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Protective efficacy of classical vaccines and vaccination protocols against an exotic Newcastle disease virus genotype VII.2 in Belgian layer and broiler chickens

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ABSTRACT

Vaccination against Newcastle disease (ND) has been routinely implemented in the Belgian professional poultry sector since 1993, using genotype I and II vaccines. Despite this, an outbreak of genotype VII.2 avian paramyxovirus 1 (APMV-1) occurred in 2018, with 20 reported cases over the course of 3 months. Although the economic impact on the professional poultry sector was limited, this epizootic raised questions regarding the efficacy of implemented classical genotype I and II vaccines against phylogenetically distant exotic velogenic strains.

The present study provides insights into the protective efficacy of standard vaccination programs applied in layer and broiler flocks against the introduction and transmission of this velogenic APMV-1 VII.2 strain.

For fully field-vaccinated 26-week-old layer chickens, high levels of specific antibodies were measured at the time of the velogenic APMV-1 challenge, resulting in good clinical protection. However, despite the observed humoral immunity, viral excretion was not prevented, leading to transmission of the virus to non-infected sentinel birds.

In fully field-vaccinated 4-week-old broiler chickens, assessment of vaccine uptake and coverage revealed low levels of ND specific antibodies despite double vaccination at day 1 and day 14. Consequently, poor protection against velogenic APMV-1 infection was observed, with both clinical signs and viral excretion occurring in both infected and sentinel birds.

This study demonstrates that the introduction of velogenic APMV-1 VII.2 can lead to its dissemination among the Belgian avian poultry population despite the implementation of standard vaccination.

Introduction

Paramyxoviridae of the subfamily Avulavirinae, are linear, negative sense, single stranded RNA viruses (Rima et al., 2019). Based on hemagglutination and neuraminidase inhibition assays, 21 serotypes have been identified, within 3 genera: meta-, ortho- and para-avulaviruses (Amarasinghe et al., 2019). Avian paramyxoviruses of the serotype 1 (APMV-1), are the causative agents of Newcastle disease (ND) and are divided into 2 classes, named I and II. The velogenic strains (vAPMV-1) of class II induce high degrees of morbidity and mortality in Galliformes species (Alexander et al., 2012; ICTV, 2024; WOAH, 2024). ND remains a globally significant disease with substantial socioeconomic implications and a wide range of susceptible hosts across all avian species (Alexander, 2000; Samrawit and Asrat, 2018). Newcastle disease viruses (NDV) are subject to strict regulation (EU-Regulation 2016/429, 2021), obligatory notification and compulsory prophylactic vaccination in many European countries.

Within class II of APMV-1, 21 different genotypes (I-XXI) are described (Dimitrov et al., 2019). Subgenotype VII.2, regrouping the previous VIIi, VIIh and VIIk subgenotypes, includes the strains responsible for the 5th ND panzootic, affecting Asia, the Middle East, Western and Eastern Europe, and Africa (Diel et al., 2012; Miller et al., 2015;

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Dimitrov et al., 2019; Steensels et al., 2020; Tsaxra et al., 2023). Strains from this subgenotype were first identified in Indonesia throughout the 1980s, followed by Italy, Spain, the Netherlands, Belgium, and Germany in the early 1990s. Two decades later, the subgenotype VII.2 became the most frequent cause of ND outbreaks in the Middle East and Asia, gradually replacing subgenotypes VII.1.1 and VII.1.2, responsible for the 4th panzootic (Aldous et al., 2003; Miller et al., 2015). Since 2020, strains from subgenotype VII.2 have been the cause of outbreaks in Bangladesh and have been isolated in Iran, Israel, and Zambia (Nooruzzaman et al., 2022; Wiseman et al., 2022; Abdoshah et al., 2024; Kalonda et al., 2024). Viral dispersion from endemic regions to new areas has been observed, resulting in localized epidemics (Hicks et al., 2019). Viruses of genotype VII have the potential to cause high mortality and viral shedding in vaccinated flocks and have an increased host range, both as reservoirs and sensitive species (Yi et al., 2011; Wang et al., 2012; Sultan et al., 2020; Eid et al., 2022).

The over 15 kb polycistronic APMV-1 genome encodes up to 8 proteins, of which the fusion and hemagglutinin neuraminidase surface glycoproteins are considered the most immunogenic (Steward et al., 1993; Kapczynski et al., 2013; Ganar et al., 2014). Vaccination implementation helps to control the impact of exotic vAPMV-1 introduction and complements primary control measures, such as biosecurity and hygiene. The currently most widely used vaccines are the conventional whole virus inactivated or attenuated live vaccines of genotypes I and II (Dimitrov et al., 2017). Although APMV-1 can be differentiated into a wide array of genotypes, all belong to the same serotype, implying that some degree of cross-protection should be established (Miller et al., 2007). These conventional vaccines have been used since the 1960′s without regular updates or monitoring of their protection against distant exotic ND viruses (Lim, 2014; Hu et al., 2022).

