Experimental Multiple Virus-Infections of Commercial Layers with Avian Influenza, Subtype H9N2 and Velogenic Newcastle Disease Viruses

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ABSTRACT

Avian Influenza and Newcastle disease viruses (NDV) are economically very important diseases of the poultry industry worldwide. While the majority of research has been dedicated to each virus in particular, including biological, molecular and vaccine aspects of development, the impact of dual-virus infection has attracted less attention. To fill this gap, we conducted the present study on NDV vaccinated commercial adult layers that were housed in isolators for challenge with velogenic NDV (vNDV). The assessment of commercial birds is innovative, as no studies have been performed to resolve this issue in commercial birds using experimentally-controlled challenge and molecular survey. The dual-virus infection was carried out with avian influenza, subgroup H9N2, 4 days before the challenge with vNDV, which is the most prevalent scenario in Israeli commercial poultry, at the time of conducting the study. The first trial employed SPF chicks to titrate the vNDV challenge; the second trial explored the efficacy of the commercial vaccination against NDV in 30 weeks-old Lohmann layers, and the third experimental infection trial analyzed systematically the impact of dual virus-infection on 35 week-old Hyline vaccinated layers with NDV vaccine. In the trials that employed commercial layers the birds were protected against morbidity and mortality, but they still shed vNDV at low levels, probably reflecting the actual situation in many commercial flocks in Israel. No effect by the prior infection of commercial layers with AIV H9N2, followed by vNDV were observed, neither on the NDV shedding, antibody titers to both viruses and nor the rates of mortality and morbidity.

Keywords: Avian Influenza Virus-subtype H9N2; Velogenic Newcastle Disease Virus; Commercial Layers; Experimental Co-Infection.

INTRODUCTION

Avian Influenza (AI) (1) and Newcastle disease (ND) (2) are the most economically significant infectious diseases of poultry worldwide. Most studies have been focused on disease pathologies, virus characterization, and vaccine development. Compared to the numerous studies conducted on each separate virus, co-infections with both Avian Influenza Virus (AIV) and NDV have been explored to a much lesser extent. Increasing knowledge on the interactions of these two virus infections, often co-circulating in the poultry population, is important in light of the high prevalence of AIV subgroup H9N2 and velogenic NDV (vNDV) in Israel as singular (3, 4, 5) or as dual viral-infections (6). While vaccination against vNDV is mandatory in Israel (7), vaccination against AIV H9N2 is voluntary and has gained amplified attention in recent years (3, 4).

The first attempt to characterize the mutual impact of AIV and NDV co-infection was explored by Burnett (8) who

showed interference for cell agglutination by the two viruses. Furthermore, interference of chick infection with the two viruses was demonstrated in AIV and NDV co-infections of chicken embryos (9-12) as depending on the viral load and the interval of infection with the two viruses. The elegant recent study of Ge *et al.* (13) on Specific Pathogen Free (SPF) embryonated eggs co-infected with avirulent AIV and virulent NDV revealed their interaction complexity. While by simultaneous co-infection with different virus doses, the NDV replication was affected, upon sequential infection with equal viral doses of both viruses, the degree of interference was dependent upon the time of super-infection and the virus virulence. Costa-Hurtado *et al.* studied (14-16) dual experimental infections of SPF chickens and turkeys. Co-infection of chickens and turkeys with avirulent AIV and NDV affected their replication dynamics but not the clinical signs (14). Only virulent NDV affected the replication of pathogenic AIV, depending on the virus load and the infection schedule (15, 16). In contrast, dual infection of mallards with apathogenic AIV and NDV was not altered compared to singular infections (17). Duck co-infection with velogenic NDV and avirulent AIV resulted in mutual virus-replication interference without change of the clinical signs (18).

The complexity of interference patterns existing in dual-virus infections depends on the combination of variables, rendering the clinical and virological outcome multidimensional. Influencing factors include the host, the infecting virus virulence, dose, timing and sequence of inoculation. Ge *et al.* (13) revealed that the infectivity of both viruses might interfere as a result of competition for binding to cellular sialic acid-containing glyconjugates, which is the receptor for both viruses. The replication machinery and interferon expression by the same tissues are also involved in AIV and NDV infections. Prior infection with AIV H9N2 was a common situation for most commercial poultry flocks in Israel and since the year 2000 AIV H9N2 is considered endemic in Israel (4). This occurrence was evaluated in experimental infections of SPF chicks by Bonfante *et al.* (19) and also in the present study on commercially NDV-vaccinated layers. By conducting experimental co-infection trials of SPF chicks it became evident that the AIV H9N2 infection synergized and interfered with simultaneous vNDV infection in a dose-dependent mode. The greatest effects were noted with low vNDV infective doses, probably due to competition of both viruses for cell receptors and replication (19). We sought to evaluate the impact of the aforementioned infection with AIV H9N2 and subsequent infection with vNDV in order to evaluate alterations in pathogenicity, viral spread and production that might lead to obscurity.

