Protection conferred by a live avian metapneumovirus vaccine when co-administered with live La Sota Newcastle disease vaccine in chicks

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Abstract
This paper examines the effects on specific pathogen-free (SPF) chicks when avian metapneumovirus (aMPV) and Newcastle disease virus (NDV) La Sota strain vaccines are co-administered. Day-old SPF chicks were divided into five groups. The first group was inoculated with sterile water (SW) and the rest of the groups were inoculated with live NDV vaccine VG/GA by the orculo-oral route. At 21 days-old, the unvaccinated chicks were again inoculated with SW. The four VG/GA-vaccinated groups were further inoculated with (i) SW, (ii) live aMPV vaccine, (iii) live NDV La Sota, or (iv) combined live NDV La Sota and live aMPV, respectively. Chicks were monitored for post-vaccination reactions and oropharyngeal swabs were collected for vaccines detection. Blood samples were collected to detect aMPV ELISA and NDV haemagglutination-inhibition antibodies. Twenty-one days following the second vaccination, six chicks from each group were challenged with virulent NDV or aMPV respectively. Chicks were monitored for clinical signs and mortality and oropharyngeal swabs collected for aMPV detection. Results showed that, when challenged with a virulent aMPV, both chicks previously vaccinated with VG/GA and subsequently given aMPV vaccine singly or in combination with La Sota were equally protected against clinical signs. Chicks that were vaccinated against NDV either once with VG/GA or followed by La Sota (singly or in combination with aMPV) were fully protected when challenged with velogenic NDV. We concluded that simultaneous administration of live aMPV and NDV La Sota vaccines have no adverse effects on protection conferred by either live vaccine.

Introduction
In the last two decades, avian metapneumovirus (aMPV) infection has become an important component of respiratory disease in chickens. It is associated with swollen head syndrome which also causes drop in egg quality and production (Cook, 2000; Cook and Cavanagh, 2002; Sugiyama et al., 2006; Alexander and Jones, 2008; Cecchinato et al., 2011). Newcastle disease (ND), which is caused by paramyxovirus serotype 1 (APMV-1), remains as one of the most economically important poultry diseases worldwide (Alexander and Jones, 2008). For control and prevention of ND, live and inactivated vaccines are available for more than half a century (Alexander and Jones, 2008). In contrast, live and inactivated vaccines for aMPV control and prevention were only available in the last two decades and restricted to certain countries (Cook, 2000; Cook and Cavanagh, 2002; Alexander and Jones, 2008). It was reported that when live aMPV and ND virus (NDV) vaccines were given in combination in day-old chicks, both singly and dually vaccinated chicks were equally protected against aMPV challenge (Ganapathy et al., 2005, 2007). In addition, protection against virulent NDV was not affected in single and dual vaccination. It has also been demonstrated that live aMPV and NDV can be safely administered to broiler chicks with NDV maternal antibodies (Ganapathy et al., 2006). Working with another important respiratory pathogen, Cook et al. (2001) found that when aMPV and infectious bronchitis virus (IBV) vaccines were co-administered, the latter virus inhibited the replication of aMPV vaccine virus, resulting in reduced humoral antibody response to aMPV vaccine, though protection against aMPV or IBV challenges were not affected. There have not been reports on co-administration of aMPV and NDV La Sota vaccines, where the latter vaccine is widely used in ND endemic countries, mostly after priming with another lentogenic ND vaccine. This paper reports on the protection conferred by live aMPV or NDV La Sota vaccines applied singly or dually in chicks already primed with a live NDV VG/GA vaccine at day-old.

Materials and methods

Chickens
Eighty white Leghorn day-old specific pathogen-free (SPF) chicks (Lohmann Animal Health GmbH & Co., Cuxhaven, Germany) were randomly allocated into five groups of sixteen birds each and were placed in separate isolators in an experimental house. Food and water were provided ad libitum.

