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Evaluation of an indirect ELISA to study the specific humoral immune response of Muscovy ducks (*Cairina moschata*) and domestic geese (*Anser anser var. domestica*) after vaccination against Newcastle disease virus

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Abstract

In this study an indirect NDV enzyme-linked immunosorbent assay (ELISA) for waterfowl was evaluated concerning its efficiency and its suitability to monitor the antibody response in Muscovy ducks (*Cairina moschata*) and domestic geese (*Anser anser var. domestica*) following vaccination with a commercial inactivated NDV vaccine for chickens. Already three weeks after vaccination seroconversion was evident in the ELISA. Comparison of the ELISA results with those of the haemagglutination inhibition (HI) test provided a positive linear correlation between both tests (Pearson's product-moment correlation; $r = 0.652$; $p\text{-value} < 0.001$). However, a discrepancy of test results was evident in week seven and ten, with ten sera of vaccinated birds evaluated negative by HI test but positive by ELISA. Eight of these sera were confirmed to yield avian paramyxovirus (APMV) specific reactivity by Western blot (WB) analysis.

Relative diagnostic sensitivity (rDSe) and specificity (rDSp) were determined to be 100.0 % and 91.7 % for the ELISA, compared to 91.1 % and 97.2 % for the HI test. Thus, the established ELISA represents a suitable alternative to the HI test in the monitoring of the immune response of waterfowl after vaccination, particularly for the analysis of high sample numbers. Further on, the results emphasize the immunogenicity of the inactivated NDV vaccine in domestic geese and Muscovy ducks.

Introduction

Newcastle disease (ND) is one of the most important viral diseases of commercial poultry. The highly contagious character and fatal course with subsequent restrictions on trade result in an enormous economic impact on the poultry industry worldwide. The infectious agent, avian paramyxovirus serotype 1 (APMV-1), belongs to the genus *Avulavirus* of the subfamily *Paramyxovirinae* in the family *Paramyxoviridae* (Lamb *et al.*, 2005). Apart from APMV-1 other serotypes (APMV 2-9) are described, but are of minor importance.

Clinical manifestation of APMV-1 infection is highly variable, ranging from inapparent infection to severe disease with high morbidity and mortality. Whereas chickens and turkeys are highly susceptible to the clinical disease, waterfowl is known to usually overcome infection even with highly virulent APMV-1 strains, without showing any symptoms (Bolte *et al.*, 2001; Alexander *et al.*, 2003).

Although birds like geese and ducks are considered more resistant, they bear the risk to harbour and shed APMV-1 for a prolonged period of time (Winmill & Haig, 1961; Vickers & Hanson, 1982) and velogenic NDV could be transmitted to chickens, put into contact with infected geese or with their droppings (Wan *et al.*, 2004).

In addition, ND outbreaks affecting geese in China are reported and a “goose” strain induced disease in experimentally infected geese, with clinical signs like depression, anorexia, white diarrhoea, ocular or nasal discharges and deaths after variable periods of time (Wan *et al.*, 2004).

Possible preventive measures to reduce the risk of transmission of APMV-1 from inapparently infected geese and ducks to more susceptible domestic birds may include, next to indispensable biosecurity measures, vaccination of domestic waterfowl. Immunisation of

commercial meat type geese by conjunctival route, with a live La Sota type vaccine indicated that geese do not readily respond to vaccination with live lentogenic strains (Bolte *et al.*, 2001).

However, application of an inactivated oil emulsion NDV vaccine, alone or 10 weeks after initial immunisation with the live vaccine, ameliorated intensity and duration of antibody response.

Immunogenicity of an inactivated La Sota vaccine in geese is corroborated by a recent vaccination study (Dai *et al.*, 2008), which demonstrated complete protection 21 but not 140 days after immunisation. In this study best results, concerning immune response and duration of protection, were achieved using live La Sota vaccine priming, followed by a booster vaccination with a mesogenic strain (Muktsewar) 2-3 weeks later. Next to an efficient vaccine or vaccination regime, the subsequent control of the immunisation program with a suitable test system is of fundamental importance.

The HI test, used as current serological standard test in waterfowl, is laborious and relatively subjective in its evaluation. Indirect enzyme-linked immunosorbent assays (ELISA) in contrast, as commercially available for chickens and turkeys (Office International des Epizooties, 2008), can be standardised more easily, have the advantage of a possible high degree of automation, concerning the operation and evaluation process, and are thus suitable for a high sample throughput.

The aim of this study was to evaluate an indirect NDV ELISA for waterfowl (Kothlow *et al.*, 2008), concerning its potency in comparison with the HI test as conventional standard test method as well as its suitability to monitor the antibody response after vaccination.