The assessment of commercial birds is innovative, as no studies have been performed to resolve that issue, except that of Fayoumi chickens that were co-infected with lentogenic NDV and AIV H9N2 (20). The two viruses did not synergized for the development of clinical signs and lesions, but in co-infected birds the AIV horizontal spread was enhanced while the NDV replication was repressed. That finding suggested the occurrence of a viral interference in dual-infections that might have impact in diagnosis and control of the two diseases and the emphasized need to extend our present understanding.

MATERIALS AND METHODS

Chickens

The first trial employed SPF chicks (SPAFAS, Ltd., Victoria, Australia) which were hatched at the Kimron Veterinary Institute (KVI) and transferred immediately to isolators in a BSL3 room.

The second trial employed commercial Lohmann layers at 30 weeks-old from a commercial farm which were transferred to KVI for the experimental infection.

The third trial employed commercial layers, Hyline at 35 weeks-old that were grown at the experimental farm at Bet Dagan and transferred to KVI for the experimental infection.

Viruses

The Israeli isolate AIV H9N2 – A/chicken/Israel/1163/2011 was grown in SPF (SPAFAS, Ltd., Victoria, Australia) embryonated eggs. The allantoic fluid was negative for NDV and IBV, as those viruses also can replicate in embryonated eggs by a similar inoculation route. The Israeli vNDV isolate was the Chicken/Israel/ Maale-Hachamisha/998/2011 genotype VII. The infectivity titration of both viruses was performed by the Reed and Muench method (21).

RNA purification and amplification

RNA purification from trachea and cloaca swabs was performed using the QIAmp Viral RNA kit, (Qiagen, Ltd., Hilden, Germany) and from organ tissues was performed with the Maxwell 16 LEV simplyRNA Tissue kit, (Promega

Ltd., Madison, WI, USA) both according to the manufactures instructions. AIV and NDV real-time amplification was performed as described by Das and Suarez (22), and Wise *et al*. (23), respectively.

Hemagglutination inhibition assay

Hemagglutination inhibition (HI) assay was conducted with standard procedure (24). Briefly, 2-fold serial dilutions of 25 µl of serum were made in 25 µl of phosphate buffered saline (PBS). Diluted sera were incubated for 30 min at room temperature with 4HAU/25 µl of antigen (AIV H9N2 1163/2011 isolate or vNDV isolate 998/2011 genotype VII), and then 25 µl of 1% chicken red blood cells were added. The test was evaluated after 30 minutes of incubation at room temperature. Titers were calculated as the highest HI positive serum dilution reciprocal and HI titers of 3 or below were considered negative.

Experimental design

Trial I: SPF chicks (SPAFAS, Ltd., Victoria, Australia) were hatched at KVI and transferred immediately to isolators in a BSL3 room.

AIV infection: The chicks were infected by choanal inoculation (0.1 ml/bird) with 10^6 EID_{50} A/H9N2 at 21 days of age. An uninfected control group was included. The birds were examined daily for morbidity and mortality. Trachea and cloaca swabs were collected at 0, 3, 5, 7 and 12 days post infection (dpi). Organs (trachea, lungs, brain, liver, spleen and other organs, according to pathological changes) were sampled from 2 birds at each time points.

NDV infection: Infection with vNDV was performed by the oculo-nasal inoculation at 21 days of age with doses ranging from 10^1 to 10^6 EID_{50} per bird. The inoculum (0.1 ml/bird) was applied to the nostrils and eyes. The horizontal transmission was evaluated by introducing 2 uninfected birds to each isolator at 3 dpi. NDV shedding was evaluated by sampling trachea and cloaca swabs at 3, 5, 7 and 12 dpi. The systemic spread was evaluated by rtRT-PCR of RNA from organs (liver, spleen, brain, proventriculus, lungs, heart, kidney and intestine) and by introducing 2 uninfected chicks to each isolator at 3 dpi.

Trial II: Commercial Lohmann layers at 30 weeks-old from a commercial farm were transferred to KVI. The chickens were vaccinated at the commercial farm by the schedule shown in Table 1, and then transferred to 6 isolators that were located in a BSL3 room. The birds were acclimatized in isolators for 7 days before inoculation, and then divided into 2 groups of 10 birds, an uninfected control group and a vNDV inoculated group. Challenge with NDV was performed at the infective dose of EID_{50} $1.0x10^6$ per bird. Both groups were examined twice daily to follow the morbidity and mortality. To assess the vNDV shedding trachea and cloaca swabs were taken at 2, 4 10 and 15 days post challenge (DpCh). Sera for assessing the level of NDV antibodies was obtained at the beginning of the trial and at its termination at 15 DpCh. Shedding was evaluated by introducing two naïve uninfected chicks to each isolator at 3 DpCh, at the viremia peak, to assess the virus transmission to the contact sentinels.