Despite intensive vaccination in the commercial poultry sector, North-Western Europe experienced an ND epizootic caused by genotype VII.2 (VIIi) in 2018. The outbreak affected both poultry retailers and professional poultry flocks, where morbidity and mortality were reported. Additionally, multiple hobby flocks were affected more severely due to the lack of (re-)vaccination (Steensels et al., 2020). Similar outbreaks of genotype VII.2 in vaccinated flocks were also reported in multiple other non-European countries (Aljumaili et al., 2017; Ghalyanchilangeroudi et al., 2018; Nooruzzaman et al., 2022). The 2018 incursions prompted the need to assess the protective efficacy of current vaccines and typical western European vaccination protocols in commercial poultry. This study aimed to evaluate the existing protection of the 2 major categories of Belgian poultry against the introduction of vAPMV-1 VII.2, and the transmission potential of this genotype in typical vaccinated flocks across Western Europe.

Materials and methods

Chickens

Fifteen ND field-vaccinated Lohmann Brown layer chickens, 6 months of age, were purchased from a Belgian poultry farmer. The ND vaccination schedule implemented on the farm consisted of Avishield® ND (Genera, Croatia), a live attenuated LaSota (genotype II) vaccine, applied by spray at 2 and 7 weeks of age (average droplet diameter of 0.22 mm). Subsequently, at 12 weeks of age, an ND boost was administered using the trivalent Nobilis® RT+IBmulti +ND+EDS (Intervet International B.V, The Netherlands) vaccine (genotype II), via intramuscular route. All layer birds originated from the same flock and underwent the same vaccination program.

Forty field-vaccinated Ross 308 broiler chickens, 4 weeks of age, were purchased from a Belgian poultry farmer. The implemented ND vaccination schedule on the farm consisted of live attenuated Avishield® ND nebulization at hatch (day 0) and again at 2 weeks of age (average droplet diameter of 0.10 mm). All broiler birds originated from the same flock and underwent the same vaccination program.

Virus

Lung tissue homogenate from a NDV-positive backyard chicken was injected into 9-day-old embryonated specific pathogen free (**SPF**) chicken eggs, following standard procedures (WOAH, 2024). The collected hemagglutinating allantoic fluid was characterized by hemagglutination inhibition (**HI**) with a panel of poly- and monoclonal antisera (Meulemans et al., 1987), allowing differentiation of vaccine and pigeon specific APMV-1 strains. The lung tissue homogenate as well as the obtained allantoic fluid were tested for AI by RT-qPCR, and excluded the presence of AI in the inoculum. The viral titer of the APMV-1/Bassette_Chicken/Belgium/4096/2018 viral stock (**vAPMV-1-Be-4096-2018**) was determined by inoculating a 10-fold dilution series into 9-day-old embryonated chicken eggs (Steensels et al., 2020), at a fifty percent egg infectious dose (**EID50**) of $10^{9.66}$ per ml (Reed and Muench, 1938). The full genome sequence of the vAPMV-1-Be-4096-2018 challenge strain is available in GenBank under accession number MH432252.2.

Experimental model

Upon arrival at the animal facilities, birds were individually identified by numbered colored rings to allow individual clinical, serological, and virological follow-up. Serum samples were collected from all chickens upon arrival, 1 week before challenge, for serological analysis to evaluate ND-vaccinal uptake and coverage. In addition, virological samples, including cloacal and tracheal swabs, were taken to confirm the APMV-1 and avian influenza viro-negative status. Broilers were sampled again 2 days before infection to reassess their ND-serological status. Birds were housed in biosecurity level 3 (**BSL-3**) isolator units with water and feed distributed *ad libitum*. A one-week period of quarantine and acclimatization was implemented before challenging the birds, at 26 weeks of age for layers and 5 weeks of age for broilers.

Birds that were to be infected and non-challenged sentinel birds were randomly separated from each other by a plastic grid 1 day before the challenge. On the day of the infection, the chickens were inoculated by oculo-nasal route with 10^6 EID_{50}/100 µl of the heterologous vAPMV-1-Be-4096-2018 challenge strain. The grid was removed at 1 day post-infection (**dpi**) to allow interaction between the vaccinated-infected and vaccinated sentinel birds for the evaluation of viral transmission. In the layer experiment, 10 birds were infected and placed in contact with 5 sentinel chickens. In the broiler experiment, due to animal welfare requirements, the study was divided into 2 equal parts, comprising a total of 30 infected birds and 10 sentinel birds.

Birds were monitored daily for clinical signs and mortality during a 14-day period following vAPMV-1 VII.2 infection. A clinical score was assigned to each individual bird, with a value of 0 when no clinical signs were present, 1 for mild clinical signs, 2 when at least 2 or more mild to severe clinical signs were presented, and a maximum score of 3 for dead birds. The clinical score of the group was calculated as the average of the total observation period. Humane endpoints, described as birds unable to drink and/or eat for 8 h, were implemented over the entire duration of the experiment. When a humane endpoint was reached, birds were first anesthetized with a solution of medetomidine (100 µg/kg) and ketamin (25 mg/kg) injected intramuscularly (pectoral muscle). Once the birds were fully unconscious, they were exsanguinated by cutting the jugular veins in order to collect blood for further analysis.

Virological samples, cloacal and tracheal swabs, were collected from all the infected and sentinel birds at 2, 5, 7 and 9 dpi. Organs (brain, intestine, and lung/trachea) were sampled at death or at the end of the experiment (14 dpi). Additionally, feathers with vitalized pulp were taken as virological sample from broilers at 2, 5, 7, 9 dpi, and at the time of death or at the end of the experiment. Specifically, the base of the feather, which contains the highest concentration of pulp, was cut off. Both feather and organ samples were processed using a Stomacher® (Seward, Worthing, UK). This step ensured thorough tissue disruption

before proceeding with RNA extraction.

