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Protection in specific-pathogen-free chickens with live avian metapneumovirus and Newcastle disease virus vaccines applied singly or in combination

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Summary

This paper describes two experiments. In each, day-old specific pathogen free (SPF) chicks were divided into three groups. In Experiment 1 [avian metapneumovirus virus (aMPV) challenge], one group served as unvaccinated controls; the second group was vaccinated with live aMPV (subtype B) vaccine only and the third group received the aMPV vaccine in combination with live Newcastle disease virus (NDV) vaccine (VG/GA strain).

Oropharyngeal swabs, tissues and blood samples were collected before and after challenge with a virulent subtype aMPV at 21 days post vaccination (d.p.v.). Chicks were monitored for post challenge clinical signs. Swabs and tissues were examined for the detection of challenge aMPV by isolation (VI) and by reverse-transcriptase polymerase-chain reaction (RT-PCR). Sera were assayed for antibodies against aMPV and NDV. The single and combined vaccinated chicks were all protected against clinical signs and no challenge virus was isolated from either of the vaccinated -challenged groups. In Experiment 2 (NDV challenge), as in the Experiment 1, chicks were divided into three groups where one group remained as unvaccinated control and the other two groups were vaccinated as above, except that the second group received live NDV vaccine only, instead of aMPV. At 21 d.p.v., 15 chicks from each of the three groups were removed to a different site and challenged with a virulent NDV (Texas GB strain). Re-isolation of the challenge virus was not attempted. All chicks in both NDV-vaccinated challenged groups were protected against clinical signs and

mortality. These results show that based on parameters monitored for the respective challenge virus, simultaneous application of live aMPV and NDV vaccines did not affect the efficacy of either vaccine.

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Introduction

Turkey rhinotracheitis (TRT) is an important respiratory disease in turkeys caused by avian metapneumovirus (aMPV), and in chicks, the virus is sometimes associated with swollen head syndrome (SHS) (Cook & Cavanagh, 2002; Cook, 2000). Newcastle disease is caused by paramyxovirus serotype 1 (APMV-1) and is an economically important disease worldwide in chickens and turkeys (Alexander & Jones, 2001; Alexander, 2003). Live and inactivated vaccines are used to prevent both of these diseases (Alexander & Jones, 2001; Alexander, 2003).

The interactions between live aMPV and Newcastle disease virus (NDV) vaccines when given simultaneously in SPF chicks were reported by Ganapathy *et al.* (2005). The main findings included increased humoral and local immune responses to NDV vaccine in the presence of aMPV vaccine, decreased humoral immune response to aMPV in the presence of NDV but similar levels of local aMPV-specific IgA in lachrymal fluids, irrespective of single or dual vaccination.

In those experiments, no challenge was carried out to assess protection against virulent aMPV or NDV. This paper reports on the protection conferred by aMPV (NEMOVAC®) or NDV (AVINEW®) vaccines applied singly or dually in White-Leghorn specific pathogen free (SPF) chicks against virulent aMPV or NDV challenge.

Materials and Methods

Chickens. In each of the two experiments, White Leghorn day-old SPF chicks (Lohmann Animal Health, Cuxhaven, Germany) were randomly allocated into three groups and were

placed in separate isolation rooms in an experimental house. Food and water were provided *ad libitum*.

Vaccines. As in the previous report (Ganapathy *et al.*, 2005), commercial vaccines of NDV (AVINEW®) and avian metapneumovirus (NEMOVAC®) were used. The vaccine reconstitution and mixture preparation were the same as described (Ganapathy *et al.*, 2005). The dosages of vaccines are given in Table 1.

Experimental design. Two experiments were carried out. In each experiment, chicks were allocated into three groups (Table 1).

Experiment 1 (aMPV challenge). Chicks were randomly allocated to three groups of 24 each (Table 1). The control (unvaccinated) group was sham-vaccinated with sterile water. Each chick received 50 µl ocularly and another 50 µl orally. Using the same route of application and volumes, aMPV vaccine was administered to chicks in the single aMPV vaccination group (aMPV). For the dual-vaccinated (aMPV+NDV) group, sterile water containing both the aMPV and NDV vaccines were administered in the same way. Dosages received by each bird were as recommended by the manufacturer, and these are shown in the Table 1. The chicks were monitored daily for clinical signs. At 21 days post vaccination (d.p.v.), 12 birds from each group were transferred to another isolation room, and challenged with virulent aMPV (subtype B), which was propagated in tracheal organ cultures (TOCs) and the virus titre determined using the same culture system. TOCs were prepared from SPF chick embryos after 19–20 days incubation following the method of Cook *et al.* (Cook *et al.*, 1976). Each bird received 0.1 ml of 4.5 log₁₀ median ciliostatic doses₅₀ (CD₅₀) of the challenge virus via the ocular route.