Viruses
Live NDV VG/GA (AVINEW®), NDV La Sota (BIO LA SOTA) and aMPV subtype B (NEMOVAC®) vaccines were provided by Merial SAS (Lyon, France). On the other hand, as for aMPV challenge, a virulent subtype B aMPV (Ganapathy et al., 2007) was carried out as previously described. This virus was propagated and titrated in tracheal organ cultures (TOCs) (Cook et al., 1976). For NDV challenge, the velogenic APMV-1 chicken/Italy/3015/00 (intra cerebral pathogenicity index of 1.8) was used. This virus was kindly provided by OIE/FAO and National Reference Laboratory for Newcastle Disease and Avian Influenza, Legnaro, Italy. The virus was grown and titrated in SPF embryonated eggs.

Vaccine preparation
One vial each of VG/GA, La Sota or aMPV vaccines was thoroughly mixed with 100 mL of
sterile water (SW). For dual-vaccination, aMPV followed by NDV La Sota were both mixed in 100 mL of SW. Immediately after preparation, SW and the vaccines were placed in a cold box containing crushed ice. Each chick received 50 µL of SW or the appropriate vaccine ocularily and 50 µL orally.

**Experimental design**

Day-old chicks were randomly divided into five groups as shown in Table 1. One group was inoculated with 0.1 mL of SW and the other groups were inoculated with VG/GA. At 21 days of age, the group which received SW was inoculated with 0.1 mL of SW (Group: SW:SW). The four VG/GA-inoculated groups were further inoculated as follows: i) the first was sham-inoculated with SW (Group: VG/GASMW); ii) the second group received aMPV (Group: VG/GA:aMPV); iii) the third group received NDV La Sota (Group: VG/GA:LASOTA); and iv) the forth group (Group: VG/GA:aMPV+LASOTA) was inoculated with both aMPV and NDV La Sota vaccines simultaneously. Dosages received by each bird were in accordance with the manufacturer’s recommendations (Table 1). Three weeks after the second vaccination, six birds from each group were transferred into separate isolators and challenged with virulent aMPV. Each bird received 0.1 mL of 4.0 log_{10} CD_{50} of a virulent aMPV subtype B challenge virus via the ocular route. At the same time and in a similar manner, another six birds from each group were transferred into new isolators. These chicks were challenged by intranasal inoculation of 0.2 mL of 5.0 log_{10} CCID_{50} velegonic Italian NDV isolate. Four birds per group remained unchallenged and were used to determine aMPV and NDV post-vaccination antibody titres and NDV vaccine shedding.

**Clinical signs**

All chicks were observed every day and after aMPV challenge were examined daily for clinical signs and the severity scored as described before (Jones et al., 1992; Catelli et al., 2010). 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 exudates). Following NDV challenge, all chicks were examined daily for clinical signs and mortality. Those birds showing signs of illness such as paralysis were humanely killed.

**Sampling**

For aMPV and NDV vaccine detection wet and dry swabs were collected from the oropharynx of chicks at 0, 2, 7, 14 and 21 days post-VG/GA vaccination and 7, 14, 21 and 28 days following the second vaccination, oropharyngeal (OP) swabs were collected from vaccinated-unchallenged chicks. After virulent aMPV challenge, OP swabs were collected at 5 and 7 days for the detection by real time-polymerase chain reaction (RT-PCR) and virus isolation (VI) of aMPV. Wet swabs previously moistened in TOC medium [Eagles serum-free MEM with glutamine, streptomycin (50 µg/mL) and penicillin (50 U/mL)] were used. The dry and wet swabs were processed for RT-PCR and VI detection.

**Detection of viruses**

Oropharyngeal swabs were examined for aMPV by isolation in TOC (Catelli et al., 1998; Ganapathy et al., 2005) and for aMPV genome by RT-PCR as previously described (Cavanagh et al., 1999; Ganapathy et al., 2005). Newcastle disease virus VI was attempted following VG/GA and after the LaSota vaccinations from OP swabs by inoculation of 9 to 11 days old embryonated chicken eggs.