Serum samples from all the surviving birds were collected at the end of the experiment, 2 weeks after the infection, to perform a serological endpoint evaluation.

Animal studies were approved by Sciensano ethical and biosafety offices, respecting national and European regulations (Bioethical permit: 20180222-01, Biosafety permits: NRL_U1804096_vAPMV1: NRL-FAVV_Velogenic APMV-1_Bassette Chicken Belgium 4096 2018_Layer_BVC and _Broiler_BVC).

Serological analysis

HI and indirect ELISA (NDVS_CV, IDVet, Grabels, France) were used for serological analysis. HI tests were performed with 2 antigens, the LaSota vaccine homologous and the challenge strain (vAPMV-1-Be-4096-2018), at 4 hemagglutinating units (WOAH, 2024). In HI tests the sample was considered positive when the $\log_2$ (HI) titer is equal to or above 4. The HI titer corresponded to the last dilution of the serum inducing a complete inhibition of the hemagglutination of 4 hemagglutinating units of the antigen used for identification. A negative sample in HI-test, with a titer below 4, was assigned a value of 2 $\log_2$ for statistical analysis. The indirect ELISA was performed following the manufacturer's instruction (OD 450 nm, log10 (titer) = log10(S/P)+3, 520), sera samples were considered positive when a $\log_{10}$ (titer) above 3 was obtained.

As both the challenged and sentinel layers originated from the same flock and were randomly separated only 1 day prior to challenge, their pre-challenge serological data was represented as a single group in the graphs for clarity.

Virological analysis

Virological analysis was performed by generic real-time reverse transcription PCR (**RT-qPCR**) detection of a conserved region of the *M*-gene allowing the detection of all class II APMV-1 viruses (Wise et al., 2004). Viral RNA extraction was performed using the MagMAX™ Total Nucleic Acid Isolation Kit for organs and swabs, and the MagMAX™-96 AI/ND Viral RNA Isolation Kit (Applied BioSystems, Massachusetts, USA) kit for swabs, following the manufacturer's guidelines, on a semi-automated KingFisher Magnetic Particle Processor system (Thermofisher Scientific, Massachusetts, USA). For the RT-qPCR reaction the AgPath-ID™ One-Step RT-PCR kit (Applied BioSystems, Massachusetts, USA) was used, according to the manufacturer's instructions. The cycling conditions consisted of a 30-min reverse transcription step at 50°C, followed by a 10-min denaturation at 95°C and 50 cycles of denaturation (95°C for 15 s), hybridization (54°C for 34 s) and 30 s of elongation at 72°C. The Cp limit value was established at 38 during the method validation process. Viral RNA presence or excretion was reported as the logarithm of viral RNA copies per ml, quantified relative to an external standard curve during RT-qPCR analysis. Specifically, synthetic M-NDV RNA, produced in-house and derived from extracted RNA of an APMV-1 virus stock, was quantified using a NanoDrop™ spectrophotometer (Thermofisher Scientific, Massachusetts, USA) and used to construct the standard curve. The cut-off value, corresponding to the Cp limit, was set at 2 $\log_{10}$ viral RNA copies/ml. The mean viral excretion was calculated per group and per time point. Non-excreting birds, undetected or below 1 $\log_{10}$ RNA copies/ml in RT-qPCR, were assigned a value of 1 $\log_{10}$ viral RNA-copies/ml for statistical analysis. Birds with detected values below 2 $\log_{10}$ were depicted as such but considered negative.

Statistical analysis

Statistical analysis of survival, viral tropism, and virus shedding results were performed using Graph Pad Prism version 9.00 for Windows. Differences were considered significant at $p < 0.05$. Statistical results are represented in the figures by *** ($p < 0.001$), ** ($0.001 < p < 0.01$), * ($p < 0.05$). The absence of normal distribution and homogenous distribution of the within groups' variances were confirmed by the Shapiro–Wilk's test and the Bartlett's test, respectively. The organs' viral loads of infected and sentinel groups were compared for each organ type using the non-parametric Mann–Whitney test. The comparison of viral excretion was conducted in the same manner at each time point. Friedman paired test, followed by the Dunn's multiple comparisons test, were used to evaluate the viral tropism of both groups, as well as the kinetics of their viral excretion. The survival comparison of infected and sentinel birds was done by using a log-rank test (Mantel–Cox).

Results

Vaccinal efficacy of a classic vaccination protocol in commercial-field layer chickens

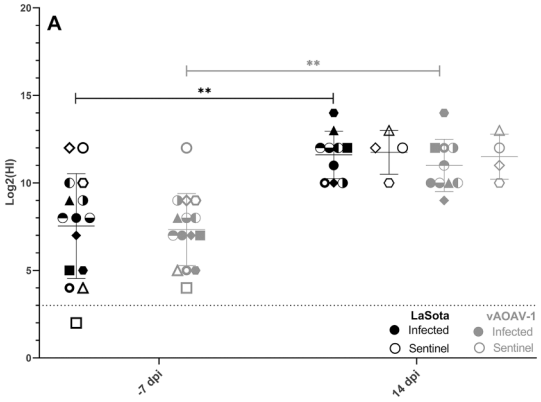
Survival and Clinical Impact. None of the 10 infected 26-week-old commercial layers demonstrated clinical signs or perished during the 14-day surveillance period. In contrast, 1 out of 5 sentinel birds exhibited clinical signs typical of vAPMV-1 at 8 and 9 dpi before succumbing at 10 dpi. The clinical signs consisted of neurological signs (shaking), conjunctivitis, and lethargy. A final average clinical score of 0.6 was calculated for the sentinel group. No statistically significant differences in clinical score or mortality were observed between infected and sentinel birds.