Post-challenge clinical signs: After challenge, birds were monitored daily for clinical signs and the severity of the clinical signs was scored as described by Jones *et al.* (1992). Briefly, a

score of 0: no signs; 1: clear nasal exudates; 2: turbid nasal exudates; 3: frothy eyes and/or swollen infraorbital sinuses in conjunction with nasal exudate.

Oropharyngeal (OP) swabs: Prior to challenge, 10 OP swabs were collected from each group. After challenge, oropharyngeal samples were obtained from 8 chicks in each group at 3, 6 and 9 days post challenge (d.p.c.) using dry swabs. In addition, another set of swabs was taken from the same chicks but using swabs previously moistened in TOC medium [Eagles serum-free MEM with glutamine, streptomycin (50 µg/ml) and penicillin (50 IU/ml)]. OP swabs were also randomly collected from the unchallenged groups but only from 5 chicks. The dry and wet swabs were processed for RT-PCR and virus isolation respectively. Swabs from the challenged birds were processed individually whilst the other swabs were pooled.

Sera: Blood samples were obtained at 21 days (pre-challenge) and 32 days (10 days post-challenge) of age from 8 chicks per group for detection of antibodies against aMPV and NDV.

Tissues: At 7d.p.v. and at 5 and 10 d.p.c., four birds per group were humanely killed and pieces of turbinate, trachea and lung were collected for aMPV RT-PCR and virus isolation.

Experiment 2 (NDV challenge). The grouping was the same as *Experiment 1* except that chicks in the single vaccination group received live NDV, instead of aMPV vaccine. The volume and administration of inocula were the same as described above and the dosages per bird are shown in Table 1. The chicks were monitored daily for clinical signs and mortality. At 21 d.p.v., 15 birds from each group were brought to the OIE/FAO/EU Reference Laboratory for Avian Influenza and Newcastle Disease, Veterinary Laboratory Agency, Weybridge, Surrey. The chicks were housed in isolation rooms for 16 hours then challenged with a virulent NDV (Texas GB strain). Prior to challenge, OP swabs were collected from 10 chicks in each group. Each bird received 0.2 ml of 4.0 log₁₀ median embryo infectious doses₅₀ (EID₅₀) of the challenge virus via the ocular route.

Post-challenge clinical signs: After challenge, birds were monitored daily for clinical signs and mortality.

Sera: Blood samples were obtained from all the groups (unvaccinated or vaccinated-unchallenged) at 21 (pre-challenge) and 28 (7 d.p.c.) days old. Eight sera per group were tested for antibodies against aMPV and NDV.

Detection of viruses. For Experiment 1 only, OP swabs and selected homogenised tissues from the chicks were examined for aMPV challenge virus by isolation in tracheal organ cultures (TOC) (Cook et al., 1976; Ganapathy *et al.*, 2005). OP swabs and tissues were also examined for aMPV by RT-PCR as described (Ganapathy *et al.*, 2005).

Detection of vaccinal antibodies. NDV antibodies were detected by haemagglutination-inhibition (HI) (Allan & Gough, 1974). For aMPV antibodies, sera were tested by an in-house ELISA as described by Worthington *et al.* (2003), but the coating antigen was subtype B aMPV (Ganapathy *et al.*, 2005). In both assays, the mean titre at each sampling point was calculated. For aMPV ELISA, titres above 6 (\log_2) are considered significant for the presence of the aMPV antibodies.

Statistics. The mean antibody titres at each sampling point were compared using a Student's *t*-test.

