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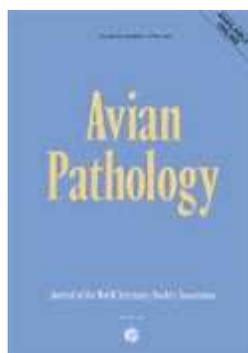
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Investigations on the protective role of passively transferred antibodies against avian Metapneumovirus (aMPV) infection in turkeys

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Abstract

The Avian Metapneumovirus (aMPV) is the causative agent of an acute respiratory disease in turkeys, which causes considerable economic losses to the poultry industry. Currently attenuated live and inactivated vaccines are widely used to control the disease, but vaccine breaks are frequently observed. For improvement of current vaccination strategies it is necessary to gain enhanced knowledge of the immune mechanisms against aMPV infection. Field observations suggest, that vaccine induced aMPV-specific antibodies are not indicative for protection. In this study we investigated the role of antibodies in protection of turkeys against aMPV. In two experiments commercial turkey poult received aMPV-specific antibodies by intravenous injection. The antibody transfer resulted in increased antibody levels in the sera. Virus-specific antibodies were also detected on mucosal surfaces such as trachea, conjunctivae and gall bladder. Turkeys were subsequently challenged with a virulent aMPV subtype A strain. Development of clinical signs, virus detection by polymerase chain reaction (PCR) and histopathological changes of tracheal mucosa in challenged turkeys with and without passively transferred antibodies were comparable with each other. Our results suggest that humoral immunity does not provide sufficient protection against aMPV-infection. Thus, the measurement of vaccine-induced aMPV antibody response may not be considered as an adequate indicator of vaccine efficacy. Further research on the protective role of cell-mediated immune mechanisms is necessary to improve current vaccine strategies

Introduction

The Avian Metapneumovirus (aMPV) is a negative sense, single stranded RNA-virus and a member of the subfamily of *Pneumovirinae* within the family of *Paramyxoviridae* (Gough, 2003).

Since its first detection in South Africa in the late 1970s (Buys *et al.*, 1980), the virus has been found in many countries worldwide with the exception of Australia (Gough, 2003). To date four subgroups (A to D) have been classified based on the nucleotide sequence of the attachment (G) protein gene (Juhász & Easton, 1994; Bâyon-Auboyer *et al.*, 2000; Toquin *et al.*, 2006). In Europe subgroups A and B are the dominating subtypes (Cavanagh *et al.*, 1997; Cavanagh *et al.*, 1999; Catelli *et al.*, 2004; Catelli *et al.*, 2006a).

aMPV replicates in the epithelium of the upper respiratory tract of a number of gallinaceous bird species (Gough *et al.*, 1988; Majo *et al.*, 1995). In susceptible hosts the virus causes an acute disease referred to as Turkey Rhinotracheitis (TRT) in turkeys or Avian Rhinotracheitis (ART) in other bird species. The disease is characterized by respiratory symptoms such as sneezing, ocular discharge and swelling of the infraorbital sinus (Gough, 2003).

Virus replication in the respiratory epithelium results in influx of lymphoid cells and mucosal damage such as epithelial desquamation and loss of ciliary activity (Majo *et al.*, 1995; Liman & Rautenschlein, 2007). A systemic immunosuppression has been proposed as an additional consequence of aMPV-infection by several authors, demonstrated by reduced *ex vivo* mitogen response of leukocytes (Chary *et al.*, 2002a), inhibited phytohemagglutinin skin test response (Timms *et al.*, 1986) and impaired efficacy of Hemorrhagic Enteritis Virus (HEV) vaccination (Chary *et al.*, 2002b). By these means aMPV-induced disease supports establishment and manifestation of secondary respiratory infections in chickens and turkeys, as experimentally demonstrated for a number of bacterial pathogens (Naylor *et al.*, 1992; Van

de Zande *et al.*, 2001; Marien *et al.*, 2005; Van Loock *et al.*, 2006). aMPV is therefore considered as a cause of major economic losses to the turkey industry worldwide (Gough, 2003).

Current vaccination regimes against aMPV infection are mainly based on attenuated live or inactivated vaccines and have proven to be useful tools for the control of the disease (Jones, 1996). Nevertheless, they often do not provide sufficient protection, resulting in field infections of vaccinated flocks, and they also remain to have considerable drawbacks. Mild disease caused by residual virulence of attenuated live vaccines has been reported, as well as reversion to full virulence after several passages of vaccine strains in turkeys or chickens (Cook *et al.*, 1989a; Naylor & Jones, 1994; Catelli *et al.*, 2006b). The necessity for parenteral application of inactivated vaccines makes them inconvenient for use in large commercial poultry operations. Parenteral application of vaccines is also thought to fail to induce local and cell-mediated immune mechanisms on respiratory surfaces (Sharma *et al.*, 2002; Sharma *et al.*, 2004). Therefore efforts to overcome these problems by development of recombinant and subunit vaccines are increasing (Qingzhong *et al.*, 1994; Kapczynski & Sellers, 2003; Kapczynski, 2004; Chary *et al.*, 2005; Liman *et al.*, 2007). The development of new and improved vaccines and vaccination regimes depends on a broadened knowledge of the immune mechanisms responsible for protection against aMPV infection and disease development. The role of aMPV-specific antibodies in protection against challenge infection is not well known. Field observations as well as experimental results suggest only a poor correlation between vaccine induced serum antibody levels and actual protection of the flock (Cook *et al.*, 1989b; Sharma *et al.*, 2004; Kapczynski *et al.*, 2008). In experimental studies even high levels of maternally derived antibodies did not prevent virus replication and clinical disease (Naylor *et al.*, 1997), nor did they interfere with development of protection induced by attenuated live vaccines (Cook *et al.*, 1989b).

In this study we examined the protective effect of passively transferred antibodies against experimental aMPV-infection. Naïve turkeys received a defined amount of aMPV-specific antibodies by intravenous injection. The antibody distribution was measured in serum and on mucosal surfaces. Turkeys with and without transferred aMPV-specific antibodies were subsequently challenged with a homologous virulent aMPV subtype A strain. The development of the disease and the course of virus clearance were compared.

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Material & Methods

Turkeys. Day-old commercial Big 6 turkey poults, which were negative for maternal aMPV-antibodies, were obtained from a commercial hatchery and housed on wood shaving litter in positive pressure isolation units of the Clinic for Poultry, University of Veterinary Medicine, Hannover, following animal welfare guidelines. Water and commercial feed were provided *ad libitum*.

aMPV strains. The virulent aMPV subtype A strain BUT 8544 (Wilding *et al.*, 1986) was kindly provided by Prof. R. C. Jones, Liverpool, UK. The strain was propagated and titrated in chicken tracheal organ culture (TOC) following standard protocols (Cook *et al.*, 1976). Titres were calculated as median ciliostatic doses (CD₅₀) by the method of Reed & Muench (1938).