Trial III: Commercial layers, Hyline at 35 weeks-old were grown at the experimental farm at Bet Dagan where vaccination was carried out against vNDV by the schedule shown in Table 1. The chickens were transferred to KVI, where they housed in isolators in a BSL3 room. The birds were divided into groups: uninfected group (4 chickens), AIV-infected group (8 chickens), NDV-infected group (8 chickens) and AIV+NDV-double infected group (8 chickens). The birds were acclimatized in isolators for 2 days prior to infection. AIV was inoculated 4 days prior to vNDV challenge. Infection with both AIV and NDV was performed at the infective dose EID_{50} $1.0x10^{6.0}$ per bird. Swabs were sampled for AIV and NDV amplification day 0, 4, 6, 8, 14 and 19 dpi, corresponding to 0, 2, 4, 10 and 15 DpCh for vNDV. At the day of AIV inoculation and at the trial termination, (0 and 19 dpi for AIV and 0 and 15 DpCh for NDV), the birds were bled to obtain sera for examination of AIV and NDV antibodies by the HI assay. The birds were monitored daily for morbidity and mortality.

RESULTS AND DISCUSSION

Titrations of the vNDV isolate infectivity and pathogenicity in SPF chicks

The first aim of this study was to define the baseline of pathogenicity, virology and spreading parameters following single virus infection of SPF chicks with vNDV or with AIV H9N2. As infection with vNDV is lethal for the chicks, the dose-response effect of vNDV was assessed by administration of 6 infective doses (10^1-10^6 EID_{50}).

Table 1: Vaccination Schedule against vNDV on the two farms, as compared to the requirements of the Israeli Veterinary Services

Type of NDV vaccine/ Age of application	Trial I Lohmann layers 30 weeks-old	Trial II Hyline layers 35 weeks-old	Veterinary Services Guidelines
Live/ one day-old	+	+	+
Inactivated/10-12 day-old	+	+	+
Live / 17 days-old	+	-	-
Live/ 4 weeks-old	-	+	+
Live/5-6 weeks/old	+	-	-
Live/9 weeks old	+	+	-
Inactivated/9 weeks-old	-	+	-
Live + Inactivated/12 weeks-old	+	+	+
Inactivated/14-16/ weeks-old	+	-	+
Live/19-20 weeks-old	+	+	-
Live/28 weeks-old	+	-	-

Table 2: Morbidity and mortality of SPF chicks caused by AIV H9N2 at one infective dose (10^6 EID_{50}) and by vNDV infection at 6 infective doses (10^1-10^6 EID_{50})

Group (Infective dose)	Morbidity		Mortality		
	No. of sick/ Total	Day of appearance of symptoms	No. Dead/ Total	Day of death	Median day of death
Uninfected control (for AIV)	0/9	NA	0/9	NA	NA
Uninfected control (for NDV)	0/9	NA	0/9	NA	NA
AIV (10^6 EID_{50})	0/8	NA	0/8	NA	NA
NDV (10^1 EID_{50})	0/10	NA	0/10	NA	NA
NDV (10^2 EID_{50})	10/10	7 days	3/10	7, 9, 12	9.3
NDV (10^3 EID_{50})	10/10	5 days	7/10	6, 6, 6, 7, 7, 7, 12	7.3
NDV (10^4 EID_{50})	10/10	5 days	5/10	6 ,7, 7, 7, 8	5.8
NDV (10^5 EID_{50})	10/10	4 days	4/10	6, 6, 7, 7	6.5
NDV (10^6 EID_{50})	10/10	2 days	6/10	5, 5, 5, 5, 7, 7	5.7

NA – not applicable

The vNDV-associated morbidity could be detected in chicks that were inoculated with the infective dose of 10^2 EID_{50}, where the clinical and pathological symptoms appeared at 7 dpi (Table 2). By increasing the infective dose the symptoms appeared earlier, on 5 dpi for the infective doses 10^3 EID_{50} and 10^4 EID_{50}, on 4 dpi for the infective dose 10^5 EID_{50} and on day 2 dpi for the highest dose of inoculum, i.e., 10^6 EID_{50}. The pathological findings included hemorrhages in intestines, proventriculus, caecal tonsils and brain, as well as kidney and caecal tonsils hypertrophy. Mortality was recorded in groups inoculated with the infective dose of 10^2 EID_{50} and higher. Accordingly, by the increasing the vNDV infective dose, the median day of death decreased from 9.3 to 5.7 days. Not all the inoculated birds died, even with the highest vNDV infective dose. The mortality rate of chicks that received the lowest inoculum was about half than that recorded in the groups that received the highest inoculum. However, not all the chicks died in these groups, probably due to individual differences in susceptibility. These findings corresponded with the parallel study of Bonfante *et al.,* (2017) who titrated the same vNDV inoculum in SPF chicks for performing a similar dual-infection trial of AIV H9N2 and vNDV in SPF chicks (19).