Results

Clinical signs and post mortem lesions. *Experiment 1 (aMPV challenge).* After vaccination, single (aMPV) and dual (aMPV+NDV) vaccinates showed no clinical signs. Following

aMPV challenge, chicks in both vaccinated groups (aMPV-Ch or aMPV+NDV-Ch) remained free of clinical signs. The unvaccinated-unchallenged chicks remained normal throughout. In the unvaccinated-challenged (Unvac-Ch) group, a clear to turbid nasal discharge was seen at different times in chicks. Figure 1 shows the mean clinical scoring in this group. No post mortem lesions were found in any birds examined at 7 d.p.v. or 10 d.p.c. For the necropsy carried out at 5 d.p.c., 3 chicks of the Unvac-Ch birds showed a bilateral turbid nasal discharge. In the vaccinated-challenged birds, clear nasal discharge was seen in 2 and 3 chicks in the aMPV-Ch and aMPV+NDV-Ch group respectively.

Experiment 2 (NDV challenge). In the Unvac-Ch chicks, signs of dullness, depression, ruffled feathers, watery eyes, inappetence and paralysis were recorded 3 days after NDV challenge. All birds in this group died or were humanely killed by 7 d.p.c. Chicks in the NDV-vaccinated groups showed no clinical signs and none died. No necropsy was carried out.

Detection of a MPV virus. *Experiment 1 (aMPV challenge).* All OP swabs from the unvaccinated, aMPV and aMPV+NDV- vaccinated birds were negative for aMPV by RT-PCR and VI (data not shown). For the Unvac-Ch birds, 8, 7 and 8 swabs were positive for aMPV at 3, 6 and 9 d.p.c. respectively by RT-PCR but virus was isolated from all swabs at 3 d.p.c. but not later (Table 2). In the aMPV-Ch and aMPV+NDV-Ch chicks, 7 and 8 swabs respectively were RT-PCR positive at 3 and 6 d.p.c., and this was reduced to 3 or 4 at 9 d.p.c. All of these swabs were negative by VI.

When the tissues were examined, no virus was detected in the trachea in any group by either method. Using RT-PCR virus was detected in turbinates (5 and 10 days) in the Unvac-Ch group (Table 3). Both of the other groups were negative.

Experiment 2. OP swabs taken at 21 d.p.v. (pre-challenge) from the unvaccinated, aMPV and aMPV+NDV vaccinated birds were negative for aMPV by RT-PCR.

Serology. *Experiment 1 (aMPV challenge).* There were no significant differences in the pre-challenge aMPV ELISA antibody titres between the aMPV-vaccinated groups (Table 3). The post-challenge antibody titres in each group were significantly higher than the respective unchallenged groups.

For NDV, HI titres in the control and single aMPV-vaccinated groups remained below the detectable level (Table 3). In the combined vaccination group, there were no significant differences in the pre or post aMPV-challenged NDV HI titres.

Experiment 2 (NDV challenge). For NDV HI antibodies, at 21 d.p.v. (pre-challenge) the titre remained below detectable level in the unvaccinated group (Table 3) and there were no significant differences between the two NDV-vaccinated groups. The post-challenge antibody titres in each group were significantly higher than the respective unchallenged groups.

For aMPV ELISA antibodies, there were no significant differences in the pre (7.90 ± 1.56) and post (9.27 ± 0.97) NDV-challenge titres in the simultaneously vaccinated chicks.

Discussion

This study was designed to complement our previous work (Ganapathy *et al.*, 2005) where interactions between live aMPV and NDV vaccine viruses in SPF chicks were examined. In that work, systemic vaccine virus distribution and immune responses were studied but no challenge was carried out with virulent aMPV or NDV. From findings reported in this paper,

it is clear that simultaneous vaccination with aMPV and NDV confers protection against virulent aMPV challenge that is no less than the level provided by aMPV vaccine alone. The aMPV vaccinated chicks were not only protected against clinical signs but the challenge virus was not isolated, even though most unvaccinated-challenged chicks were positive by RT-PCR. This may suggest that in the aMPV-vaccinated chicks, the challenge virus was unable to colonise the respiratory epithelium. However, necropsy at 5 d.p.c. revealed nasal discharge in few of the aMPV-vaccinated-challenged chicks, which may suggest that sterile vaccination was not completely achieved. The difficulty in recovering infectious virus from aMPV-vaccinated chicks is well known but factors such as sampling time and low virus titre may have influenced the outcome. Whilst our results appear to indicate a discrepancy between the molecular detection and virus isolation, Hess *et al.* (2004) have shown that the aMPV genome was still detectable as late as 28 d.p.c., long after the infectious virus was eliminated.