A subtype A strain attenuated to chicken embryo fibroblasts (CEF) was kindly provided by Prof. E. F. Kaleta, Gießen, Germany and used in the virus neutralization test (VNT). This strain, designated BUT/CEF, originates from strain BUT 8544. This strain was propagated and titrated on CEF cultures and titres were calculated as median culture infectious doses (CID₅₀) by the method of Reed & Muench (1938). A commercially available inactivated aMPV vaccine, also based on a CEF-adapted BUT 8544 strain, was used for the preparation of anti-aMPV hyperimmune turkey sera.

Antibody preparations. Turkey serum free of detectable aMPV-specific antibodies was collected from turkey poults reared under isolated conditions. For the production of anti-aMPV hyperimmune serum three male turkeys were inoculated with virulent BUT 8544 at the age of 8 weeks. At the age of 11, 13, 15 and 17 weeks they received booster vaccinations with a commercially available inactivated aMPV subtype A vaccine by intramuscular injection.

One week after the last booster vaccination all turkeys were sacrificed. Their serum was harvested and stored at -70°C until further use. Virus neutralizing (VN) log-2 titres of the individual hyperimmune sera ranged from 7.0 to 9.6.

The turkey sera were heat inactivated at 56°C for 30 minutes in a water bath. Total Immunoglobulin (Ig) concentration was increased by ammonium sulphate precipitation. Briefly, serum was supplemented stepwise with an equal volume of saturated ammonium sulphate (SAS) solution to achieve a final ammonium sulphate saturation of fifty percent. The proteins were precipitated under continuous stirring at 4°C for 6 hours. Precipitated proteins were pelleted by centrifugation at $3,000 \times g$ for 30 min. and resuspended in PBS, pH 7.4 (0.2-fold the original serum volume). Antibody preparations were dialysed thoroughly against PBS, pH 7.4 to remove the ammonium sulphate. The resulting concentrated antibody preparations obtained from the hyperimmune sera (aMPV-Ab+) had VN log-2 titres of 10.3 (Exp. 1) and 9.0 (Exp. 2). Preparations from aMPV-antibody free turkey sera (aMPV-Ab-) were confirmed to be free of aMPV-specific antibodies by VNT and ELISA.

As described previously for chicken sera, a 50% saturated ammonium sulphate solution precipitates the three poultry Ig-isotypes IgG, IgM and IgA (Lebacq-Verheyden *et al.*, 1974).

Sample collection for aMPV antibody detection. The following samples were collected for serological examination: serum, lacrimal fluid, bile and tracheal washings.

Bile was taken at necropsy from the gall bladder, centrifuged at $10,000 \times g$ for 10 min and the supernatant was harvested. For the collection of tracheal washings 10 cm of the middle part of the trachea were removed and washed with $500 \mu\text{l}$ PBS, pH 7.4. The collected washings were vortexed thoroughly and centrifuged at $10,000 \times g$ for 10 min to remove mucus. Blood contaminated samples were excluded from further analysis. Lacrimal fluid was collected with filter papers (6 mm discs, Schleicher & Schüll, Dassel-Einbeck / Germany),

which were carefully placed underneath the eyelid of the turkey for several seconds until the discs were completely soaked. The discs, containing 24 μ l fluid, were stored in 400 μ l ELISA dilution buffer (BioChek, Gouda / The Netherlands), resulting in an 18-fold dilution of the sample. All samples were stored at -20°C until further use.

Serology. aMPV-specific IgG antibodies were detected by Avian Rhinotracheitis Antibody Test Kit (BioChek, Gouda / Netherlands) following the manufacturers' instructions. The different samples were diluted in the provided dilution buffer as follows: serum 500-fold (Exp. 1 & 2), tracheal washings 5-fold (Exp. 1 & 2), bile 10-fold (Exp. 1) and lacrimal fluid 18-fold (Exp. 1 & 2). ELISA-results are presented as sample to positive control (S/P) ratios. VN antibodies were detected by VNT as previously described (Baxter-Jones *et al.*, 1989). Briefly, replicates of 50 μ l of two-fold dilution series of serum samples or tracheal washings were generated in 96-Well cell culture plates and incubated for one hour at 37°C with 50 μ l medium containing 100 CID_{50} of aMPV-strain BUT/CEF. Subsequently 100 μ l medium containing 7.5×10^4 CEFs was added to each well. After incubation for 7 days at 37°C and 5% CO_2 atmosphere, cytopathic effects were recorded. VN titres were calculated using the method of Reed & Muench (1938).

Detection of aMPV by RT-PCR. RNA was isolated from choanal swabs with 500 μ l Trifast GOLD (Peqlab, Erlangen / Germany) per sample according to the manufacturers' instructions. The RT reaction was performed using the ImProm-II© RT system (Promega, Madison / USA) according to the manufacturer's directions with random primers (Invitrogen, Karlsruhe / Germany). The first PCR step was performed with primers G6- (5'-CTGACAAATTGGTCCTGATT-3'), G1+A (5'-GGGACAAGTATCTCTATG-3') and G1+B (5'-GGGACAAGTATCCAGATG-3') (Cavanagh *et al.*, 1999) with the following thermocycler profile: initial denaturation at 94°C for 5 min, 30 cycles of denaturation at 94°C

for 20 sec, primer annealing at 54°C for 45 sec and prolongation at 72°C for 45 sec, followed by a final prolongation step at 72°C for 10 min. Subsequently a nested PCR was performed with primers G5- (5'-CAAAGAA/GCCAATAAGCCCA-3'), G8+A (5'-CACTCACTGTTAGCGTCATA-3') and G9+B (5'-TAGTCCTCAAGCAAGTCCTC-3') (Cavanagh et al., 1999). The thermal profile setup was similar to that of the previous step with the exception of a shorter prolongation step of only 35 sec. For both PCRs 2 µl sample obtained from the previous step were added to the reaction mix to reach a final volume of 25 µl. All primers were used at a final concentration of 200 nM. SAWADY Taq-DNA-Polymerase (Peqlab, Erlangen / Germany) was used for both PCRs at an amount of 0.625 units per reaction.

PCR products were separated by agarose gel electrophoresis and visualized by ethidium bromide staining and ultraviolet transillumination.

Clinical score. Clinical signs were recorded as individual scores per animal. A score of 0 (no signs) to 3 (severe signs) was assigned to each of the following respiratory symptoms: nasal exudate, ocular discharge and infraorbital swelling (Table 1). The sum of these scores resulted in a total score of 0 to 9 for every turkey.

Histopathology. Trachea samples were fixed in 10% phosphate-buffered formalin and embedded in paraffin. Tissue sections were stained with haematoxylin and eosin (H&E). The identity of the sections was blinded before analysis by light microscopy. Mononuclear and heterophilic infiltrations of the mucosa and deciliation and desquamation of respiratory epithelial cells were considered as conspicuous patho-histological lesions (Majo *et al.*, 1995; Liman & Rautenschlein, 2007).