Serological Response (Fig. 1). Prior to infection, the average HI-titer of the commercial layers was statistically comparable between the lentogenic homologous vaccine and the velogenic-heterologous challenge antigens (7.5±3.0 versus 7.3±2.1, respectively) (Fig. 1A). The sentinel bird that succumbed at 10 dpi was the only bird that did not have a measurable HI response with the vaccine antigen and was negative by indirect ELISA (Fig. 1B), while a minimal HI response was detected with the velogenic challenge antigen. All other birds tested positive by both HI and indirect ELISA before infection (Fig. 1A-C).

Two weeks after vAPMV-1 infection, no significant differences were identified between infected and sentinel birds, either by HI for both antigens or by ELISA. When comparing data before and after the infection, a significant increase was observed in infected birds using both HI for both antigens and indirect ELISA. Although an increasing trend is observed, no statistically significant increase could be demonstrated for the sentinels following APMV-1 infection, due to high variability in humoral immunity before the challenge for both antigens.

Viral Kinetics (Fig. 2). Following APMV-1 infection, the main route of viral replication and excretion was identified to be the respiratory tract for both the infected and sentinel birds, starting at 5 and 7 dpi, respectively. The tracheal viral excretion period was short, with a peak in the number of excreting birds at 5 dpi (50%) for infected birds and at 9 dpi (60%) for sentinel birds. Cloacal viral excretion remained extremely limited throughout the study with 1 infected bird excreting at 7 dpi and 1 sentinel bird at 9 dpi (Fig. 2A). The birds excreting through the gastro-intestinal route presented prior respiratory viral excretion. Viral excretion was not detected in 5 infected (50%) and 2 sentinel (40%) birds at any timepoint.

Viral RNA in organs was mainly detected in the trachea/lung (9/15 birds), while only limited viral presence in the intestine (2/15) and brain (1/15) of dead or euthanized infected and sentinel birds was found, indicating a primary respiratory tropism of the vAPMV-1 VII.2 strain (Fig. 2B). The only bird for which the virus was detected in both the intestine and the brain was the sentinel bird that was seronegative prior to APMV-1 infection. In addition, only 1 out of the 10 infected birds demonstrated viral presence in the intestine, while no virus was detected in the lung/trachea or brain.



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	-7 dpi						14 dpi					
	HI				ELISA		HI				ELISA	
	LaSota		vAOAV-1 VII.2				LaSota		vAOAV-1 VII.2			
	Infected	Sentinel	Infected	Sentinel	Infected	Sentinel	Infected	Sentinel	Infected	Sentinel	Infected	Sentinel
% birds seroconverted	100 (10/10)	80 (4/5)	100 (10/10)	100 (5/5)	100 (10/10)	80 (4/5)	100 (10/10)	100 (4/4)	100 (10/10)	100 (4/4)	100 (10/10)	100 (4/4)
Average Titer ± StDev	7.3±2.0	8.0±4.7	7.1±1.3	7.8±3.3	4.09±0.2	3.85±0.82	11.6±1.3	11.8±1.3	11.0±1.5	11.5±1.3	4.31±0.04	4.32±0.04
overall	7.5±3.0		7.3±2.1		4.0±0.5							

Fig. 1. Humoral immune responses in layer birds against APMV-1 before and after infection, measured by HI (A) and indirect ELISA (B). A summary of the obtained data is represented in C. Data are represented at the individual level and as average group value ± standard deviation of $\log_2$ (HI-titer) or $\log_{10}$ (ELISA-titer). The HI geometric mean titers were expressed as reciprocal $\log_2$, and titers ≥ 4 $\log_2$ were considered positive, this cutoff is indicated by the dotted line. Although the challenged and sentinel birds are represented together at the pre-challenge time point, their values were not mixed for statistical comparisons.