Materials and Methods

Sera. APMV-1 positive waterfowl sera were derived from immunised adult Muscovy ducks (*Cairina moschata*) and adult domestic geese (*Anser anser var. domestica*) that received an inactivated APMV-1 vaccine for chickens (Nobilis Newcavac®) subcutaneously. Depending on the dosage, ducks as well as geese were divided into two groups. The first group of 9 ducks and 11 geese was immunised with the single chicken dose (0.5 ml; 1×), the second one of 9 ducks and 7 geese with the two-fold chicken dose (1.0 ml; 2×) at the start of the trials. Sera obtained before immunisation and at the indicated time points after vaccination were stored at -20°C. They were initially tested by HI test and ELISA. All questionable sera, i.e. sera with positive reactivity before vaccination as well as sera from vaccinated birds with negative or differing results in HI test and ELISA were additionally analysed by Western blot. Only those samples that yielded a sufficient amount of serum for the performance of all tests were included in analysis of the test systems. Therefore, the numbers of sera can vary in part between the different time points of sampling.

Enzyme-linked immunosorbent assay. The ELISA was performed as described previously (Kothlow *et al.*, 2008). All sera of the immunisation study were tested in parallel. Briefly, Polysorp Immuno Plates (Nunc) coated with sucrose step-gradient purified antigen of APMV-1 strain La Sota (5 µg/ml) (Lamontagne *et al.*, 1975) were incubated for 30 minutes with the test sera, diluted 1:200 in blocking buffer. To reduce nonspecific binding, test sera were pre-treated with negative chicken embryo amnio-allantoic fluid for one hour at room temperature, before they were added to the wells. After removal of the test sera and three successive washing steps, monoclonal antibody 14A3 (mab 14A3), directed against the duck immunoglobulin light chain (Kothlow *et al.*, 2005), was added (1:10 in blocking buffer). Incubation took place at room temperature for 30 minutes. Plates were washed again and horseradish peroxidase-conjugated

goat-anti-mouse immunoglobulin (Ig)-specific polyclonal antibodies (Sigma) (1:250 in blocking buffer) were added for half an hour. Subsequent to the washing procedure, plates were incubated in darkness with the substrate (*ortho*-phenyldiamidihydrochloride; Sigma) [1 mg/ml] in citrate buffer, containing 0.15 % v/v hydrogen peroxide. The reaction was stopped after ten minutes with sulphuric acid [2M] and optical density (OD) was measured at 490 nm. For the evaluation of the ELISA the cut off was defined as the arithmetic mean of the OD of all sera taken before vaccination, plus the threefold standard deviation.

Haemagglutination inhibition test. The HI test was performed according to standard protocols (Office International des Epizooties, 2008). Titres are defined as the highest serum dilution that inhibited haemagglutination and are expressed as \log_2 of the reciprocal values. HI end-points of four or greater were considered positive.

Western blot analysis. Sucrose step-gradient purified APMV-1 antigen was subjected to 10 % glycine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis (glycine-SDS-PAGE) under denaturing conditions ((Laemmli, 1970) using a preparative comb of a minigel system (Peqlab, Biotechnologie GMBH, Erlangen, Perfect Blue, 10x10 cm) with 1.67 μ g purified APMV-1 antigen per mm running front of the gel. Subsequently proteins were transferred to nitrocellulose (NC) membranes (Amersham Biosciences) by semi-dry blotting. Membranes were blocked for 60 minutes with 2 % w/v skim milk in phosphate-buffered saline (PBS, pH 7.4) containing 0.1 % Tween20, cut into stripes (2-3 mm) and stored at -20°C until their utilisation. Test sera were pre-incubated for one hour with negative chicken embryo amnio-allantoic fluid in a dilution of 1:2, to diminish unspecific reactivity, centrifuged for 10 minutes at 8000 rpm (Heraeus Biofuge pico, rotor 3325 B, 6077xg) and then brought to a final dilution of 1:1000 with TBST buffer (Tris-

buffered saline: 50 mM Tris, pH 7.5; 0.1 % Tween20) plus 3 % skim milk (TBST-M buffer). NC-strips were then incubated overnight with the diluted test sera at 4°C, on a shaker. After three 5-min washes with TBST buffer, mab 14A3 cell culture supernatant was added at a dilution of 1:100 in TBST-M buffer. Following 30 minutes of incubation at room temperature on a shaker and three subsequent washing steps, stripes were covered with horseradish peroxidase-conjugated goat-anti-mouse Ig-specific polyclonal antibodies (Sigma), at a dilution of 1:5000 in TBST-M buffer. They were incubated for another 30 minutes at room temperature under agitation. After three further washing steps, membranes were finally covered for five minutes with a horseradish peroxidase substrate (ECL plus Western Blotting Detection System; Amersham Biosciences) and reaction was visualised by autoradiography on a medical X-ray film (AGFA Curix HT 1.000 G+).

