in the field (Miller *et al*., 2010), especially Class II genotype VII, one of the genotypes most responsible for recent outbreaks in Asia (Tan *et al*., 2010; Choi *et al*., 2013; Choi *et al*., 2014).

Outbreaks of ND caused by the genotype VII virus strains have been frequently reported in the flocks vaccinated with the conventional NDV Lasota strain or other commercially available vaccines in Asian countries including China and Korea (Cho *et al*., 2008; Liu *et al*., 2008). This poor vaccinal protection against some VII viruses has been supported by several studies on *in vivo* protection efficacy (Kapcynski and King, 2005; Miller *et al*., 2009). The genotype VII virus has currently at least eight subtypes (VIIa to VIIh) in Asia and shows genetic diversity among subtypes (Choi *et al*., 2013; Choi *et al*., 2014). VIIh viruses, in particular, are highly divergent from other known VII subtypes and emerged in SE Asia in the mid-2000s. Since the late 2000s, these VIIh viruses have been the cause of substantial economic loss, even in large-scale poultry farms using conventional NDV vaccination programs in Malaysia. This has raised the question that vaccination failure might be due to antigenic divergence between the vaccine strains and virulent field

viruses (Kapczynski and King, 2005; Miller *et al*., 2007, 2009; van Boven *et al*., 2008; Hu *et al*., 2009).

Accordingly, we examined whether vaccines developed from locally isolated apathogenic NDV vaccine strain can protect chickens from highly virulent NDVs (VIIh viruses as well as the VIId virus). Here, we used single NDV vaccine strain NDRL0901 instead of using multiple commercial NDV vaccine strains. The NDRL0901 strain is one of the NDV vaccines traditionally used in Korea and it was expected that the genetically divergent field strains of genotype VIIh or VIId viruses may get better neutralized from the protection developed by NDRL0901. As the protection efficacy of vaccine from Korean strain was further assessed by challenge with highly virulent NDVs through two different challenge routes, the natural intranasal exposure route and the i.m. injection route (used internationally for potency testing), the results clearly showed that both the natural route and i.m route resulted in full protection from the disease.

In the study, none of the vaccinated birds showed clinical signs of disease or mortality. No vaccinated birds showed viral shedding regardless of challenge virus strains when exposed to challenge viruses via the natural route. Similar results were also observed in vaccinated birds challenged via i.m. injection. Our results indicate that the NDRL0901 vaccine provides complete protection against challenge viruses regardless of the challenge route. These results are in contrast with several previous studies arguing that current commercially available vaccines prevent disease but cannot stop viral shedding (Hu *et al*., 2009; Miller *et al*., 2009; Xiao *et al*., 2012b). The degree of protection offered by vaccination to SPF chickens could be affected by many factors, including commercial vaccine variables (strain, dose, vaccination route, etc.), challenge virus variables (strain, dose, challenge route, etc.) and chicken variables (breed, age, environment, etc.). Here, we used the optimal dose (approximately 10^6 EID_{50} per dose) of the NDRL0901 virus contained in the developed vaccine. All challenge viruses (approximately 10^6 EID_{50} per dose) killed all birds with viral shedding within five days. This means that the dose of viruses used in the challenge was sufficient for protection efficacy.

In general, the HI antibody titers are considered to correlate well with protection efficacy against virulent NDV (van Boven *et al*., 2008). The NDRL0901 strain was highly immunogenic in three-week-old chickens, provoking a strong humoral immune response (mean HI titers of $2^{6.3}$ at 14 dpv), although antibody response was relatively low in week-old chickens (mean HI titers of $2^{4.1-4.7}$at 7 dpv) due to their immature immune systems. As a result, it is reasonable to infer that antibody titers of protective levels induced by the NDRL0901 vaccine, rather than antigenic variation due to genetic divergence provide good protective efficacy against virulent NDVs. In fact to make the claim of its commercial potential, further study needs to perform to verify its efficacy under field condition in comparison with other commercially available live/killed vaccines, in the presence of maternal antibodies and in the presence of other live vaccines such as infectious bronchitis virus.

Conclusions: The NDRL0901 vaccine provided complete protection against the VIIh viruses as well as the VIId virus. This indicates that new VIIh NDVs as well as the VIId virus may not be antigenic variants to such a degree as to escape vaccinal immunity, despite the genetic divergence between the vaccine strain and the challenge viruses. This also suggests that traditionally used NDV vaccine (NDRL0901 strain) could protect birds from fatal exposure to VIIh viruses in the field if properly vaccinated.

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Authors contribution: JYK, HJL and SK executed the animal experiments and analyzed the sera and viral samples. SCJ and KSC analyzed the data. All authors interpreted the data, critically revised the manuscript for important intellectual contents and approved the final version.

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