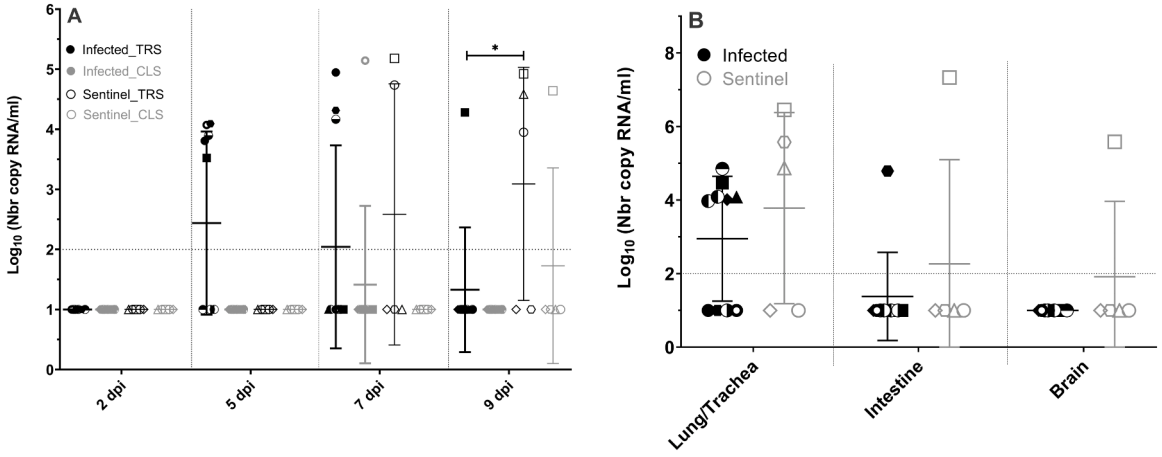


Fig. 2. (A) Viral RNA excretion by infected and sentinel layer birds at different points after infection, by the respiratory and gastrointestinal tract. (B) Viral presence in the organs of infected and sentinel birds at time of death or the end of the experiment (14 dpi), determined by RT-qPCR. CLS, cloacal swab; TRS, tracheal swab.

Vaccinal efficacy of a classic vaccination protocol in commercial-field broiler chickens

Survival and Clinical Impact. After vAPMV-1 VII.2 infection at 5 weeks of age, 3 weeks after the boost vaccination, both infected and sentinel broilers exhibited a gradual onset of clinical signs, starting with redness of the eyes and conjunctivitis, progressing to lethargy and neurological signs such as loss of balance and lack of coordination, ultimately leading to mortality (Fig. 3). Mortality among infected broilers started at 3 dpi and peaked at 5 dpi. For the sentinels, mortality started at 6 dpi and reached a maximum level at 8 dpi. By the end of the study (14 dpi), mortality reached 43% in infected and 60% in sentinel birds (Fig. 3). Two of the 17 surviving infected birds still presented neurological signs, with a twisted neck and lack of coordination (clinical score of 2), while the other 15 survivors had recovered (n = 6) or never showed any clinical signs (n = 9). In contrast, all sentinels displayed clinical signs at various time points. Among the 4 surviving sentinels, only mild clinical signs were observed for a limited period, with 2 birds still displaying mild signs at the end of the study. The evolution of the

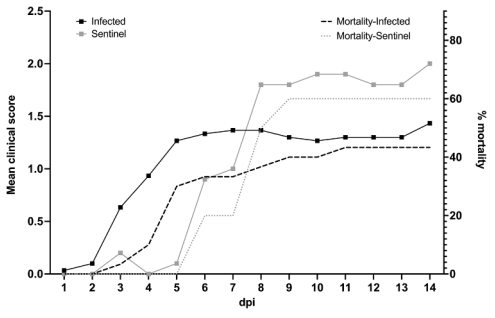


Fig. 3. Representation of mean clinical scores and mortality rates for infected (black) and sentinel (grey) field vaccinated broiler chickens after infection.

mean clinical scores demonstrated a faster progression from the onset of clinical signs to death for sentinels, compared to infected birds (Fig. 3). Both mortality rates and clinical profiles between infected and sentinel

birds were statistically equivalent.

Serological Response (Fig. 4). One week before infection (-7 dpi) at 4 weeks of age, only 55% (22/40; 3.5±0.7) and 15% (6/40; 2.9±0.7) of the birds tested positive in the HI test for the vaccine and challenge antigens, respectively, with a statistically significant difference between the 2 antigens. By indirect ELISA 32.5% (13/40; 2.9±0.4) of the broilers tested positive at -7 dpi (Fig. 4C). Analysis of a second blood sampling 2 days before the challenge (-2 dpi) demonstrated a significant reduction in the number of HI positive birds, down to only 7.5% (3/40; 2.3±0.6) and 2.5% (1/40, 2.1±0.4) with the vaccine and challenge antigens, respectively (Fig. 4A-C). The difference between both antigens was no longer statistically significant due to the negligible number of seropositive birds in both HI-tests. However, the indirect ELISA showed an increasing trend in seropositivity with 45% of the birds testing positive (18/40, 3.0±0.4) (Fig. 4B, C).

Two weeks after the infection, a significant increase was measured for both the infected and sentinel birds by HI (Fig. 4A-C), with both antigens, and only for the infected birds by ELISA (). This increase in HI-titer was less pronounced for the challenge antigen compared to the vaccinal antigen.

Viral Kinetics. Two days post vAPMV-1 infection, 90% (27/30) of the infected broiler chicken demonstrated viral excretion through the respiratory tract, whereas only 43% (13/30) excreted virus through the gastrointestinal tract. Viral excretion started at 5 dpi for the sentinel birds by both the respiratory and gastrointestinal tract. The peak of viral excretion was observed at 5 dpi for infected birds and at 7 dpi for sentinels, regardless of the excretion route. In surviving birds, viral excretion gradually decreased, with only trace amounts detected by 14 dpi (Fig. 5A).

Viral material was detected in feather pulp (Fig. 5B) at 2 and 5 dpi from infected and sentinel birds, respectively. These measurements increased until 5 dpi in infected birds and 7 dpi in sentinels, reaching plateaus that persisted until 9 dpi for both groups. Among the birds that survived until the end of the experiment (14 dpi), 29% (5/17) of infected and 100% (4/4) of sentinel birds displayed significant amounts of viral material in feathers. Additionally, feathers collected at the time of death from the birds that did not survive the experiment did all show the presence of viral content.