Statistical evaluation. The relative diagnostic sensitivity (rDSe) and specificity (rDSp) of the established ELISA in comparison to different reference tests and the vaccination status, were calculated following standard procedures (Miller *et al.*, 1991).

Statistical calculations and diagrams were designed using R project, a free software environment for statistical computing and graphics (www.r-project.org). To determine the significance of correlation the Pearson's product-moment correlation coefficient was used. To test the significance of differences between the ELISA and the HI test, the Wilcoxon rank-sum test was performed (Sachs, 2004). HI titres smaller than 2 are, for the sake of completeness, contained only as virtual values ($\log_2 = 0.5$) in Figures 1 and 3. They are not taken into account for regression and not included in the evaluation of the significance of correlation and the significance of differences between ELISA and HI test. The level of significance ($\alpha = 0.05$) was

corrected, when necessary, with the help of the Bonferroni correction and in the following is given behind the calculated p-value.

Blood samples taken from ducks before application of the 1× vaccine dose were used for the 1x as well as the 2× dosage group, to evaluate the differences in reactivity between sera taken before and after vaccination.

The sample size, necessary to determine the success of vaccination in a flock, was estimated with the help of Win Episcope 2.0 (<http://www.clive.ed.ac.uk/winepiscope/>).

Results

Test sera were derived from adult Muscovy ducks and domestic geese, immunised with either a 1× or 2× chicken dose of an inactivated APMV-1 vaccine. All animals were tested negative before vaccination by HI test (Figure 1 B and D). By ELISA one serum lay marginally above the cut off (Figure 1 A). Three weeks after vaccination birds of both dosage groups exhibited an APMV-specific antibody response, detected by HI test (Figure 1 B and D) as well as ELISA (Figure 1 A and C). The APMV-specific reactivity was still evident seven and ten weeks after immunisation. Concerning the ELISA values, significant differences between sera taken before vaccination and those taken three, seven and ten weeks thereafter were calculated (Wilcoxon rank-sum test; highest p-value of all tests ≤ 0.0021 ; $I_s = 0.0083$).

In the group receiving the 1× chicken dosage, the highest median of the HI titres was obtained three weeks after immunisation for geese as well as ducks, and then decreased continuously seven weeks and ten weeks after vaccination (Figure 1 B). Three, seven and ten

weeks post vaccination, 89.5 % (n: 17/19), 73.7 % (n: 14/19) and 66.7 % (n: 12/18) respectively of birds vaccinated with the 1× chicken dosage were identified NDV-positive by HI test.

By ELISA, the median of the initial antibody reactivity, three weeks after immunisation with the 1× chicken dose, seemed weak compared with later time points (Figure 1 A). Nevertheless, 84.2 % of the vaccinated birds (n: 16/19) were determined APMV-positive at that time. The highest median of the ELISA-reactivity was obtained seven weeks after vaccination, with 94.7 % positive reactants (n: 18/19), but declined 10 weeks after immunisation, when 83.3 % (n: 15/18) of vaccinated birds were detected ELISA-positive.

In the group receiving the 2× chicken dosage, visible HI- and ELISA-reactivity was measured three weeks after vaccination, but had dropped seven weeks after immunisation. However in both, domestic geese and Muscovy ducks, a second increase in antibody response became evident ten weeks after vaccination. Three weeks after immunisation the HI test identified 93.8 % (n: 15/16) of the vaccinated birds APMV-positive. The same birds were seropositive by ELISA. Seven weeks after vaccination 78.6 % of the animals were tested positive by HI test (n: 11/14), while the ELISA identified 100.0 % as positive (n: 14/14). At the time of the second increase in antibody response, ten weeks after immunisation, both tests achieved positive results in 100.0 % (14 out of 14) of vaccinated birds. At that time point the highest median of HI titres as well as ELISA OD-values was measured. Statistical evaluation revealed that for geese the increase in antibody response ten weeks after immunisation was significant by HI test (Wilcoxon rank-sum test; p-value ≤ 0.0019 ; Is = 0.01666) as well as by ELISA (Wilcoxon rank-sum test; p-value = 0.00058; Is = 0.0083), whereas for ducks the increase was only significant by HI test and only from week seven to week ten (Wilcoxon rank-sum test; p-value = 0.002; Is = 0.01666). Statistical comparison of each of the three time points after vaccination between the two dosage groups revealed that the antibody response 10 weeks after vaccination

for both, the ELISA as well as the HI test, was significantly higher in the 2× dosage group (Wilcoxon rank-sum test; p -value ≤ 0.0041 ; $ls = 0.05$).