Amplification of vNDV from trachea and cloaca of inoculated SPF chicks

The vNDV infectivity was titrated in SPF chicks inoculated with 6 vNDV infective doses (10^1-10^6 EID_{50}), by determining the vNDV presence in the trachea and cloaca (Table 3) and in organs (Table 4). Tables 3 and 4 show results for the inoculated chicks (Table 3a and Table 4a) and the in-contact sentinels (Table 3b and Table 4b). The infectivity positivity was determined by amplification below C_T < 36.0. Table 3a revealed that the infective dose of 10^2EID_{50} was the lower infective dose, peaking at 7 dpi, and from the dose of 10^3EID_{50} and above the chick infectivity rate was 100% beginning at 3 dpi. The trachea and the cloaca shed vNDV similarly. In the in-contact infected sentinels (Table 3b) the vNDV could be amplified beginning from 5 dpi in both trachea and cloaca, as the chicks were introduced to the isolators only 2 days before, at 3 dpi.

Tables 4 and 5 show the vNDV presence in the various organs sampled from birds that died of disease or at the trial termination. NDV could be detected in the organs starting from a NDV challenge dose of $EID_{50}10^2$. The NDV isolate was shown to cause a systemic infection and to replicate efficiently in all organs tested. The rRT-PCR results and histopathological analyses confirmed the classical pathological picture of a multi-organ vNDV infection (Haddas R., Personal communication) and those of the parallel study of Bonfante *et al.,* (2017) (19).

Table 3: Amplification of vNDV in trachea and cloaca of SPF chicks

a. Inoculated groups

	3 dpi		5 dpi		7 dpi		12 dpi	
	Trachea	Cloaca	Trachea	Cloaca	Trachea	Cloaca	Trachea	Cloaca
Control	0/9	0/9	0/9	0/9	0/7	0/9	0/3	0/3
10^1 EID_{50}	0/10	0/10	0/10	0/10	0/10	0/10	0/3	0/3
10^2 EID_{50}	2/10	2/10	5/10	4/10	10/10	9/10	4/4	3/4
10^3 EID_{50}	10/10	9/10	10/10	10/10	7/7	7/7	2/2	1/2
10^4 EID_{50}	10/10	10/10	10/10	10/10	8/8	8/8	1/1	1/1
10^5 EID_{50}	10/10	10/10	10/10	10/10	7/9	8/9	2/2	2/2
10^6 EID_{50}	10/10	10/10	10/10	10/10	4/4	4/4	2/4	4/4

b. In-contact infected groups

	3 dpi		5 dpi		7 dpi		12 dpi	
	Trachea	Cloaca	Trachea	Cloaca	Trachea	Cloaca	Trachea	Cloaca
Control	NA	NA	0/2	0/2	0/2	0/2	0/2	0/2
10^1 EID_{50}	NA	NA	0/2	0/2	0/2	0/2	0/2	0/2
10^2 EID_{50}	NA	NA	0/2	0/2	2/2	2/2	1/1	1/1
10^3 EID_{50}	NA	NA	2/2	2/2	2/2	2/2	1/1	1/1
10^4 EID_{50}	NA	NA	2/2	1/2	2/2	2/2	1/1	1/1
10^5 EID_{50}	NA	NA	2/2	1/2	2/2	2/2	2/2	2/2
10^6 EID_{50}	NA	NA	2/2	1/2	2/2	1/2	1/1	1/1

NA – not applicable

Table 4: NDV in organs of SPF chicks

a. Inoculated groups

Group	Sampling day	Liver	Spleen	Brain	Proventriculus	Lungs	Trachea	Else
Control	14	0/1	0/1	0/1	NT	0/1	0/1	NT
10^1 EID_{50}	14	0/1	0/1	0/1	NT	0/1	0/1	NT
10^2 EID_{50}	10, 14	2/2	2/2	2/2	NT	2/2	2/2	$1/1_1$
10^3 EID_{50}	5, 6, 6, 6, 7	4/4	4/4	5/5	1/1	4/4	4/4	$1/1_3$
10^4 EID_{50}	6, 7, 14	2/2	3/3	3/3	NT	3/3	3/3	$2/2_{2+3}$
10^5 EID_{50}	6, 6, 7, 14	4/4	4/4	4/4	NT	4/4	4/4	NT
10^6 EID_{50}	5, 5, 5, 14	4/4	4/4	4/4	2/2	4/4	4/4	$2/2_{2+3}$