Experiment 1. Eighty-three 14-day-old commercial female Big-6 turkey poults, which were confirmed to be free of maternal antibodies against aMPV, were randomly assigned to four groups of 18 to 24 turkeys. Birds of two groups (AC-1, 24 birds and AV-1, 18 birds) were intravenously inoculated with aMPV-specific antibodies by two consecutive injections of 1.4 ml of the concentrated aMPV-specific antibodies (aMPV-Ab+) at days 14 and 15 of life. The two remaining groups (CC-1, 23 birds and CV-1, 18 birds) received a similar treatment with the the aMPV-Ab negative preparation (aMPV-Ab-). Turkeys of groups CV-1 and AV-1 were oculonasally inoculated with 10^3 CD₅₀ of the virulent aMPV-strain BUT 8544 per bird fifteen minutes after the second antibody injection. Groups CC-1 and AC-1 received virus-free TOC-supernatant. At the same time, five and six turkeys of groups CC-1 and AC-1, respectively, were sacrificed for necropsy. Six turkeys of each group were sacrificed at days 5, 9 and 14 post inoculation (pi). At necropsy tracheal washings and bile samples were collected for serological examination, and samples of the middle part of the trachea were taken for histopathological examination. In addition choanal swabs were taken for aMPV-genome detection and serum and lacrimal fluid was collected for antibody detection. Choanal swabs and serum samples were collected immediately before the first antibody injection, fifteen minutes after the second antibody injection and at days 1, 3, 5, 7, 9, 11 and 14 pi (n = 6-10). Lacrimal fluid was collected fifteen minutes after the second antibody injection and at days 5, 9 and 14 pi (n = 6-10). The experiment is summarized in Table 2.

Experiment 2. A second experiment was conducted with a comparable experimental design. For this experiment 31 female, 18-day-old commercial Big-6 turkey poults, were divided into four groups of 6 to 10 turkeys. The turkeys were free of maternally derived anti-aMPV antibodies, but different to Exp. 1 they had been spray vaccinated with an attenuated aMPV subtype B vaccine at the hatchery. Birds of groups AC-2 (8 birds) and AV-2 (10 birds) were intravenously inoculated with anti-aMPV-antibodies by two consecutive injections of 2 ml

aMPV-Ab+ each at days 18 and 19 post hatch, whereas groups CC-2 (6 birds) and CV-2 (7 birds) received aMPV-Ab-. Turkeys of groups CV-2 and AV-2 were oculonasally inoculated with $10^{2.7}$ CD₅₀ per bird of the virulent aMPV-strain BUT 8544 fifteen minutes after the second antibody injection. At the same time three and five turkeys of groups CC-2 and AC-2, respectively, were sacrificed for necropsy. The remaining turkeys of all groups were sacrificed at day 9 pi. At necropsy tracheal washings were collected for antibody detection and samples of the middle part of the trachea were taken for histopathological examination. Choanal swabs and serum samples were collected from all turkeys immediately before the first antibody injection, fifteen minutes after the second antibody injection and at days 1, 3, 5, 7 and 9 pi. Lacrimal fluid was collected from all turkeys 15 minutes after the second injection and at days 3 and 9 pi. The experiment is summarized in Table 2.

Statistical analysis. Statistical analysis of ELISA and VNT results was performed with Statistix 7.0 software, using One-way analysis of variants (ANOVA) and comparison of means by Tukey Test. *P*-values of $P < 0.05$ were considered to indicate significant differences.

Results

Serum antibody levels. aMPV-specific serum antibodies were detected by ELISA and VNT (Fig. 1). In Exp. 1 turkeys were free of anti-aMPV antibodies at the beginning of the experiment. Turkeys of Exp. 2 had low levels of VN antibodies before the passive antibody transfer, presumably due to the vaccination with the aMPV subtype B vaccine. No ELISA IgG antibodies were detected in this experiment before the passive immunization.

In both experiments significantly increased anti-aMPV ELISA and VN antibody titres ($P<0.05$) were detected in sera of groups AC-1, AV-1 (Exp. 1) and AC-2, AV-2 (Exp. 2) after the second antibody injection at the time of virus-inoculation. The antibody levels declined gradually in group AC-1 and AC-2 over the following days, but levels of group AC-1 were still significantly higher than in the unchallenged control group CC-1 at day 14 pi ($P<0.05$). aMPV-challenged birds without passively transferred anti-aMPV antibodies (CV-1) developed significantly increased ELISA antibody levels starting at day 9 pi in Exp. 1 (Fig. 1A; $P<0.05$). In turkeys with passively transferred aMPV-specific antibodies (AV-1 and AV-2) ELISA-IgG levels did not significantly increase after challenge infection. Starting at day 7 pi the aMPV-inoculated groups CV-1, AV-1 (Exp. 1) and CV-2, AV-2 (Exp. 2) showed significantly enhanced virus neutralizing antibody titres compared to the respective uninfected groups in both experiments (Fig. 1C & D; $P<0.05$).

Antibody levels on mucosal surfaces. Anti-aMPV IgG antibodies were measured by ELISA in lacrimal fluids (Fig. 2), tracheal washings (Fig. 3A & B) and bile (Fig. 4). In tracheal washings also VN antibodies were measured (Fig. 3C & D). VNT was not performed on lacrimal fluid and bile samples.

Fifteen minutes after the second antibody injection significantly enhanced ($P<0.05$) aMPV-specific ELISA antibody levels were detected in lacrimal fluids of groups AC-1, AC-2

and AV-1 (Fig. 2) and bile of group AC-1 (Fig. 4). Statistical analysis of tracheal washing ELISA results was not possible at day 0 pi in Exp. 1 due to a high number of blood contaminated samples. However, at day 5 pi ELISA IgG levels in tracheal washings were significantly higher in group AC-1 compared to group CC-1 (Fig. 3A, $P<0.05$). A significant increase of VN antibodies after the antibody transfer was not observed in tracheal washings (Fig. 3C & D). Throughout both experiments antibody levels of group AC-1 and AC-2 declined gradually on all mucosal surfaces. However, ELISA S/P-ratios of lacrimal fluid in group AC-1 were still significantly higher than those of group CC-1 at day 14 pi (Fig. 2A; $P<0.05$). Following aMPV-challenge of birds without passively transferred aMPV-specific antibodies (CV-1), ELISA and VN antibody responses were significantly increased at day 9 pi in all mucosal samples compared to the unchallenged group CC-1 ($P<0.05$). These levels had already partially declined by day 14 pi. In contrast to this, aMPV-challenge of antibody positive birds (AV-1 and AV-2) did not result in significantly increased antibody levels compared to the respective unchallenged group AC-1 or AC-2 in any sample type tested.