Testing repeatability of the ELISA, using the same sera under the same working conditions, provided comparable results (Figure 2). Statistical evaluation by calculation of the correlation coefficient “ r ” and the p -value revealed a positive linear correlation (r) of 0.982 (Pearson’s product-moment correlation; p -value < 0.001 ; $ls = 0.05$).

Comparison of ELISA-reactivity and HI titres (Figure 3) also proved a positive linear correlation of the established ELISA with the HI test as the current serological standard test (Pearson’s product-moment correlation; $r = 0.652$; p -value < 0.001 ; $ls = 0.05$). Similar correlations could be determined, when statistical analysis was performed separately for sera of the different time points after vaccination (Pearson’s product-moment correlation; week three: $r = 0.619$; $p < 0.001$; week seven: $r = 0.774$; $p < 0.001$; week ten: $r = 0.699$; $p < 0.001$). Striking was the observation that sera with the same HI titre yielded different ELISA values. For example, several sera collected seven weeks after vaccination, with an HI titre of seven, varied by their ELISA OD-values between 0.74 and 2.03 (Figure 3). Analysing these sera by WB confirmed APMV-specific reactivity (Figure 4). In accordance with the ELISA results, the sera showed differences, concerning the strength and pattern of proteins recognised. Especially sera with high ELISA-reactivity resulted in stronger bands and additional reactivity with the matrix protein (M).

To determine efficacy of the ELISA to monitor the antibody response, the results were compared more detailed to those of the HI test, as serological standard test (Table 1). Using the HI test as reference resulted in a relative diagnostic sensitivity (rDSe) of 98.8 % and a relative diagnostic specificity (rDSp) of 74.4 % for the ELISA. Analysed separately for the different time points, the rDSe was continuously 100.0 %, with the exception of week three after immunisation (96.9 %), when one of the 32 HI test positive sera was evaluated negative by ELISA. The rDSp

was 96.2 % before vaccination and 100.0 % three weeks thereafter, but poor in week seven (12.5 %) and ten (50.0 %) following immunisation, when a total of ten sera was determined positive only by ELISA. However, WB analysis verified APMV-specific antibodies in eight of these ten sera. Therefore, all questionable sera were additionally analysed by WB and rDSe as well as rDSp of ELISA and HI test were subsequently determined, using the HI test in combination with the WB results as reference (Table 1). In this calculation, the ELISA yielded a higher rDSe (100.0 %) than the HI test (91.1 %), with most abundant differences at week seven and ten after vaccination. Concerning the rDSp, the HI test was overall more specific (ELISA: 91.7 %; HI test: 97.2 %). The rDSp of the ELISA was particularly weak seven weeks after vaccination, when two of three WB negative sera yielded a false positive reactivity in the ELISA. However, in week three and ten the ELISA reached with 100.0 % an equal or even higher specificity than the HI test. Furthermore, it is interesting that seven sera from vaccinated birds were negative by HI test, ELISA and WB analysis. Therefore, statistical evaluation of the results on the basis of the vaccination status was done, assuming that after successful ND vaccination all birds should be APMV-positive (Table 1). This evaluation provided a higher diagnostic sensitivity for the ELISA (92.0 %) than for the HI test (83.0 %). Calculated separately for the different time points, the diagnostic sensitivity of the ELISA was lowest in week three, that of the HI test in week seven and ten after vaccination. Concerning the diagnostic specificity, the HI test surpassed the ELISA with 100.0 % to 96.0 %.

Discussion

In this study, an indirect whole virus ELISA for waterfowl was evaluated, concerning its potency in comparison with the HI test as conventional test-method and its suitability to monitor the antibody response of domestic geese and Muscovy ducks after immunisation with an APMV-1 vaccine.

Statistical comparison of ELISA results with those of the HI test, performed in parallel, provided a positive linear correlation between both tests. However, in the course of seroconversion some differences became evident, especially within the 1× dosage group. Here the highest median of the HI titres is reached already three weeks after vaccination, whereas ELISA results provide a more moderate increase, with the highest median determined seven weeks after immunisation. A similar time dependent discrepancy between HI test and ELISA has been observed for the detection of antibodies to a haemagglutinating duck adenovirus (Duck adenovirus 1, DAdV-1) in chickens (Piela & Yates, 1983): While all hens developed significant HI antibody titres already seven days after inoculation, they were negative by an indirect ELISA at that time. It was suggested that this phenomenon was due to different test methods: IgG as well as IgM, which dominates the first two weeks of a primary immune response, are able to agglutinate viral particles (Keller, 1994; Kuby, 1997) and therefore are likely to be measured by the HI test. The ELISA of the above study however, was limited to the detection of IgY, the functional equivalent to mammalian IgG. As the monoclonal antibody 14A3, directed against the light chain of anamid serum IgY, is capable to detect IgY, IgA and presumably also binds to IgM (Kothlow *et al.*, 2005), the above explanation is unlikely for the established NDV ELISA for waterfowl.