– Heart, 2 – Kidney, 3 – Intestine

b. In-contact infected groups

Group	Sampling day	Liver	Spleen	Brain	Proventriculus	Lungs	Trachea	Else
Control	14	0/1	0/1	0/1	NT	0/1	0/1	NT
10^1 EID_{50}	7	0/1	0/1	0/1	NT	0/1	0/1	NT
10^2 EID_{50}	14	1/1	1/1	1/1	NT	1/1	1/1	$2/2_{1+2}$
10^3 EID_{50}	NT	NT	NT	NT	1/1	NT	NT	NT
10^4 EID_{50}	10	1/1	1/1	1/1	NT	1/1	1/1	$1/1_2$
10^5 EID_{50}	10	1/1	1/1	1/1	NT	1/1	NT	$2/2_{1+2}$
10^6 EID_{50}	9	1/1	1/1	1/1	NT	1/1	1/1	$1/1_3$

1 – Kidney, 2– Intestine

Table 5: AIV in organs, trachea and cloacal swabs of inoculated SPF chicks

Group	Dpi	Swabs		Organs				
		Trachea	Cloaca	Liver	Spleen	Brain	Trachea	Lung
Control	3	.0/10	0/10	NT	NT	NT	NT	NT
	5	0/7	0/7	NT	NT	NT	NT	NT
	7	2/5 35.2*, 34.4*	1/5 35.7	0/1	0/1	0/1	0/1	0/1
	12	0/3	0/3	0/1	1/1 33.6*	1/1 35.6*	1/1 34.3*	0/1
AIV	3	22/22	21/22	2/2	2/2	2/2	2/2	2/2
	5	18/18	14/18	1/2	0/2	2/2	2/2	2/2
	7	9/14	4/14	1/2	0/2	1/2	2/2	1/2
	12	1/8	0/8	0/2	1/2	1/2	1/2	1/2

* C_T value

AIV H9N2 was inoculated at one infectious dose, EID_{50} 10^6 per bird, according to the previously established infection model (Davidson et al., 2016). Table 5 shows the AIV H9N2 amplification as single infection of SPF chicks, as compared to the uninfected control group, where the rRT-PCR positivity limit was C_T< 36.0. None of the H9N2 infected birds died. At 3 dpi, 22/22 and 21/22 infected birds shed virus from the trachea and the cloaca, respectively. The AIV H9N2 presence was demonstrated also in organs (liver, spleen, brain, and lungs). Swabs were sampled at 3, 5, 7 and 12 dpi at each time point and two birds were sacrificed for organ sampling. AIV replication was detected in the trachea from 3 dpi and the virus persisted in most chicks for 7 days both in the trachea and the cloaca. It seems that the AIV H9N2 virus used in the present trial has enhanced *in vivo* systemic replication ability, compared to previous AIV H9N2 isolates, as the AIV H9N2 could be detected in the liver, spleen, brain and lungs.

NDV infection in Lohmann commercial layers

Aiming to reflect the actual situation in commercial flocks that received all NDV vaccines that were required until the 30 weeks of age, the birds were experimentally challenged with the highest vNDV infective dose of EID_{50} 10^6. Table 6 shows the amplification of velogenic and/or lentogenic NDV, representing the challenge and the vaccine virus, respectively. Amplification was conducted in two stages, first NDV detection was based on the M gene, and positive samples were then subjected for sub-typing. The M gene positivity threshold (C_T) was 36.0, while for the sub-typing it was 40.0. Trachea and cloaca swabs were sampled at 2, 4, 10 and 15 DpCh. The uninfected control group was mostly negative or borderline for NDV, probably due to vaccine NDV. Due to the low amplicon amount the subsequent amplification for the sub-type determination was impossible. In the challenged group the NDV was detected from 2-10 DpCh, and in several birds sub-typing was successful. The velogenic NDV was detected in the trachea of 2 and 3 birds at 2 and 4 DpCh, respectively. In one bird both velogenic and lentogenic viruses were detected. These low virus recoveries indicated that the commercial vaccination induced a good immunity, that prevented massive velogenic NDV shed. It is notable to mention that no morbidity and mortality were recorded throughout the experiment, indicating that the commercial vaccine protected against a massive experimental NDV challenge.

The HI antibody titers to vNDV of sera were obtained from all birds bled on the first day of their acclimatization in isolators, before challenge and on 14 DpCh at the termination of the trial. The NDV HI titer (log_2±standard deviation) of the commercial flock was 7.8±1.27 which is considered a good level of humoral immunity. At 14 DpCh the NDV HI titers in the uninfected control group and in the NDV-challenged groups was statistically similar (7.89±1.27 and 10.8±0.92, respectively), although in the challenged group the titer elevation was slightly higher by one log_2. That similarity could be attributed to the vaccination with the last booster that was performed at the farm several days before commencing the experimental trial at 14 DpCh, thus a raise in the NDV HI titer was observed in both groups.