As for NDV, no clinical signs or mortality were seen in NDV-vaccinated chicks (alone or dually with aMPV) but all the unvaccinated-challenged chicks died or were humanely killed (due to illness) within 7 days of challenge. This result shows that irrespective of single or dual NDV vaccination, complete protection was induced against virulent NDV challenge.

With respect to the serological results, in contrast to our previous study (Ganapathy *et al.*, 2005), between the single and dual vaccinated groups, there were no significant differences in the levels of antibodies with respect to each vaccine. The reasons for this discrepancy are not clear but factors such as group size (larger here compare to the small and reducing numbers in the previous work), perhaps the male-female ratio (unknown in both) and other unknown factors may have influenced the outcome. For aMPV, previous studies have emphasized that local and cell mediated immune responses are important in resisting and clearing the virus (Cook *et al.*, 1989; Jones *et al.*, 1992; Khehra *et al.*, 1999; Ganapathy *et al.*, 2005). For NDV, levels of protection closely correlate with the levels of humoral HI antibodies (Alexander, 2003). In this study, at the time of NDV challenge, the mean NDV HI

titres in the single and dual vaccinated groups were \log_2 4.75 and 5.06 respectively. All birds in these groups were protected against clinical signs and mortality.

In conclusion, for live aMPV (NEMOVAC) vaccine, as judged by post challenge clinical signs and virus detection, its efficacy was not compromised with simultaneous application of live NDV (AVINEW) vaccine in SPF chicks. In the NDV challenge study, due to logistical reasons, detection of residual viruses was not carried out. However, based on post challenge clinical signs and mortality, we have shown that the dual vaccination did not interfere with the efficacy of NDV (AVINEW) vaccine.

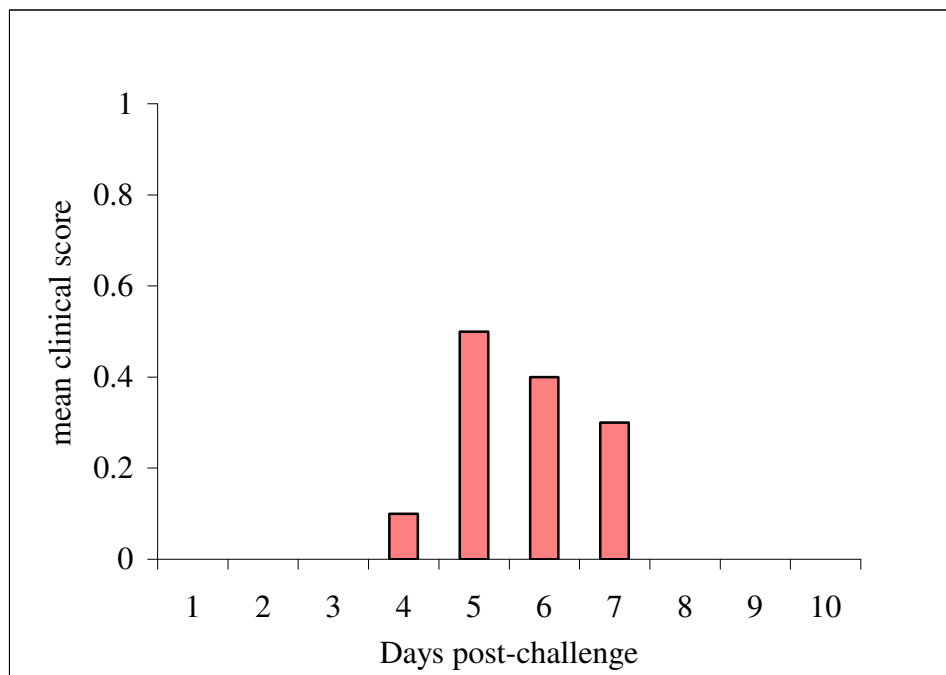


Figure 1. *Experiment 1. Mean clinical score in the unvaccinated-challenged chicks following aMPV challenge. No clinical signs were observed in the vaccinated-challenged groups.*

Table 1. *Experimental design for protection against virulent aMPV or NDV challenge in SPF chicks offered by live aMPV or NDV vaccines given either alone or simultaneously*