However, the different test methods of HI test and ELISA implicate that antibodies to different antigenic determinants of the virus are measured in each assay. While the former test specifically detects antibodies directed against epitopes of the haemagglutinin neuraminidase

(HN) protein of APMV-1 (Kaleta, 1992), the ELISA measures a broad spectrum of antibodies specific for different viral proteins (Charan *et al.*, 1981; Snyder *et al.*, 1983). Onset and amount of antibody production against the different proteins of NDV might vary with the time after vaccination. For example this phenomenon is known for hepatitis C virus (HCV) and bovine respiratory syncytial virus (BRSV) infections: Antibody responses to non-structural proteins of HCV were detected earlier and were of greater magnitude than those to structural proteins (Netski *et al.*, 2005). In infections with BRSV, the G antibody titres declined significantly more rapidly than the F antibody titres (Schrijver *et al.*, 1996). Therefore referring to our study, positive NDV ELISA-values at a time point, when none or only a low HI titre is measured, can indicate the single or dominating presence of antibodies directed against viral proteins other than the HN protein. This consideration is corroborated by WB analysis. Eight of ten ELISA positive but HI test negative and therefore questionable sera from week seven and ten after vaccination could be verified APMV-positive by WB analysis, whereas the single HI test positive but ELISA negative serum of a vaccinated goose was determined negative. In addition, WB analysis revealed that even sera taken at the same time point after vaccination, from different individuals can show variations in virus protein specific antibody pattern. This phenomenon points to the collateral occurrence of individual variations in the antibody pattern or the antibody kinetic. Differences in decline of antibody titres in individual animals are also mentioned for BRSV (Schrijver *et al.*, 1996).

Thus, our study indicates that time dependent dominance of antibodies against different viral proteins may be of diagnostic relevance. The WB analysis proves that differences in the antibody pattern lead to a greater number of false negative results in the HI test than in the ELISA. Especially at later time points after virus contact the ELISA provides a higher detection level than the HI test, which is advantageous in surveillance programs. Several other working

groups suggested that the ELISA detects more NDV seroreactants than the HI test (Snyder *et al.*, 1983; Hlinak *et al.*, 1992), implying that the immunoassay has a higher methodical sensitivity in the detection of NDV-specific antibodies.

RDS_e and rDS_p calculated in our study taking the vaccination status or, instead of the HI test alone, a combination of HI test and WB as reference reflect these facts and underline the effect of early and late immune reaction on the efficiency and correlation of ELISA and HI test. In a former study, subsequent to a comparative evaluation of ELISA and HI test to detect immunity against measles virus, it was concluded that the HI test should not be used as a reference method for evaluation of the sensitivity of ELISA IgG kits, because of the different antigenic determinants of the virus involved in each assay (Duvdevani *et al.*, 1996). The rDS_p of the established NDV ELISA for ducks and geese in both cases lay under that of the HI test, but reached 91.7 % taking HI test and WB as reference.

The overall aim of this study was to analyse the suitability of the ELISA, to monitor the antibody response of domestic geese and Muscovy ducks after subcutaneous immunisation with an inactivated APMV-1 vaccine for chicken, administered in two different doses. Results indicate that the established ELISA distinguishes clearly and in a reproducible manner between sera of vaccinated and non-vaccinated birds. 96.2 % of the birds were tested APMV seronegative by ELISA before vaccination and 92.0 % of birds were determined APMV seropositive after vaccination. ELISA evaluation demonstrates that the inactivated APMV-1 vaccine for chicken induces a measurable antibody response in domestic geese and Muscovy ducks. Seroconversion became evident three weeks post vaccination in both dosage groups and persisted until week ten after immunisation. These results confirm the good immunogenicity of inactivated NDV vaccines not only in domestic geese, as already described (Bolte *et al.*, 2001), but also in Muscovy ducks. Moreover, the humoral immune response was stronger and longer lasting in domestic geese and

Muscovy ducks receiving the 2× chicken dose, with a significantly higher reactivity ten weeks after vaccination compared to the 1× dosage group. In addition, the majority of non-responders (six of seven) and a higher number of sera with no HI titre and low ELISA-reactivity (seven of ten) were found in the 1× dosage group (Figure 3). Considering these facts, it is recommendable to immunise waterfowl with the two-fold chicken dosage. Nevertheless, vaccine efficacy yet has to be verified by challenge experiments.