**Detection of vaccinal antibodies**

Newcastle disease antibodies were detected by HI (Allan and Gough, 1974). For detection of aMPV antibodies, a commercial ELISA kit (BioChek B.V., Gouda, The Netherlands) was used and the assay was carried out as recommended by the manufacturers.

**Statistics**

The antibody titres within the groups were log-transformed and then examined by analysis of variance (ANOVA) with MINITAB® for WINDOWS® 14 (MINITAB Ltd., Coventry, UK). A P value of <0.05 was considered statistically significant.

**Results**

**Clinical signs**

No clinical signs were observed after the first or the second vaccinations. After aMPV challenge, no clinical signs were seen in the groups that received aMPV vaccine either alone (VG/GAaMPV) or in combination with NDV La Sota (VG/GA:aMPV+LASOTA). In contrast, nasal exudate and watery eyes were observed in the groups not given aMPV vaccine the following mean daily scores resulted:

| Groups               | Vaccination age, days | Challenge virus | Day-old |
|----------------------|----------------------|----------------|---------|
| SW:SW               | 16                   | 6              | aMPV    |
| VG/GASMW            | 16                   | 6              | NDV     |
| VG/GA:aMPV          | 16                   | 6              | aMPV    |
| VG/GA:LASOTA        | 16                   | 6              | NDV     |
| VG/GA:aMPV+LASOTA   | 16                   | 6              | aMPV    |

SW: sterile water; aMPV, avian metapneumovirus; NDV, Newcastle disease virus. 1.52 log_{10} EID_{50}, 2.4 log_{10} CCID_{50}, 7.34 log_{10} EID_{50}.

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Table 1. Experimental design for protection against virulent avian metapneumovirus or Newcastle disease virus challenge in specific pathogen-free chicks vaccinated singly or in combination.

| Groups               | Vaccination age, days | Challenge virus | 21       |
|----------------------|----------------------|----------------|---------|
| SW:SW               | 16                   | 6              | aMPV    |
| VG/GASMW            | 16                   | 6              | NDV     |
| VG/GA:aMPV          | 16                   | 6              | aMPV    |
| VG/GA:LASOTA        | 16                   | 6              | NDV     |
| VG/GA:aMPV+LASOTA   | 16                   | 6              | aMPV    |

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SW:SW (0.5, day 5 post challenge), VG/GA:SW (0.83, day 5 post challenge; 0.5, day 6 post challenge); VG/GA: LASOTA (0.5, day 6 post challenge). Also, no clinical signs were seen in any of the NDV-vaccinated groups. However, within five days post-NDV challenge, five out of six chicks in the NDV-unnovcinated control group (SW:SW) died and one was humanely killed.

Detection of avian metapneumovirus

Groups that did not receive aMPV vaccine remained negative for this virus (Table 2). In aMPV-vaccinated chickens, the combined (VG/Ga:aMPV+LASOTA) vaccinated group was positive for aMPV by RT-PCR at 7, 14 and 21 days post-vaccination, but the group that received aMPV vaccine (VGGa:aMPV) alone was positive at only 7 days post-vaccination. aMPV was only isolated from both aMPV-vaccinated groups at day 7 post-vaccination.

Five days following challenge with a virulent aMPV, four out of six birds in the aMPV-unnovcinated groups were aMPV positive by VI, but all six were positive by RT-PCR (Table 2). In contrast, there was no detection of aMPV in the aMPV-vaccinated groups except 1 of 6 in the single aMPV-vaccinated group (VG/Ga:aMPV) which was positive by RT-PCR only. Seven days after challenge, OP samples from all groups were negative for aMPV by VI and RT-PCR except for the VG/Ga:LASOTA group where one of six birds was positive for aMPV by both methods.

Detection of Newcastle disease virus

Newcastle disease vaccine VI was attempted following VG/GA and after the La Sota vaccinacions. No NDV virus was detected in the control group (SW:SW). In the NDV-vaccinated groups, vaccine was isolated at two and seven days post-VG/GA vaccination (Table 3), but not at all following the NDV La Sota vaccination.