Clinical signs. Clinical signs were recorded daily throughout the experiments using a scoring system (Table 1). In Exp. 1 virus-inoculated turkeys developed respiratory symptoms, such as nasal and ocular discharge and swelling of the infraorbital sinus. Symptoms were first observed at day 3 pi and were most severe at days 6 and 7 pi. Clinical signs had completely vanished by day 12 pi. Both infected groups (CV-1 and AV-1) showed comparable symptom development (Fig. 5).

In Exp. 2 aMPV-challenge infection of vaccinated turkeys did not result in considerable clinical signs. Individual birds from both challenged groups expressed mild nasal exudation or watery eyes, resulting in low peak clinical mean scores of 0.43 and 0.60 at day 6 pi in groups CV-2 and AV-2, respectively (data not shown). No clinical signs were observed

in the uninfected groups CC-1, AC-1 (Exp. 1) and CC-2, AC-2 (Exp. 2) throughout both experiments.

Detection of aMPV by RT-PCR. aMPV was detected from choanal swabs by a subtype specific RT-PCR (Cavanagh *et al.*, 1999). In Exp. 1 all samples collected from the aMPV-inoculated groups (CV-1 and AV-1) were positive for aMPV subtype A between day 3 and 7 pi. Detection rates declined starting at day 9 pi in both challenged groups. At day 14 pi only one sample from group CV-1 was aMPV-positive by RT-PCR (Fig. 6A).

In Exp. 2 aMPV detection rates in the aMPV-inoculated groups were much lower than in Exp. 1 (Fig. 6B). The overall incidence of aMPV subtype A positive swabs was similar in both challenged groups (CV-2: 20 %; AV-2: 22 %).

Swabs from uninfected groups (CC-1, CC-2, AC-1, AC-2) as well as samples collected before aMPV challenge were negative for aMPV subtype A throughout both experiments (data not shown). In Exp. 2 aMPV subtype B genome was sporadically detected in all groups.

Histopathology. Samples of the middle part of the trachea were prepared for histological examination. In both experiments mild lesions, such as lymphoid and heterophilic infiltration of the mucosa and epithelial deciliation and desquamation, were observed in all aMPV-inoculated groups up to day 9 pi. In the first experiment 33 to 50 % of aMPV-inoculated turkeys with (AV-1) or without (CV-1) passively transferred aMPV-specific antibodies showed mucosal lesions at days 5 and 9 pi (Table 3). At day 14 pi the lesions had completely declined in both groups. In Exp. 2 fourteen to 30 % of groups CV-2 and AV-2, respectively, showed mild mucosal lesions in the trachea at day 9 pi. No histopathological changes were observed in turkeys not challenged with aMPV subtype A (groups CC-1, CC-2, AC-1 and AC-2) throughout the experiments.

Discussion

The presented study was designed to investigate the role of antibodies in protection of turkeys against aMPV infection and disease development. For this purpose formulations of concentrated aMPV-specific antibodies were intravenously transferred to 14- to 18-day-old turkey poults in two experiments. The treatment resulted in increased anti-aMPV antibody titres in sera and on different mucosal surfaces, detected by IgG-specific ELISA and VNT. Following challenge with a virulent aMPV subtype A strain, turkeys with and without passively transferred aMPV-specific antibodies showed no difference in development of clinical signs, histopathological lesions and frequency of virus detection.

Increased anti-aMPV ELISA and VN antibody levels were detected in the sera following passive antibody transfer and remained significantly higher than those of control groups for up to two weeks. Following two intravenous injections, anti-aMPV IgG was also detected by ELISA on the mucosal surfaces of the respiratory tract in tracheal washings and lacrimal fluid as well as in gall bladder fluid. This is in agreement with previous studies conducted with turkeys and chickens, in which intravenously administered IgG was transferred to the mucosal surfaces of conjunctivae and trachea within five to ten minutes (Toro *et al.*, 1993; Suresh & Arp, 1995). Due to low sample numbers and high titre variations the increase of VN antibodies in tracheal washings after passive immunisation was not significant. The antibody precipitation method used in this study does generally precipitate all poultry Ig-isotypes, IgA, IgG and IgM (Lebacq-Verheyden *et al.*, 1974). Thus all antibodies present in the hyperimmune turkey sera were likely to be transferred to the passively immunized birds. However, IgG may be more effectively transported from serum to the respiratory surfaces than IgA and IgM, leading to detectable ELISA titres, but possibly not to significantly increased VN antibodies.

Experimental aMPV-infections of turkeys and chickens in this and other studies showed, that detection of VN antibodies in the serum occurs earlier and is of shorter duration, than antibody titres detected by IgG-specific ELISA (Baxter-Jones *et al.*, 1989; Aung *et al.*, 2006; Liman & Rautenschlein, 2007). This suggests that not IgG, but other immunoglobulin isotypes confer the major neutralizing activity against aMPV in poultry.

Virulent aMPV challenge infection of turkeys without aMPV-specific antibodies resulted in a peak of VN and ELISA antibody response at day 9 pi, which had already markedly declined at day 14 pi. Virus induced ELISA IgG antibodies were detectable at the site of virus replication in respiratory secretions. aMPV-specific IgG was also detectable in bile after aMPV-inoculation. Previous studies with aMPV subtype C have also demonstrated the induction of aMPV-specific IgA in bile (Cha *et al.*, 2007). The role of these antibodies in aMPV-infection and disease control is not clear.

Antibodies induced by spray vaccination of day-old turkeys were barely detectable in Exp. 2, which is in congruence with previous experiences obtained from vaccinated day-old chicks (Ganapathy & Jones, 2007). In all sample types antibody levels achieved by antibody transfer at the time of aMPV challenge were comparable to or even higher than peak antibody responses induced by aMPV challenge infection in this study. This indicates that antibody levels achieved by passive transfer in this model should be considered to be protective, if antibodies were a major protective mechanism against aMPV.

However, our results revealed that, despite the presence of high levels of local and circulating antibodies, turkeys were not protected against oculonasal challenge with $10^{2.7}$ or 10^3 CD_{50} of a homologous aMPV-A strain. Development of clinical scores, frequency of virus detection and histopathological lesions in the trachea were comparable in challenged turkeys with and without transferred anti-aMPV antibodies in both experiments. Turkeys of Exp. 2 developed comparably milder signs of aMPV-induced disease than observed in Exp. 1. This may be attributable to partial protection obtained by spray vaccination with a heterologous

subtype B vaccine in the hatchery. Passively transferred antibodies did not provide additional protection.