All in all, the presented ELISA would allow monitoring the antibody response in geese and ducks after immunisation with an inactivated NDV vaccine on a routine basis. Diagnostic sensitivity and specificity as obtained in our study can, in addition, be used for the statistical verification of test regimes. On the basis of a population of 1000 vaccinated birds and a sensitivity of the ELISA of 92.0 %, we can propose that with a level of confidence of 95.0 % a sample size of 29 birds has to be tested positive, to be certain that the vaccination induced antibodies. For the HI test a sample size of 52 birds would have to be tested positive, due to a correspondent sensitivity of 83.0 %. These facts indicate that the established ELISA would be more suitable to give a yes-no-answer concerning the humoral immune response after vaccination.

In conclusion, the established ELISA with its high diagnostic sensitivity, especially concerning later time points after vaccination, and its only marginally lower diagnostic specificity, represents an adequate alternative to the HI test, particularly for the analysis of high numbers of samples from vaccinated geese and ducks. Whether the ELISA may be suitable as diagnostic test in epidemiological monitoring programs or not has to be evaluated. In combination with serotyping by HI test of ELISA seroreactants, this would allow a more detailed analysis of the APMV-1 seroprevalence in waterfowl.

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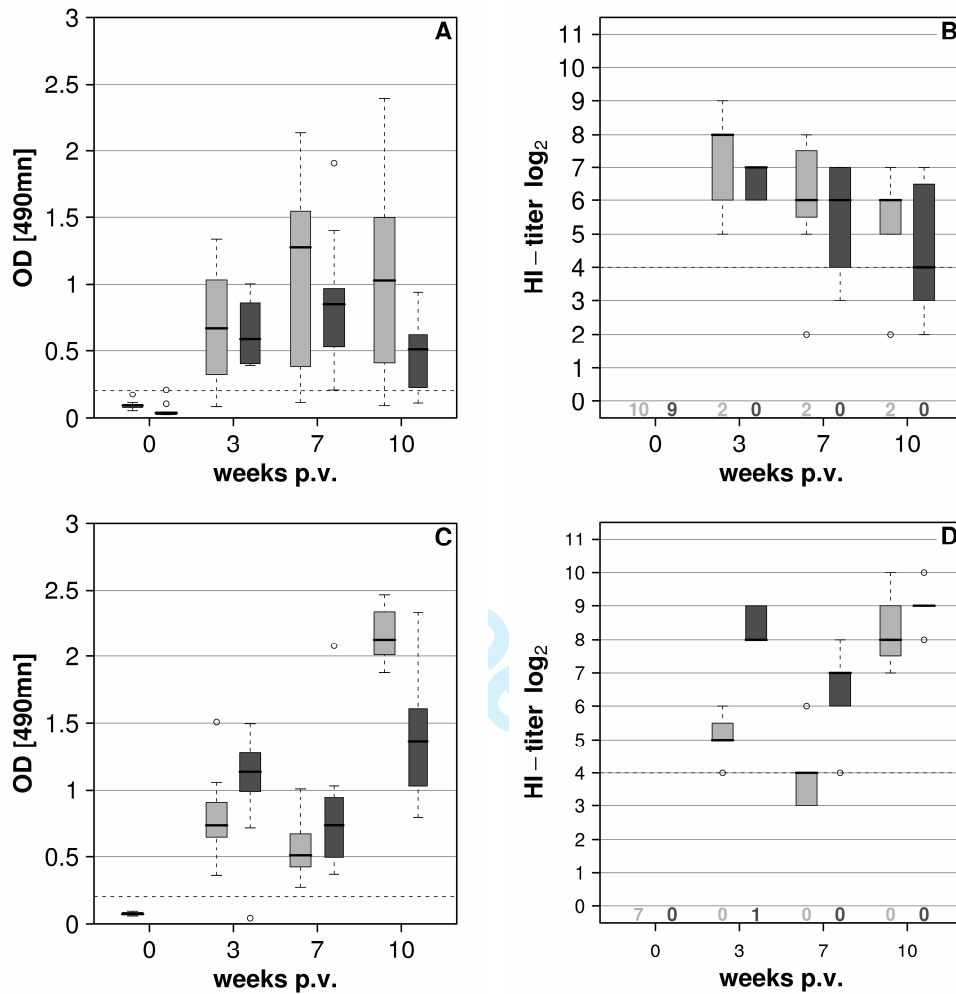


Figure 1. Antibody response of ducks and geese after ND-vaccination. Sera from domestic geese and Muscovy ducks, taken at different time points after immunisation with an APMV-1 vaccine, were tested in parallel by ELISA (A,C) and HI test (B,D). Shown are results of geese (□) and ducks (■) vaccinated with a single (A and B) or a two-fold (C and D) chicken dose. The Box Plot evaluation provides the median (bolded line), the lower and upper hinge (25 % and 75 % quartile, respectively), the extreme of lower and upper whisker, as well as the outliers (○). The

number of HI \log_2 titres smaller than 2, at the different time points, is mapped above the x-axis.