Table 6: NDV in trachea and cloaca swabs of Lohmann layers

Sampling Day (dpi)	Uninfected Control		NDV Challenged	
	Trachea	Cloaca	Trachea	Cloaca
2	1/10* (35.0)**	1/10 (35.7)	6/10 (35.9, 35.0, 35.4, 35.2, 33.8, 35.3)	0/10
4	0/9	1/9 (35.7)	9/10 (34.1, 35.5, 32.8***, 31.3, 36.0, 32.5, 34.6, 35.0, 35.9)	1/10 (32.7)
10	2/9 (35.6, 35.8)	0/9	1/10 (35.3)	0/10
15	0/9	0/9	0/10	0/10

* Positive chickens by real-time PCR
** C_T
*** Sub-typed as both velogenic and lentogenic
Underlined – Sub-typed as velogenic NDV

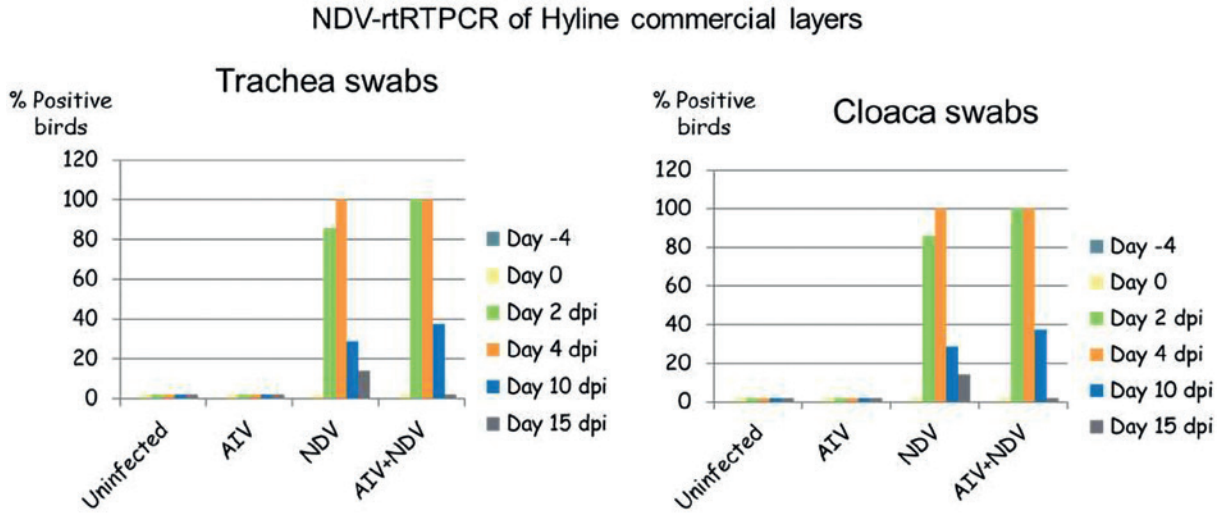

Figure 1: Shedding of vNDV in the trachea and cloaca swabs of Hyline commercial layers, as expressed by the percent of positive birds.

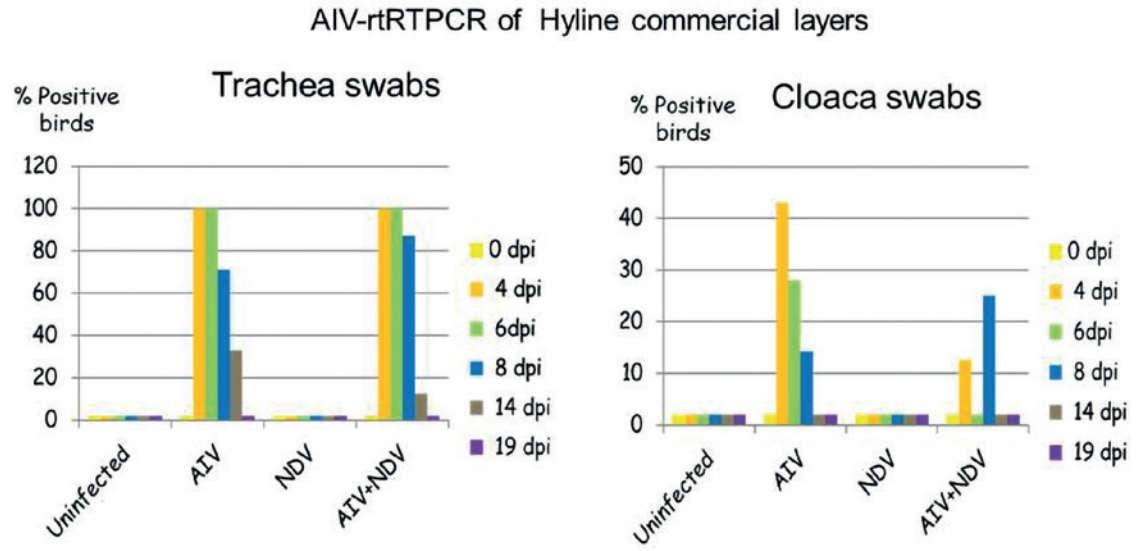

Figure 2: Shedding of AIV H9N2 in the trachea and cloaca swabs of Hyline commercial layers, as expressed by the percent of positive birds.