Serology

No aMPV antibodies were detected in groups that did not receive either aMPV vaccine or the challenge virus. Following aMPV vaccination, there were no significant differences in the levels of aMPV antibodies between the groups given aMPV alone (VGGa:aMPV) and those given aMPV in combination with La Sota vaccine (VGGa:aMPV+LASOTA), except at 7 days, where the titre in the latter group was signifi- cantly higher than the singly vaccinated group (Figure 1). Following the challenge, levels of aMPV antibodies in the challenged groups increased significantly than those of the unchallenged chickens (Table 4). No NDV HI antibodies were detected in the NDV-unnovcinated (SW:SW) group. In all other groups, antibodies were detected either in chicks that were vaccinacated only with NDV VG/GA or those fol-

Discussion

In our previous work (Ganapathy et al., 2005, 2007), it was demonstrated that when live aMPV and VG/GA vaccines were given simultaneously to day-old SPF chicks, the efficacy of the vaccines was not affected. In addi-
tion, local and humoral immune responses in SPF (Cook et al., 2001; Ganapathy et al., 2005, 2007; Ganapathy and Jones, 2007; Tarpey et al., 2007) and broiler chicks (Ganapathy et al., 2006) have been reported before. Those reports concentrated on single or dual vaccinacion of young chicks. In some Asian, African and Latin American countries where ND is endemic, the live aMPV vaccines are often administered simultaneously with live La Sota vaccines, which are normally given to boost the protection against ND field challenge. This study was undertaken to examine the potential effect of live aMPV and La Sota vaccines in such situations when simultaneously adminis-
tered to chicks already vaccinacated with another lentogenic NDV vaccine. Chicks that had already been primed with VG/GA were used to vaccinacation.

Table 2. Detection of vaccine or challenge aMPV virus in the swabs by reverse transcriptase-polymerase chain reaction or passage in tracheal organ cultures.

| Groups                        | Methods of detection | Days post-aMPV vaccination | Days post-virulent aMPV challenge |
|-------------------------------|----------------------|---------------------------|-----------------------------------|
|                               |                      | 0  | 7  | 14 | 21 | 28° | 5  | 7   |
| SW:SW                         | RT-PCR              | -  | -  | -  | -  | -   | 6  | 0   |
|                               | VI                  | -  | -  | -  | -  | -   | 4  | 0   |
| VG/Ga:SW                      | RT-PCR              | -  | -  | -  | -  | -   | 6  | 0   |
|                               | VI                  | -  | -  | -  | -  | -   | 4  | 0   |
| VGGa:aMPV                     | RT-PCR              | -  | +  | -  | -  | -   | 1  | 0   |
|                               | VI                  | -  | +  | -  | -  | -   | 0  | 0   |
| VGGa:LASOTA                   | RT-PCR              | -  | -  | -  | -  | -   | 6  | 1   |
|                               | VI                  | -  | -  | -  | -  | -   | 4  | 1   |
| VGGa:aMPV+LASOTA              | RT-PCR              | -  | +  | +  | -  | -   | 0  | 0   |
|                               | VI                  | -  | +  | -  | -  | -   | 0  | 0   |

Table 3. Detection of Newcastle disease virus in the swabs by passage in embryonated chicken eggs.

| Groups                        | Days post-NDV VG/GA vaccination | Days post-NDV La Sota vaccination |
|-------------------------------|---------------------------------|----------------------------------|
|                               | 0  | 2  | 7  | 14 | 21 | 7 (28)° | 14 (35) | 21 (42) | 28 (49) |
| SW:SW                         | -  | -  | -  | -  | -  | -       | -       | -       | -       |
| VG/Ga:SW                      | +  | +  | +  | -  | -  | -       | -       | -       | -       |
| VGGa:aMPV                     | +  | +  | +  | -  | -  | -       | -       | -       | -       |
| VGGa:LASOTA                   | +  | +  | -  | -  | -  | -       | -       | -       | -       |
| VGGa:aMPV+LASOTA              | +  | +  | -  | -  | -  | -       | -       | -       | -       |

aMPV, avian metapneumovirus; SW, sterile water; RT-PCR, reverse transcriptase-polymerase chain reaction; VI, virus isolation. *From unchallenged groups, attempted by TOC, by oropharyngeal swabs.
mimic the common field practices in ND endemic countries. In some countries, a milder live lentogenic vaccine (e.g. B1, VG/GA) are used within first few days of hatching and 14-21 later flocks are re-vaccinated with a live La Sota vaccine to boost the protection levels against field ND challenge.