Thresholds for the tests (HI-titre > 4, ELISA-OD > 0,207) are given as dashed lines.

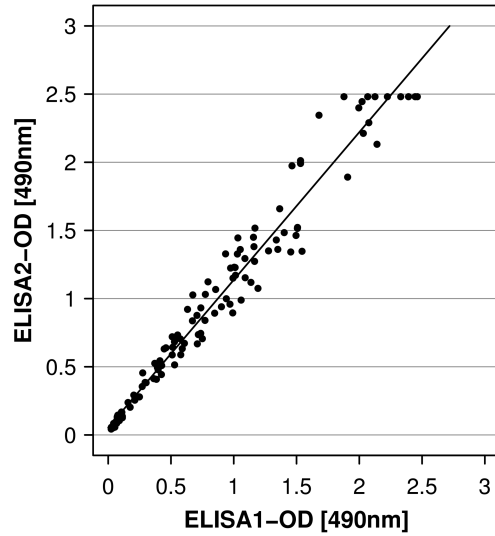
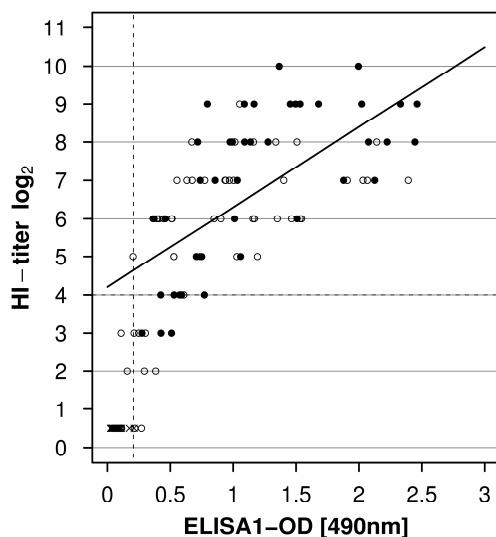


Figure 2. *Repeatability* of the APMV-ELISA. Sera from domestic geese and Muscovy ducks, immunised with an APMV-1 vaccine for chickens, were analysed by ELISA twice, under the same working conditions. Sera of both dosage groups and all sample dates are included. Shown is the comparison of the obtained OD-values by regression.

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Figure 3. Correlation of ELISA- and HI test-results. Sera of adult domestic geese and Muscovy ducks from an immunisation study with an APMV-1 vaccine for chickens were analysed in parallel by ELISA and HI-test. The diagram includes sera obtained at the day of vaccination (×) as well as three, seven and ten weeks thereafter, from animals immunised with the single (0,5 ml; ○) and the twofold (1,0 ml; ●) chicken dose respectively. Sera with HI titres smaller than 1 are for the sake of completeness included as virtual values ($\log_2 = 0.5$), but are not taken into account for the correlation. Thresholds for the tests are given as dashed lines.

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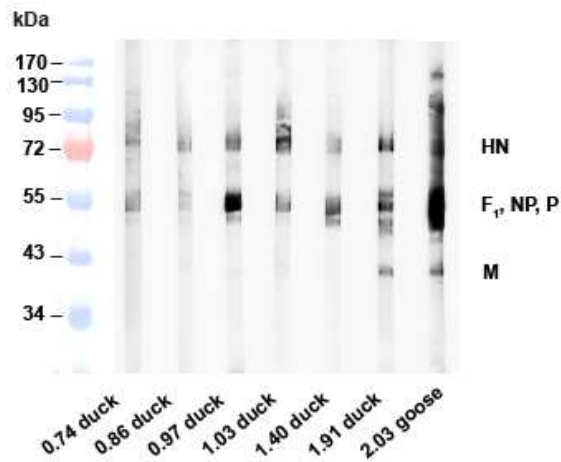


Figure 4. Western blot analysis of sera with the same HI titre but different ELISA-reactivity.

Seven sera taken seven weeks after vaccination with an HI titre of seven but differing ELISA-reactivity were investigated by WB analysis. Reactivity is shown after an exposure time of 10 seconds. (HN = haemagglutinin-neuraminidase, P = phosphoprotein, NP = nucleoprotein, F₁ = fusion protein 1, M = matrix protein).