Our results support previous observations that serum antibodies acquired after vaccination were not indicative for actual protection (Cook *et al.*, 1989b; Williams *et al.*, 1991a; Williams *et al.*, 1991b; Sharma *et al.*, 2004; Kapczynski *et al.*, 2008). Naylor *et al.* (1997) also demonstrated that maternally derived antibodies were not protective against challenge with a low dose of virulent aMPV. However, it is not known whether the maternally derived antibodies were directed against the same aMPV subtype as the virulent strain used for challenge infection. Also neutralizing activity of the maternal antibodies and their presence at the sites of infection were not subject of the study, leaving open questions about the possible reasons for the observed lack of protection. In our experiments all strains used for production of hyperimmune serum and for challenge infection originated from the same aMPV-A strain BUT 8544 (Wilding *et al.*, 1986).

Failure of maternally derived or passively transferred antibodies to give full protection against infection and disease has already been demonstrated for other members of the subfamily of *Pneumovirinae*, such as Bovine Respiratory Syncytial Virus (BRSV), human Respiratory Syncytial Virus (hRSV) and human Metapneumovirus (hMPV) in natural and experimental hosts (Kimman *et al.*, 1987; Kimman *et al.*, 1988; Belknap *et al.*, 1991; Larsen, 2000; Alvarez & Tripp, 2005). Although the severity of the disease was often reduced in the seropositive individuals, distribution and replication of the virus were barely affected. Explanations for this lack of protection and the role of other immune mechanisms have to be subject of further research.

Ciliated cells of the respiratory epithelium are considered to be the major target cells for replication of the *Pneumovirinae* subfamily. It has been reported that BRSV and hRSV additionally possess the ability to replicate in leukocytes, such as monocytes and alveolar macrophages (Panuska *et al.*, 1990; Midulla *et al.*, 1993; Schrijver *et al.*, 1995; Sharma &

Woldehiwet, 1996). *In vitro* experiments of Goris *et al.* (2009) in primary bovine lung organ cultures demonstrated, that BRSV initially replicates in subepithelial cells, possibly dendritic cells, whereas the respiratory epithelium was infected only at high inoculation titres. Preliminary results of Sharma *et al.* (2004) indicated, that aMPV-replication in macrophages may also play a role in the pathogenesis of aMPV in turkeys. Antibody binding may promote uptake of the virus by macrophages. Replication in phagocytosing cells would enable the virus to spread to neighbouring cells via cell-to-cell fusion without antibodies gaining access to the virus particles. Thus in the initial phase of the infection the virus may need to be controlled by other immune mechanisms than humoral immunity, including cytotoxic T-lymphocytes or T-helper cell activities.

The idea of cell mediated immune mechanisms rather than antibodies playing the major role in the protection of turkeys against aMPV-infection gets additional support by various studies on attenuated live vaccines, which are offering good protection against infection and disease despite the absence of detectable vaccine-induced seroconversion (Cook *et al.*, 1989b; Williams *et al.*, 1991b). In studies of Jones *et al.* (1992) experimentally B-cell-compromised and vaccinated turkeys developed full protection against aMPV-infection.

Cell-mediated immune mechanisms are known to play an important role in the protection of chickens against other viral pathogens of the respiratory tract, such as Infectious Bronchitis Virus (IBV; Seo & Collisson, 1997; Seo *et al.*, 2000) or Newcastle disease virus (NDV; Al-Garib *et al.*, 2003). IBV-specific T-lymphocytes of infected chickens have been detected via cytotoxicity assay (Seo & Collisson, 1997), and transfer of IBV-specific T-lymphocytes to naïve chicks resulted in protection against virulent challenge infection (Seo *et al.*, 2000). NDV-specific T-cell activity has been quantified by *ex vivo* recall stimulation of leukocytes and subsequent detection of antigen-induced interferon gamma production via ELISA, ELISPOT or intracellular cytokine staining (Lambrecht *et al.*, 2004, Ariaans *et al.*, 2008). However, methods to detect aMPV-specific T-lymphocytes in poultry and especially in

turkeys remain to be developed. The effect of T-lymphocytes on aMPV-specific immunity have to be target of future research.

In summary, the presented study demonstrates that intravenously administered antibodies did not protect turkeys from aMPV-infection and respiratory disease, although they were readily transferred to the mucosal surfaces. In agreement with previous reports, our results show that antibody detection is an insufficient parameter for estimating the degree of protection in vaccinated poultry flocks. Neither the absence of antibodies can be interpreted as lack of protection, nor is the presence of high levels of vaccine-induced serum antibody an indication of protection. The development of new and improved vaccines and control strategies should therefore focus on the induction of local and cell-mediated immune mechanisms. Further research has to focus on broadening the knowledge of the aMPV-pathogenesis and the role of T-lymphocytes in aMPV infection

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Figure legends

Figure 1: *Detection of aMPV-specific serum ELISA IgG (A, B) and VN antibodies (C, D) after intravenous administration of aMPV-antibodies and subsequent inoculation with virulent aMPV in Exp. 1 (A, C) and Exp. 2 (B, D).*

CC-1 and CC-2: turkeys received an aMPV-Ab- formulation and remained aMPV-free; CV-1 and CV-2: turkeys received an aMPV-Ab- formulation and were subsequently challenged with virulent aMPV subtype A; AC-1 and AC-2: turkeys received an aMPV-Ab+ formulation and remained aMPV-free; AV-1 and AV-2: turkeys received an aMPV-Ab+ formulation and were subsequently challenged with virulent aMPV subtype A. Serum samples were collected immediately before the first antibody injection at day -1; samples at day 0 were collected 15 minutes after the second antibody injection. Presented are mean values per group and day with standard deviation. Values marked with different superscript letters at the same experimental day are significantly different from each other (One Way ANOVA and Tukey test, $P < 0.05$); Exp. 1: $n = 6-10$; Exp. 2: $n = 3-10$

Figure 3: *Detection of aMPV-specific ELISA IgG (A, B) and VN antibodies (C, D) tracheal washings (C-F) after intravenous administration of aMPV-specific antibodies and subsequent inoculation with virulent aMPV in two consecutive experiments.*

CC-1 and CC-2: turkeys received an aMPV-Ab- formulation and remained aMPV-free; CV-1 and CV-2: turkeys received an aMPV-Ab- formulation and were subsequently challenged with virulent aMPV subtype A; AC-1 and AC-2: turkeys received an aMPV-Ab+ formulation and remained aMPV-free; AV-1 and AV-2: turkeys received an aMPV-Ab+ formulation and were subsequently challenged with virulent aMPV subtype A. Samples at day 0 were collected 15 minutes after the second injection. Presented are mean values per group and day with standard deviation. Values marked with different superscript letters at the same

experimental day are significantly different from each other (One Way ANOVA and Tukey test, $P < 0.05$); Exp. 1: $n = 6-10$; Exp. 2: $n = 3-10$; ND: not done.