AIV and NDV infection in Hyline commercial layers

To examine experimentally the impact of AIV H9N2 and vNDV co-infection on NDV-vaccinated commercial layers, an experimental infection trial was performed employing NDV vaccinated Hyline layers as detailed in Table 1. The vNDV and AIV H9N2 shedding was evaluated by rtRT-PCR of tracheal and cloacal swabs, as shown in Figs. 1 and 2, reflecting the rate of positives, and in Table 7, showing the amplification values.

Only the groups inoculated with vNDV shed the virus in their trachea and cloaca, and there was no contamination of the uninfected control and AIV-infected chickens. vNDV trachea positive chickens in both NDV-infected and NDV+AIV-infected birds was between 2-10 days post infection (p.i.), however, few NDV-Infected chickens shed virus also on day 15 p.i.. A similar pattern was observed for the cloacal swabs. No difference was observed between the single NDV-infected and the AIV+NDV-co-infected chickens. The amplification levels in the NDV positive birds were very low, close to the negative borderline, except the trachea of the NDV-infected birds at 15 dpi. The NDV subtyping at borderline C_T levels was not feasible, and it was considered reasonable to assume that part of the NDV positive birds might reflect lentogenic NDV that originate from the live NDV vaccines. Only the groups inoculated with AIV shed the virus in their trachea and cloaca; there was no contamination of the uninfected control and NDV-infected chickens.

Table 7: Virus amplification in vNDV and AIV experimentally-infected Hyline layers

a. NDV rtRT-PCR

Group	-4dpi	0 dpi	2 dpi	4 dpi	10 dpi	15 dpi
			Trachea			
Uninfected	ND	ND	ND	ND	ND	ND
AIV	ND	ND	ND	ND	ND	ND
NDV	ND	ND	30.3*	29.3	35.5	14
AIV+NDV	ND	ND	34.0	26.4	34.0	ND
			Cloaca			
Uninfected	ND	ND	ND	ND	ND	ND
AIV	ND	ND	ND	ND	ND	ND
NDV	ND	ND	28.6	31.2	30.9	33.6
AIV+NDV	ND	ND	ND	30.2	31.7	34.8

ND – not detected

b. AIV H9N2

Group	0 dpi	4 dpi	6 dpi	8 dpi	14 dpi	19 dpi
			Trachea			
Uninfected	ND	ND	ND	ND	ND	ND
AIV	ND	20.7	27.5	34.4	33.3	ND
NDV	ND	ND	ND	ND	ND	ND
AIV+NDV	ND	22.7	28.6	33.0	35.0	ND
			Cloaca			
Uninfected	ND	ND	ND	ND	ND	ND
AIV	ND	34.7	34.5	34.0	ND	ND
NDV	ND	ND	ND	ND	ND	ND
AIV+NDV	ND	34.7	ND	33.0	ND	ND