Table 1. Statistical parameters calculated by taking the HI-test, HI-test and WB, or the vaccination status as reference standard.

	weeks post vaccination									
	0		3		7		10		total	
	n ^a = 26		n = 35		n = 33		n = 32		n = 126	
	+	-	+	-	+	-	+	-	+	-
ELISA	1 ^b	25	31	4 ^b	32	1 ^b	29	3 ^b	93	33
HI-test	0	26	32	3 ^b	25	8 ^b	26	6 ^b	83	43
WB	0	26	31	4	30	3	29	3	90	36
HI-test as standard:										
ELISA										
Sensitivity	100.0%		96.9%		100.0%		100.0%		98.8%	
Specificity	96.2%		100.0%		12.5%		50.0%		74.4%	
HI test and WB as standard:										
ELISA										
Sensitivity	100.0%		100.0%		100.0%		100.0%		100.0%	
Specificity	96.2%		100.0%		33.3%		100.0%		91.7%	
HI-test										
Sensitivity	100.0%		100.0%		83.3%		89.7%		91.1%	
Specificity	100.0%		75.0%		100.0%		100.0%		97.2%	
Vaccination status as standard:										
ELISA										
Sensitivity	96.2%		88.6%		97.0%		90.6%		92.2%	
Specificity	96.2%		88.6%		97.0%		90.6%		92.2%	
HI-test										
Sensitivity	100.0%		91.4%		75.8%		81.3%		83.0%	
Specificity	100.0%		91.4%		75.8%		81.3%		83.0%	

^a n = number of birds tested
^b sera verified by WB analysis

Supplemental information, *Statistical parameters to Figure 1.*

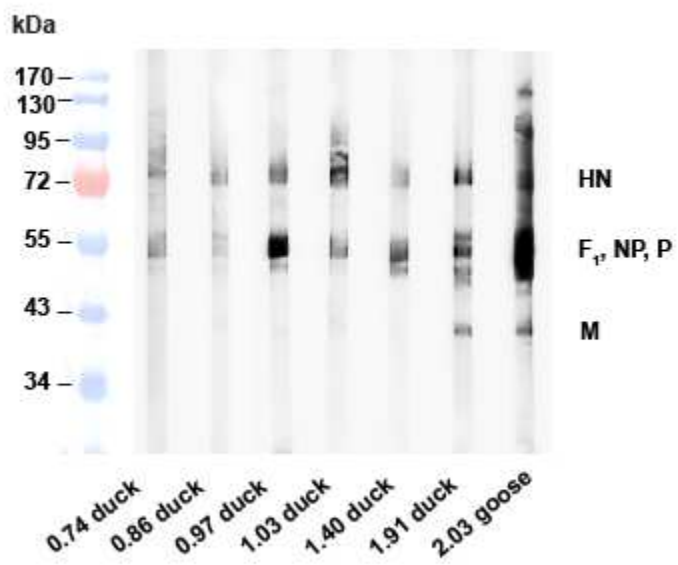
	ELISA				HI test			
	weeks post vaccination				weeks post vaccination			
	0	3	7	10	0	3	7	10
single chicken dose:								
ducks								
median	0.037	0.593	0.848	0.510	7	6	4	
extreme of lower whisker	0.024	0.392	0.212	0.109	6	3	2	
extreme of upper whisker	0.044	1.004	1.402	0.935	7	7	7	
geese								
median	0.091	0.671	1.272	1.029	8	6	6	
extreme of lower whisker	0.053	0.083	0.113	0.090	5	5	5	
extreme of upper whisker	0.113	1.339	2.141	2.392	9	8	7	
two-fold chicken dose:								
ducks								
median		1.137	0.738	1.366	8	7	9	
extreme of lower whisker		0.718	0.371	0.795	8	6	9	
extreme of upper whisker		1.496	1.033	2.330	9	8	9	
geese								
median	0.072	0.737	0.510	2.125	5	4	8	
extreme of lower whisker	0.058	0.363	0.275	1.878	5	3	7	
extreme of upper whisker	0.093	1.059	1.011	2.462	6	4	10	

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	ELISA				
	weeks post vaccination				w
	0	3	7	10	0
single chicken dose:					
ducks					
median	0.037	0.593	0.848	0.510	
extreme of lower whisker	0.024	0.392	0.212	0.109	
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HI test		
Weeks post vaccination		
3	7	10

7	6	4
6	3	2
7	7	7
8	6	6
5	5	5
9	8	7

8	7	9
8	6	9
9	8	9
5	4	8
5	3	7
6	4	10

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