Figure 2: *Detection of aMPV-specific ELISA IgG in lacrimal fluids after intravenous administration of aMPV-specific antibodies and subsequent inoculation with virulent aMPV in two consecutive experiments.*

CC-1 and CC-2: turkeys received an aMPV-Ab- formulation and remained aMPV-free; CV-1 and CV-2: turkeys received an aMPV-Ab- formulation and were subsequently challenged with virulent aMPV subtype A; AC-1 and AC-2: turkeys received an aMPV-Ab+ formulation and remained aMPV-free; AV-1 and AV-2: turkeys received an aMPV-Ab+ formulation and were subsequently challenged with virulent aMPV subtype A. Samples at day 0 were collected 15 minutes after the second injection. Presented are mean values per group and day with standard deviation. Values marked with different superscript letters at the same experimental day are significantly different from each other (One Way ANOVA and Tukey test, $P < 0.05$); Exp. 1: $n = 6-10$; Exp. 2: $n = 3-10$; ND: not done.

Figure 4: *Detection of aMPV-specific ELISA IgG antibodies in turkey bile after intravenous administration of aMPV-specific antibodies and subsequent inoculation with virulent aMPV (Exp. 1).*

CC-1: turkeys received an aMPV-Ab- formulation and remained aMPV-free; CV-1: turkeys received an aMPV-Ab- formulation and were subsequently challenged with virulent aMPV subtype A; AC-1: turkeys received an aMPV-Ab+ formulation and remained aMPV-free; AV-1: turkeys received an aMPV-Ab+ formulation and were subsequently challenged with virulent aMPV subtype A. Samples at day 0 were collected 15 minutes after the second antibody injection. Presented are mean values per group and day with standard deviation. Values marked with different superscript letters at the same experimental day are significantly

different from each other (One Way ANOVA and Tukey test, $P < 0.05$); $n = 6-10$; ND: not done.

Figure 5: *Development of clinical signs after passive transfer of aMPV-antibodies and subsequent inoculation with virulent aMPV (Exp. 1).*

Turkeys were individually examined for clinical signs on a daily base. Results are presented as mean clinical scores; observed symptoms were nasal and ocular discharge and swollen infraorbital sinus. CV-1: turkeys received an aMPV-Ab- formulation and were subsequently challenged with virulent aMPV subtype A; AV-1: turkeys received an aMPV-Ab+ formulation and were subsequently challenged with the same strain. Unchallenged birds of groups CC-1 and AC-1 remained free of respiratory symptoms throughout the experiment. $n = 6-24$

Figure 6. *Detection of aMPV subtype A by RT-PCR from choanal swabs taken after intravenous administration of aMPV-antibodies and subsequent inoculation with virulent aMPV in Exp. 1 (A) and Exp. 2 (B).*

Concentrated aMPV-specific antibodies were injected intravenously at two consecutive days (day -1; 0) and turkeys were subsequently inoculated with a virulent aMPV subtype A strain at day 0 by oculonasal route. CV-1 and CV-2: turkeys received an aMPV-Ab- formulation and were subsequently challenged; AV-1 and AV-2: turkeys received an aMPV-Ab+ formulation and were subsequently challenged. Exp. 1: $n = 6-10$; Exp. 2: $n = 7-10$. In the unchallenged groups CC-1, AC-1 (Exp. 1) and CC-2, AC-2 (Exp. 2) aMPV subtype A was not detected.

Table 1. *Clinical Score*

Nasal exudate (Score 0-3)

- 1 mild, clear nasal exudate; visible after gentle pressure on the nostrils
- 2 nasal exudate, visible before handling
- 3 extreme nasal exudate or exudate with blood

Ocular discharge (Score 0-3)

- 1 watery eyes, single bubbles after handling
- 2 watery eyes, visible bubbles already before handling
- 3 frothy ocular discharge before handling

Swollen infraorbital sinus (Score 0-3)

- 1 unilaterally, mildly swollen infraorbital sinus
 - 2 bilaterally, mildly swollen infraorbital sinuses
 - 3 extremely swollen infraorbital sinus(es) with periorbital swelling
-

The total clinical score (0-9) is composed of three individual scores (0-3) representing the TRT-typical respiratory symptoms nasal exudates, ocular discharge and swollen infraorbital sinus. Score 0 reflects the absence of these signs.

Table 2. *Experimental design*

| Experiment / Group | Passive anti-aMPV antibody transfer at days post hatch | aMPV challenge at day of life (challenge dose) | total number of turkeys | number of birds sacrificed at day pi | | | |
|--------------------|--|--|-------------------------|--------------------------------------|----|----|----|
| | | | | 0 | 5 | 9 | 14 |
| Exp. 1 | 14 & 15 | 15 | 83 | | | | |
| | | (10 ^{3.0} CD ₅₀) | | | | | |
| CC-1 | - ^a | - | 23 | 5 | 6 | 6 | 6 |
| CV-1 | - | + | 18 | ND | 6 | 6 | 6 |
| AC-1 | + ^b | - | 24 | 6 | 6 | 6 | 6 |
| AV-1 | + | + | 18 | ND | 6 | 6 | 6 |
| Exp. 2 | 18 & 19 | 19 | 31 | | | | |
| | | (10 ^{2.7} CD ₅₀) | | | | | |
| CC-2 | - | - | 6 | 3 | ND | 3 | ND |
| CV-2 | - | + | 7 | ND | ND | 7 | ND |
| AC-2 | + | - | 8 | 5 | ND | 3 | ND |
| AV-2 | + | + | 10 | ND | ND | 10 | ND |

^a = serum preparation without specific aMPV-antibodies

^b = serum preparation with specific aMPV-antibodies with titres of VN log-2 of 10.3 (Exp. 1) and 9.0 (Exp. 2).

Exp. = Experiment; ND = not done.

Table 3. *Histopathological lesions in the trachea following aMPV-inoculation of turkeys with and without passively transferred aMPV-specific antibodies*

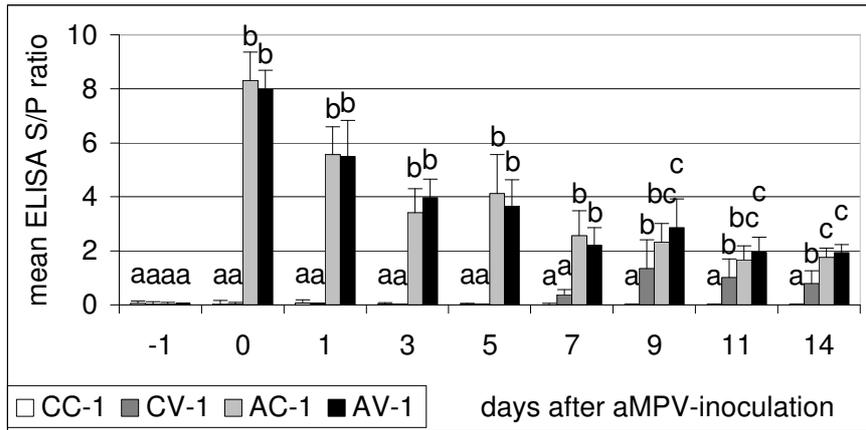
| Group | anti-aMPV antibody transfer | aMPV challenge infection | Number of animals with histological lesions ^a of the tracheal mucosa / total animals (% of positive animals) at day pi. | | | |
|--------|-----------------------------------|--------------------------------|---|------------|-----------|--------------|
| | | | Experiment 1 | | | Experiment 2 |
| | | | 5 | 9 | 14 | 9 |
| CC-1/2 | - ^b | - | 0 / 5 (0) | 0 / 6 (0) | 0 / 6 (0) | 0 / 3 (0) |
| AC-1/2 | + ^c | - | 0 / 6 (0) | 0 / 6 (0) | 0 / 6 (0) | 0 / 3 (0) |
| CV-1/2 | - | + | 2 / 5 (40) | 3 / 6 (50) | 0 / 6 (0) | 1 / 7 (14) |
| AV-1/2 | + | + | 2 / 6 (33) | 2 / 5 (40) | 0 / 6 (0) | 3 / 10 (30) |