Group	Inoculum	Dosage	Number of chicks	
			Expt 1 ^a	Expt 2 ^b
Unvaccinated (Unvac)	SW	-	24	30
NEMOVAC [®] (aMPV)	aMPV	2.4 log ₁₀ CCID ₅₀ ^c	24	ND ^e
AVINEW [®] (NDV)	NDV	5.6 log ₁₀ EID ₅₀ ^d	ND	30
NEMOVAC+AVINEW (aMPV+NDV)	aMPV+NDV	2.4 log ₁₀ CCID ₅₀ 5.6 log ₁₀ EID ₅₀	24	30

^a Experiment 1: At 21 d.p.v., 12 birds from each group were challenged intraocularly with 0.1 ml of 4.5 log₁₀ ciliostatic dose₅₀ virulent subtype B aMPV.

^b Experiment 2: At 21 d.p.v., 15 birds from each group were challenged intraocularly with 0.2 ml of 4.0 log₁₀ EID₅₀ virulent NDV (Texas GB strain).

^c cell culture infective dose

^d egg infective dose

^e Not done

Table 2. *Experiment 1. Detection of aMPV virus in the swabs and tissues by RT-PCR and passage in tracheal organ cultures.*

Samples			Days post challenge			
			3	6	9	10
OP ^a swabs						
Tissues			5			
OP swab	Unvaccinated (Unvac)	RT-PCR ^b	8 ^c	7	8	
		VI ^d	8 ^e	0	0	
	NEMOVAC (aMPV)	RT-PCR	7	7	4	
		VI	0	0	0	
NEMOVAC+ AVINEW (aMPV+NDV)	} RT-PCR		8	8	3	
		VI	0	0	0	
Turbinate	Unvaccinated (Unvac)	RT-PCR	2			1
		VI	0			0
	NEMOVAC (aMPV)	RT-PCR	0			0
		VI	0			0
NEMOVAC+ AVINEW (aMPV+NDV)	} RT-PCR		0			0
		VI	0			0
Trachea	Unvaccinated (Unvac)	RT-PCR	0			0
		VI	0			0
	NEMOVAC (aMPV)	RT-PCR	0			0
		VI	0			0
NEMOVAC+ AVINEW (aMPV+NDV)	} RT-PCR		0			0
		VI	0			0
Lungs	Unvaccinated (Unvac)	RT-PCR	0			0
		VI	0			0
	NEMOVAC (aMPV)	RT-PCR	0			0
		VI	0			0
NEMOVAC+ AVINEW (aMPV+NDV)	} RT-PCR		0			0
		VI	0			0

^a OP: oropharyngeal^b Virus detection by RT-PCR^d attempted virus isolation^c number positive of 8 OP or 4 tissues examined^e number positive of 8 OP or 4 tissues examined

Table 3. Experiments 1 and 2. aMPV and NDV mean antibody titres in groups of chicks vaccinated at day old with each vaccine alone or with both vaccines

Groups	Methods	Experiment No.			
		1 (aMPV challenge)		2 (NDV challenge)	
		Unchallenge d	Challenged ^a	Unchallenged	Challenged ^b
Unvaccinated (Unvac)	NDV HI	< 2	< 2	< 2	All died
	aMPV ELISA	4.33 ^A (0.08)	13.54 ^B (1.33)	4.23 (0.74)	All died
NEMOVAC (aMPV)	NDV HI	< 2	< 2	ND ^c	ND
	aMPV ELISA	9.12 ^A (1.66)	16.02 ^B (1.02)		
AVINEW (NDV)	NDV HI	ND	ND	4.75 ^A (0.3)	6.3 ^B (0.5)
	aMPV ELISA			4.20 (0.13)	5.26 (1.55)
NEMOVAC+ AVINEW (aMPV+NDV)	NDV HI	6.5 (1.08)	5.8 (1.03)	5.06 ^A (0.4)	6.4 ^B (1.03)
	aMPV ELISA	8.70 ^A (1.08)	14.95 ^B (1.41)	7.9 (1.56)	9.27 (0.97)

^a 10 days after aMPV challenge.

^b 7 days after NDV challenge.

^c Not done

Different superscripts between unchallenged and challenged groups indicate that the values differ significantly ($p < 0.05$).

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