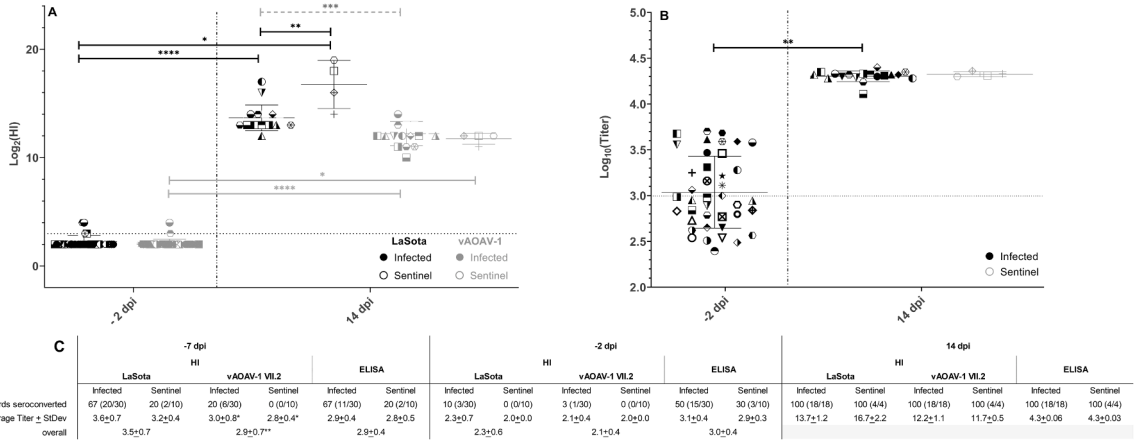
Regarding organ samples (Fig. 5C), viral presence was high and ubiquitous in all infected and sentinel birds that died during the experiment. On the contrary, viral presence was not consistent across the 3 organs in the infected birds euthanized at 14 dpi. A significant amount of viral material was detected in only 1 organ for 6 out of 17 birds, and for 1 out of 17 birds both the respiratory and gastrointestinal tracts were affected. On the other hand, 2 out of the 4 sentinels that survived were positive in all 3 organs, while the remaining 2 were positive in either the brain or the respiratory tract.

Discussion

Circulating field strains, such as those responsible for the fourth and fifth ND panzootics, have progressively diverged genetically and antigenically from genotype I and II vaccine strains (Dimitrov et al., 2017; Hu et al., 2022). As a result, these classical genotype I and II vaccines exhibit reduced efficacy in preventing viral replication and shedding following genotype VII velogenic infection (Gu et al., 2011; Samuel et al., 2013; Pandarangga et al., 2022). The emergence of genotype VII has posed major challenges due to its high virulence and diversity, causing important outbreaks since the 1980s. Similar to Belgium, subgenotype VII.2 has been recovered from vaccinated commercial flocks in Pakistan, Israel, Mozambique, South Africa, Malaysia, Indonesia, and China (Miller et al., 2015; Rehmani et al., 2015; Mapaco et al., 2016; Abolnik et al., 2018; Putri, 2018; Liu et al., 2019; Mahamud et al., 2021; Steensels et al., 2020).

The primary objective of the present study was to offer relevant insights into the current level of protection of layer and broiler flocks vaccinated with standard Belgian protocols against the introduction of VII.2 vAPMV-1 in the field, where co-infections and/or immunosuppression are part of the potential conditions that these flocks face.

In the described experiment conducted on fully vaccinated field commercial layer chickens, no mortality was observed except for 1 sentinel chicken with low seroconversion levels. Besides expected biological variation, inadequate vaccination application in the field or immunosuppression due to an underlying infection could explain this immune antibody response variability (Hanson, 1976; Rhee et al., 1998). According to the literature, despite vaccination and the resulting high HI titers, viral excretion was not sufficiently reduced to prevent transmission to vaccinated sentinel birds. (Han et al., 2017; Yang et al., 2017). Analysis of targeted organs revealed that the virus showed a clear respiratory tropism, which was further confirmed by the prevalence of viral presence in tracheal swabs, supporting previous research (Pandarangga et al., 2016; Khader et al., 2020). In addition, 40% of the infected birds presented a systemic infection (lung/trachea) without any detectable viral excretion. This could possibly be explained by brief acute shedding periods between the sampling timepoints. The relatively small number of birds for which virus was detected in the lung/trachea, intestine or brain can likely be attributed to the robust immune response elicited by the vaccination regimen.