ND – not detected
* Mean C_T values

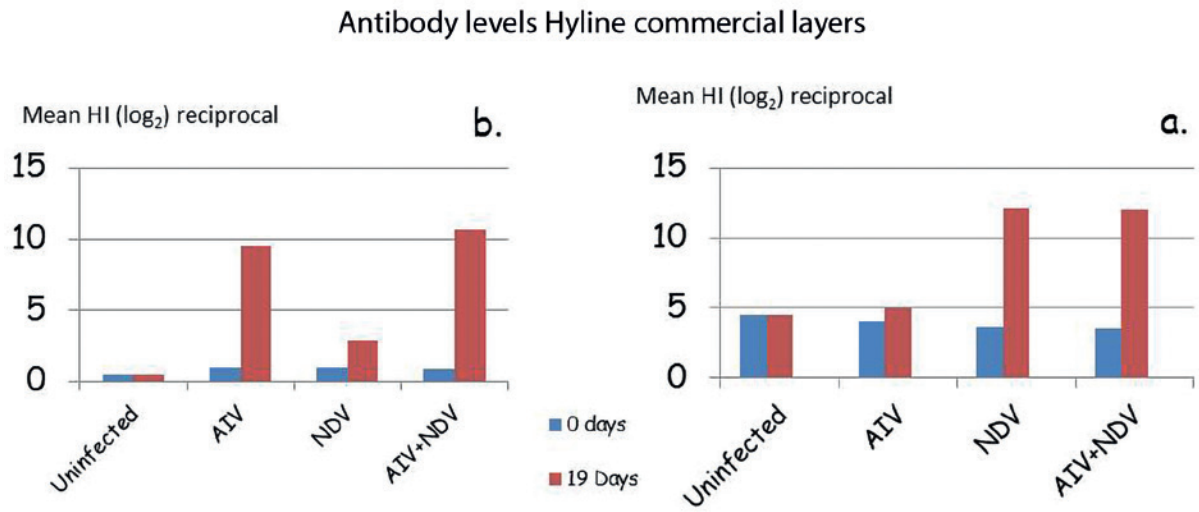

Figure 3: Antibody levels to vNDV (a) and AIV H9N2 (b) by the HI assay in sera of Hyline commercial layers.

AIV H9N2 positive chickens in both AIV-infected and AIV+NDV groups were detected between 4-8 days p.i.. On day 14 p.i. only the AIV-infected chicks showed shedding in the trachea. The reduced AIV shedding in tracheal swabs at 14 dpi and in cloaca swabs on 4-6 dpi indicated inhibition of AIV shedding by NDV co-infection, although that effect was reversed in the cloaca at 8 dpi, unlike in the SPF chicks where the replication of AIV H9N2 was unaffected in co-infected chicks (19).

In contrast to the effect of AIV H9N2 and vNDV co-infection in SPF chicks, no effects on mortality and morbidity were noticed in commercial layers. Virus shedding reflected the interaction between the two viruses in co-infected chickens by revealing the vNDV suppressive effect on AIV H9N2 shedding from trachea, as opposed to Bonfante *et al.* (2017) findings, which described the complex interaction in SPF co-infected chicks (19). On one hand the AIV H9N2 rendered the birds more susceptible to the vNDV challenge, demonstrated by lowering the vNDV minimum infective dose required to cause infection and by causing more severe clinical signs. On the other hand, the co-infection led to delayed vNDV shedding, onset of disease and death. These contrasting effects were vNDV dose-dependent.

To verify the immunological competence of the commercial layers to infection with the AIV H9N2 and the challenge with vNDV, on the background of the commercial vaccination, the chickens were bled at the experimental trial commencing and on the day of the trial termination. The sera were used in the HI test to determine the level of antibodies to NDV (Fig. 3a) and AIV H9N2 (Fig. 3b). Before the challenge with vNDV the HI titers were low, below the geometric HI $\log_2$ mean titer of 5.0. However, following the vNDV challenge the level raised significantly to above a $\log_2$ mean of 12.0, and no difference was noted in the presence or in the absence of AIV H9N2 co-infection. The level of vNDV antibodies in the two sources of commercial birds, Lohmann and Hyline differed. The Hyline birds received fewer vaccinations than the Lohmann layers that had initially the geometric HI $\log_2$ mean titer of 8.0, therefore their HI titer was as low as 5.0, and could raise significantly following the vNDV experimental inoculation. However, no significant difference was noted between the groups that were infected with AIV alone or the group that was co-infected with AIV and NDV, similarly to the findings in SPF chicks (19).

CONCLUSIONS

To delineate the impact of dual-virus infection, several parameters were evaluated, including the morbidity, mortality, virus shedding and humoral response in SPF chicks and in NDV commercially-vaccinated commercial layers. The vaccination efficacy of commercial layers was demonstrated under experimental conditions by using a lethal dose of vNDV (10^6 EID_{50}) where mortality was prevented in vaccinated birds and virus shedding was reduced to borderline levels. According to the present findings in both trials the birds were protected against morbidity and mortality, however, birds in both groups still shed vNDV at low levels of virus detection, probably reflecting the actual situation in many commercial flocks in Israel. That similarity was noted in spite of the large differences in the geometrical mean titer of vNDV antibodies, as measured by the HI assay, probably reflecting difference that can be attributed to the difference in the vaccination schedule against vNDV.

These trends indicate the occurrence of competitive effects on the chicken respiratory tract, as both viruses bind to the same sialic acid virus receptors. A further insight into dual infection in field poultry will contribute to implementation of effective protection measures. Under the physical conditions available it was difficult and almost impossible to evaluate the impact of NDV infection of NDV-vaccinated commercial layers on their laying performance.

In contrast to previous studies that evaluated the impact of dual infections with AIV and NDV (14-16, 18, 19), we could not detect any changes in commercial layers. The differences between the genetic and management conditions could explain the differences in results. The outcome of the multifaceted interaction between the two viruses in double virus-infections mirrors the summation of the birds' genetic background, the synchronicity of the flock infection, vaccination, sequence of infection with the two viruses and their infective dose, interferon expression and competition at the site of infection for cellular receptors, enzymes and replication factors. Recognizing that complexity and its manifestation on the flock basis is crucial in assessing diagnosis and control.

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