^a Observed histopathological lesions were lymphoid infiltration of the mucosa and deciliation and desquamation of respiratory epithelium. Slides were analysed as blinded samples.

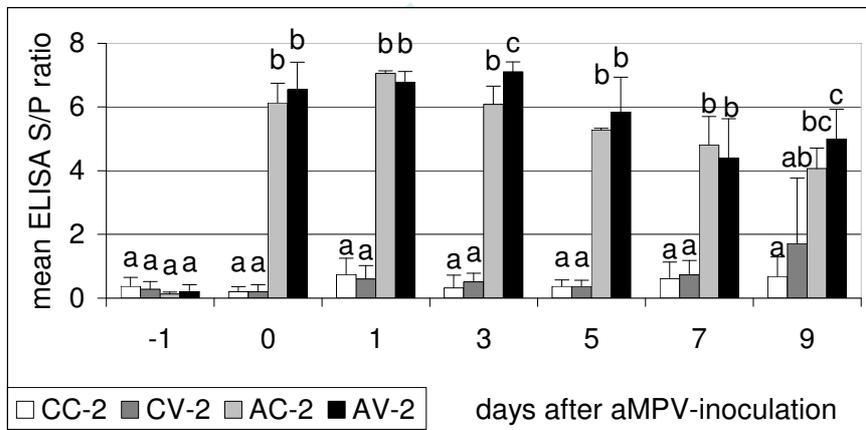
^b negative

^c positive

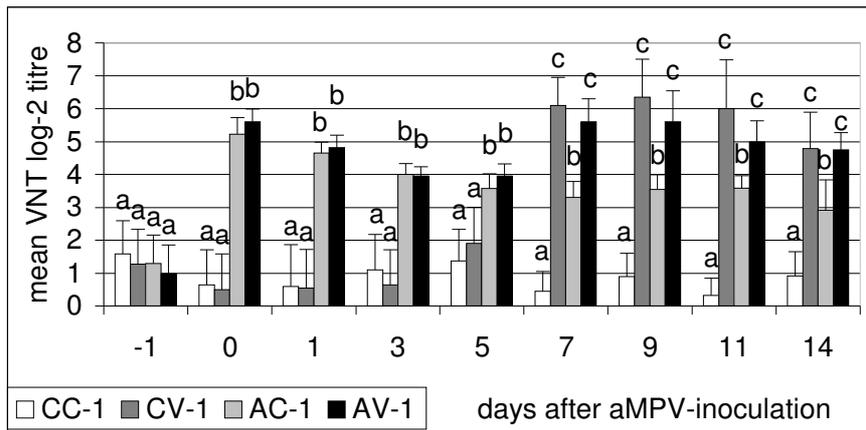
(A) ELISA antibodies (Exp. 1)



(B) ELISA antibodies (Exp. 2)



(C) Virus neutralizing antibodies (Exp. 1)



(D) Virus neutralizing antibodies (Exp. 2)

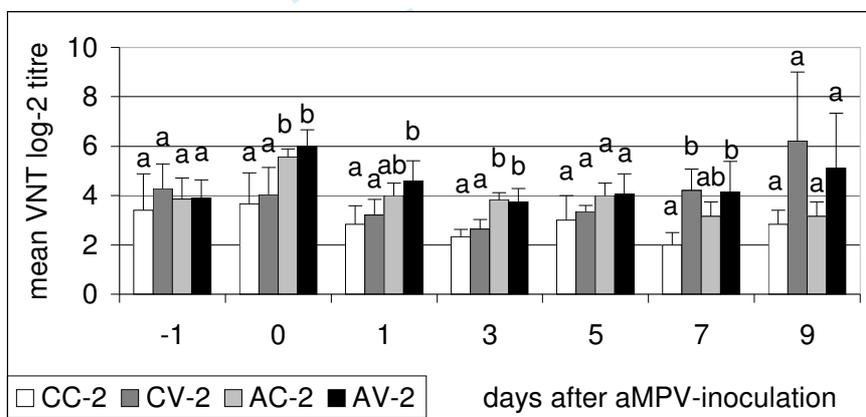
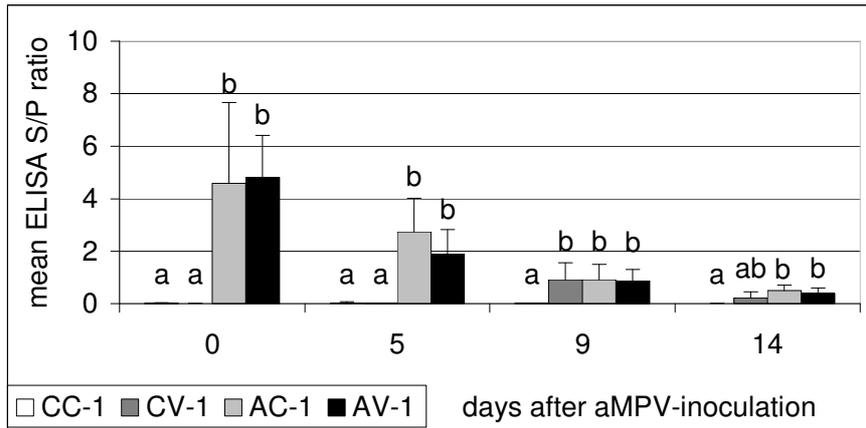


Figure 1: Detection of aMPV-specific serum ELISA IgG (A, B) and VN antibodies (C, D) after intravenous administration of aMPV-antibodies and subsequent inoculation with virulent aMPV in Exp. 1 (A, C) and Exp. 2 (B, D).