C

	-7 dpi HI LaSota Infected	-7 dpi HI LaSota Sentinel	-7 dpi HI vAOAV-1 VII.2 Infected	-7 dpi HI vAOAV-1 VII.2 Sentinel	-7 dpi ELISA Infected	-7 dpi ELISA Sentinel	-2 dpi HI LaSota Infected	-2 dpi HI LaSota Sentinel	-2 dpi HI vAOAV-1 VII.2 Infected	-2 dpi HI vAOAV-1 VII.2 Sentinel	-2 dpi ELISA Infected	-2 dpi ELISA Sentinel	14 dpi HI LaSota Infected	14 dpi HI LaSota Sentinel	14 dpi HI vAOAV-1 VII.2 Infected	14 dpi HI vAOAV-1 VII.2 Sentinel	14 dpi ELISA Infected	14 dpi ELISA Sentinel
% birds seroconverted	67 (20/30)	20 (2/10)	20 (6/30)	0 (0/10)	67 (11/30)	20 (2/10)	10 (3/30)	0 (0/10)	3 (1/30)	0 (0/10)	50 (15/30)	30 (3/10)	100 (18/18)	100 (4/4)	100 (18/18)	100 (4/4)	100 (18/18)	100 (4/4)
Average Titer ± StDev	3.6±0.7	3.2±0.4	3.0±0.8*	2.8±0.4*	2.9±0.4	2.8±0.5	2.3±0.7	2.0±0.0	2.1±0.4	2.0±0.0	3.1±0.4	2.9±0.3	13.7±1.2	16.7±2.2	12.2±1.1	11.7±0.5	4.3±0.06	4.3±0.03
overall	3.5±0.7		2.9±0.7**		2.9±0.4		2.3±0.6		2.1±0.4		3.0±0.4							

Fig. 4. Humoral immune responses in broiler birds against APMV-1 before and after infection, measured by HI (A) and indirect ELISA (B). A summary of the obtained data is represented in C. Data are represented at the individual level and as average group value ± standard deviation of $\log_2$ (HI-titer) or $\log_{10}$ (ELISA-titer). The HI geometric mean titers were expressed as reciprocal $\log_2$, and titers ≥ 4 $\log_2$ were considered positive, this cutoff is indicated by the dotted line. Although the challenged and sentinel birds are represented together at the pre-challenge time point, their values were not mixed for statistical comparisons.

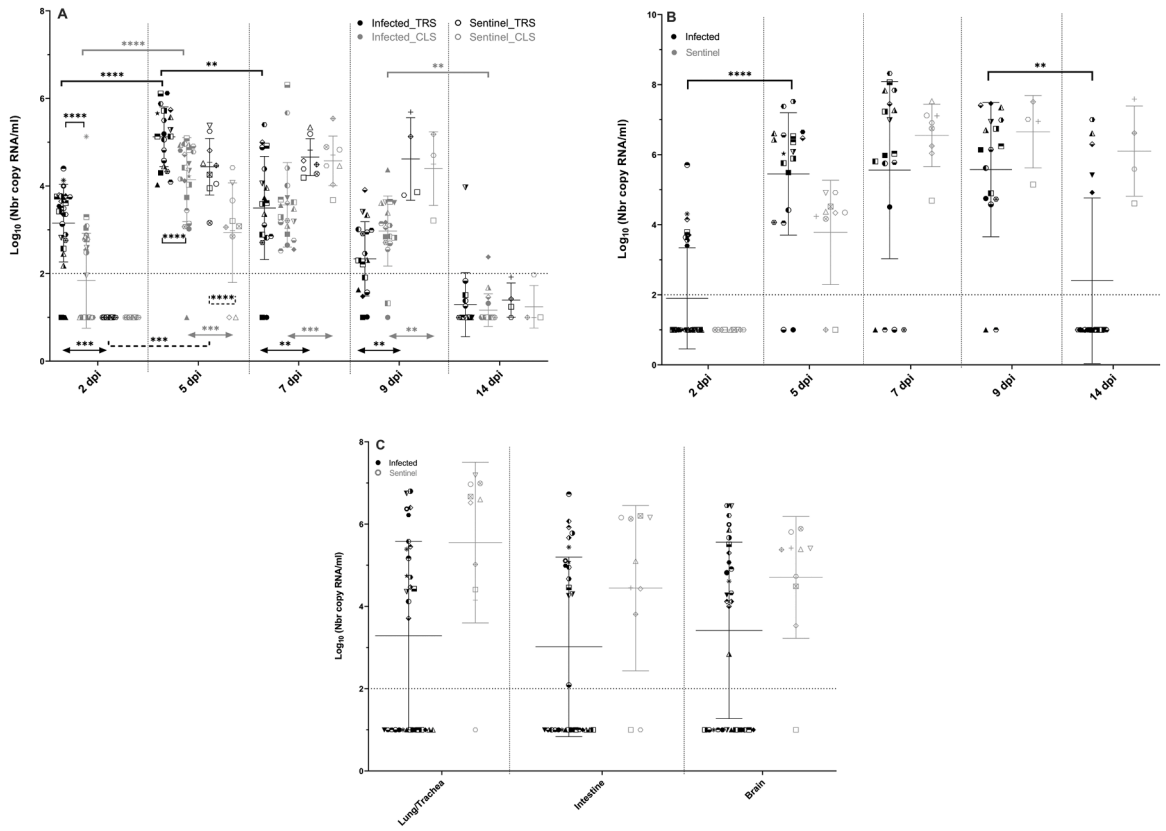


Fig. 5. (A) Viral RNA excretion by infected and sentinel vaccinated commercial field broiler chickens at different time points after infection, by the respiratory and gastrointestinal tract. (B) Viral presence in the feathers over time after infection taken from live birds. (C) Viral presence at time of death or the end of the experiment (14 dpi) in the organs of infected and sentinel birds, determined by RT-qPCR.

The results of this study confirm prior research demonstrating that poultry vaccines featuring genotypes significantly divergent from circulating velogenic strains can mitigate clinical signs and mortality caused by ND while not entirely halting viral shedding (Kapczynski and King, 2005; Miller et al., 2009; Xiao et al., 2012; Sultan et al., 2020). These findings suggest a risk of silent circulation in standard vaccinated layer flocks and highlight the overall poor predictive value of ND serological HI titers in regards to heterologous protective efficacy against viral shedding.