Based on findings from this experiment, chicks that were vaccinated either singly with aMPV or dually with NDV La Sota vaccines were protected against virulent aMPV challenge. In addition, no aMPV was isolated from the aMPV-vaccinated groups even though one of six chicks in the VG/GA:aMPV was positive for aMPV by RT-PCR. However, the detection of aMPV genome in the absence of viable virus isolation has been reported before (Hess et al., 2004; Ganapathy et al., 2007). Thus, it appears that simultaneous application of live aMPV and NDV La Sota vaccines in chicks already primed with NDV VG/GA showed no adverse effects in conferring protection against aMPV challenge.

In this experiment, it was demonstrated that both chicks that received either NDV VG/GA alone at day-old or followed by NDV La Sota at 21 days old were protected against a velogenic Italian NDV. No clinical signs or mortality were recorded in these groups but all the chicks in the NDV-unvaccinated group became ill, died or were humanely killed. Although no attempts were made to detect the NDV challenge virus in the vaccinated-challenged chicks, based on protection conferred against clinical signs and mortality, it appears that aMPV vaccine does not interfere with the protection conferred by the NDV La Sota vaccine. Previously, it has been shown that chicks simultaneously vaccinated with aMPV and NDV VG/GA vaccines gave full protection against velogenic NDV Texas GB strain (Ganapathy et al., 2007). This appears to be the first study to demonstrate protection conferred against velogenic NDV and field aMPV by simultaneous vaccination with aMPV and La Sota in chickens.

To evaluate the immune responses to live aMPV and NDV vaccines, humoral antibodies were monitored following the vaccination. The antibody levels were similar to previous report

| Groups         | Antibody assay | Challenge virus |
|----------------|----------------|-----------------|
|                | aMPV           | NDV             |
|                | Unchallenged   | CHallenged°     |
| SW:SW          | NDV III        | -               |
|                | aMPV ELISA     | 213 (50)        |
|                |                | 1456 (335)      |
|                |                | <2              |
|                |                | All died        |
| VG/GA:SW       | NDV III        | -               |
|                | aMPV ELISA     | 142 (41)        |
|                |                | 4616 (792)      |
|                |                | 4.67 (0.61)     |
| VG/GA:aMPV     | NDV III        | -               |
|                | aMPV ELISA     | 2778 (566)      |
|                |                | 5898 (1219)     |
|                |                | 4.83 (0.38)     |
|                |                | 5.17 (0.49)     |
| VG/GA:LASOTA   | NDV III        | -               |
|                | aMPV ELISA     | 98 (29)         |
|                |                | 1496 (286)      |
|                |                | 5.17 (0.38)     |
|                |                | 4.50 (0.42)     |
| VG/GA:aMPV+LASOTA | NDV III     | -               |
|                | aMPV ELISA     | 2818 (610)      |
|                |                | 5940 (1780)     |
|                |                | 5.83 (0.4)      |
|                |                | 4.67 (0.52)     |

aMPV, avian metapneumovirus; NDV, Newcastle disease virus; SW, sterile water; HI, haemagglutination-inhibition. °Blood collected seven days after aMPV or NDV challenge. Numbers in parentheses indicate standard deviations. Different upper case superscripts between unchallenged and challenged groups indicate that the values differ significantly (P<0.05).
Conclusions

In conclusion, using NDV VG/GA-primed SPF chicks, it was demonstrated that subsequent simultaneous vaccination with live NDV La Sota and aMPV vaccines does not adversely affect the protection of the chicks against viral NDV or aMPV challenges.

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