In the experiment involving fully vaccinated field commercial broiler chickens, the low seroconversion rate can most likely be attributed to vaccine neutralization due to the presence of residual maternally derived passive antibodies transmitted from vaccinated dams to their progeny at the time of vaccination. As previously described, high levels of maternally derived antibodies can neutralize vaccine antigens, reducing the ability of live vaccines to infect cells, replicate and produce more viral antigens (Murr et al., 2020; Dimitrov et al., 2021). This results in a lower uptake of infected cells by antigen-presenting cells and leaves only a small fraction of antigens for B cell differentiation and affinity maturation, thus reducing vaccine efficacy (Hamal et al., 2006; Bertran et al., 2018; Hu et al., 2020). The results of this experiment suggest that maternally derived antibodies interfered with the primo-vaccination at 1 day-old, leading to unsuccessful priming of immunity. Additionally, the proportion of seroconverted birds further decreased towards the time of the challenge. This could indicate that the second vaccination might have been hampered by residual MDAs as well, leading to suboptimal vaccine uptake and ultimately weak levels of humoral immunity boosting. Interestingly, ELISA values followed an opposite trend as they raised from 4 to 5 weeks of age. While the HI test focuses on anti-HA antibodies measurement, the selected ELISA kit measures the entire anti-NDV antibody population, likely with an emphasis on nucleoprotein (NP)-specific antibodies, as NP is the most abundantly expressed protein in NDV particles (Nath et al., 2020). This may suggest that the second vaccination induced a partial mounting of humoral immunity of antibodies measured by ELISA, but not yet by HI.

These low serological results translated into poor protection against infection, morbidity, mortality, and viral excretion for both infected and sentinel birds. As indicated by the increase in ELISA-positive birds between 4 and 5 weeks of age, it is possible that a later challenge might have yielded better results. However, given that modern commercial broiler chickens have a production cycle of 6 weeks, the primary goal of prophylactic measures is to ensure adequate protection for birds at earlier life stages, particularly as maternally-derived immunity wanes to negligeable levels around 3 weeks of age (Gharaibeh and Mahmoud, 2013; Bertran et al., 2018; Martinez et al., 2018; Tickle et al., 2018). Possibly, a challenge at 3-week-old might lead to higher survival rates due to improved virus neutralization.

As observed for layers, no clear link between humoral seroconversion and clinical protection could be identified, as 9 out of 30 challenged broilers were protected despite no or very limited ND seroconversion at the time of challenge. Possibly, specific T-cell immune responses can explain the limited protection. Indeed, as the chickens were vaccinated twice with live vaccines, cell-mediated as well as local mucosal immunity should not be overlooked (Rauw et al., 2009; Yan et al., 2011). Even though some challenged birds did not demonstrate clinical signs throughout the observation period, all broilers excreted virus at some time point. Viral excretion was primarily observed through the respiratory route at the onset of infection, with higher viral excretion via the gastrointestinal route noted towards the end of the observation period, contrasting with observations in layers. In broiler chickens, viral shedding was more prevalent than systemic viral presence, whereas the opposite trend was observed in layers.

Current research suggests that while there is no definitive evidence linking the severity of poultry diseases to compromised pulmonary defenses, genetic manipulation of poultry breeds for rapid growth and high productivity, combined with intensive farming practices, may weaken these defenses (Maina, 2023). Research has described a negative correlation between body weight and antibody titer in White Leghorn layers and in multiple broiler lines such as Ross 508. While layer-type chickens have also been selectively bred, this selection appears to have had a lesser impact on their immune systems compared to broilers (Koenen et al., 2002). While these factors may help understand the differences in viral excretion patterns between layer and broiler chickens, further research is still needed.

In the course of this experiment, feathers were evaluated as an alternative sampling method to swabbing. The results confirm a previous report on subgenotype VII.2 that feathers may be an ideal diagnostic matrix for APMV-1 as the virus appears to accumulate rather than peaking and disappearing as it does in swabs (Steensels et al., 2020). Similar results were observed in domestic ducks, waterfowls, and wild swans in the scope of highly pathogenic avian influenza virus (H5N1) infection (Yamamoto et al., 2008a; 2008b, 2009, 2010). The drawbacks of this sampling method is that, from 15 weeks of age, reduced vascularization in feathers makes it challenging to apply in long-living birds, while it is also not considered less invasive for the bird than swabbing (Turcu et al., 2023).

This study, while confirming previous observations, provides valuable insight into the current situation in Western Europe, where confidence in vaccination protocols may lead to complacency in surveillance efforts. While the current NDV vaccination program provides clinical protection in layers, it may allow silent transmission of exotic genotypes in vaccinated flocks. This highlights the need for improved surveillance and a revision of vaccine regulations. This research further supports that suboptimal protection in layers is due to poor antigenic matching between vaccine and circulating strains, rather than improper vaccination procedures, suggesting the need for vaccine updating. In vaccinated broiler birds, protection gaps stem from maternal antibody interference, emphasizing the need for new vaccination strategies to enhance early immunity.

These findings highlight the need for emergency vaccination strategies based on virus genotype matching, as well as adapted vaccination protocols to safeguard poultry throughout their life cycle.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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