CC-1 and CC-2: turkeys received an aMPV-Ab⁻ formulation and remained aMPV-free; CV-1 and CV-2: turkeys received an aMPV-Ab⁻ formulation and were subsequently challenged with virulent aMPV subtype A; AC-1 and AC-2: turkeys received an aMPV-Ab⁺ formulation and remained aMPV-free; AV-1 and AV-2: turkeys received an aMPV-Ab⁺ formulation and were subsequently challenged with virulent aMPV subtype A. Serum samples were collected immediately before the first antibody injection at day -1; samples at day 0 were collected 15 minutes after the second antibody injection. Presented are mean values per group and day with standard deviation. Values marked with different superscript letters at the same experimental day are significantly different from each other (One Way ANOVA and Tukey test, $P < 0.05$); Exp. 1: $n = 6-10$; Exp. 2: $n = 3-10$

(A) Lacrimal fluid (ELISA, Exp. 1)



(B) Lacrimal fluid (ELISA, Exp. 2)

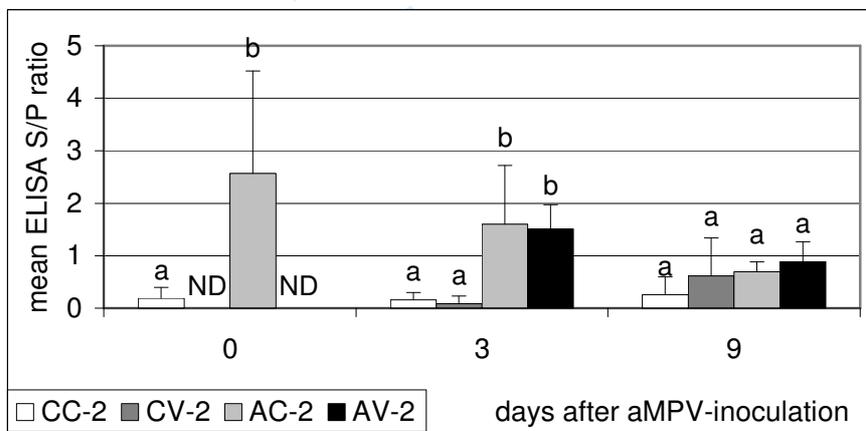
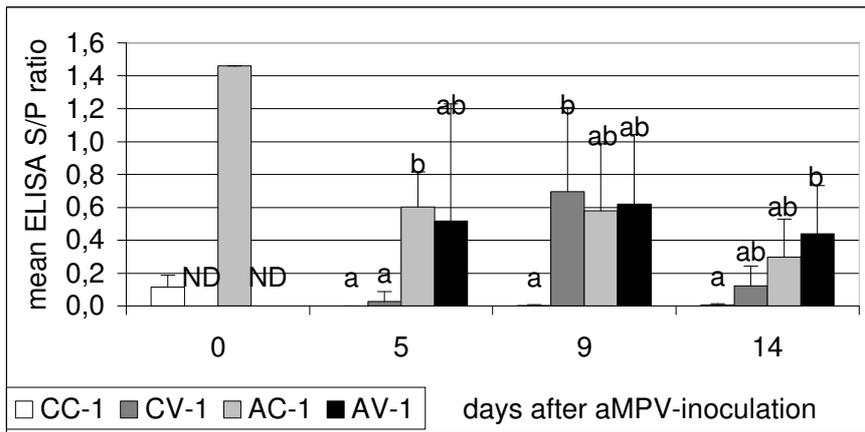


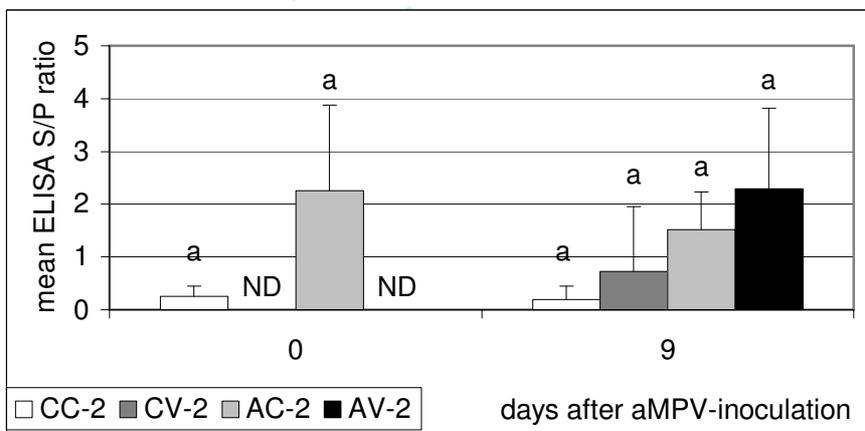
Figure 2: Detection of aMPV-specific ELISA IgG in lacrimal fluids after intravenous administration of aMPV-specific antibodies and subsequent inoculation with virulent aMPV in two consecutive experiments.

CC-1 and CC-2: turkeys received an aMPV-Ab⁻ formulation and remained aMPV-free; CV-1 and CV-2: turkeys received an aMPV-Ab⁻ formulation and were subsequently challenged with virulent aMPV subtype A; AC-1 and AC-2: turkeys received an aMPV-Ab⁺ formulation and remained aMPV-free; AV-1 and AV-2: turkeys received an aMPV-Ab⁺ formulation and were subsequently challenged with virulent aMPV subtype A. Samples at day 0 were collected 15 minutes after the second injection. Presented are mean values per group and day with standard deviation. Values marked with different superscript letters at the same experimental day are significantly different from each other (One Way ANOVA and Tukey test, $P < 0.05$); Exp. 1: $n = 6-10$; Exp. 2: $n = 3-10$; ND: not done.

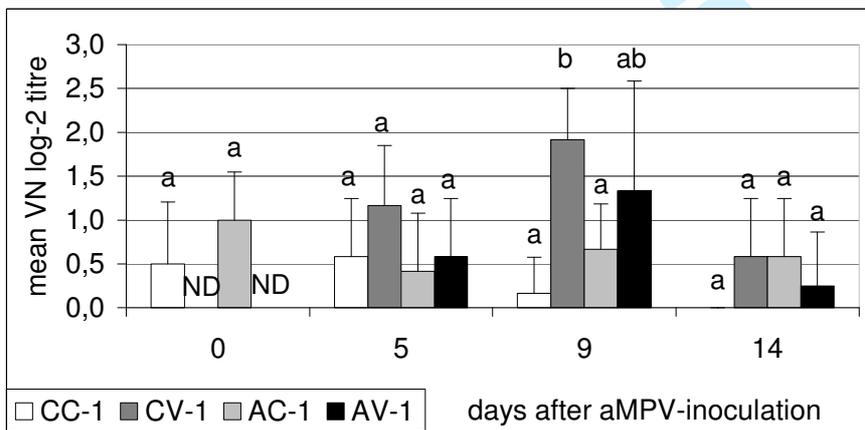
(A) Tracheal Washings (ELISA, Exp. 1)



(B) Tracheal Washings (ELISA, Exp. 2)



(C) Tracheal Washings (VNT, Exp. 1)



(D) Tracheal Washings (VNT, Exp. 2)

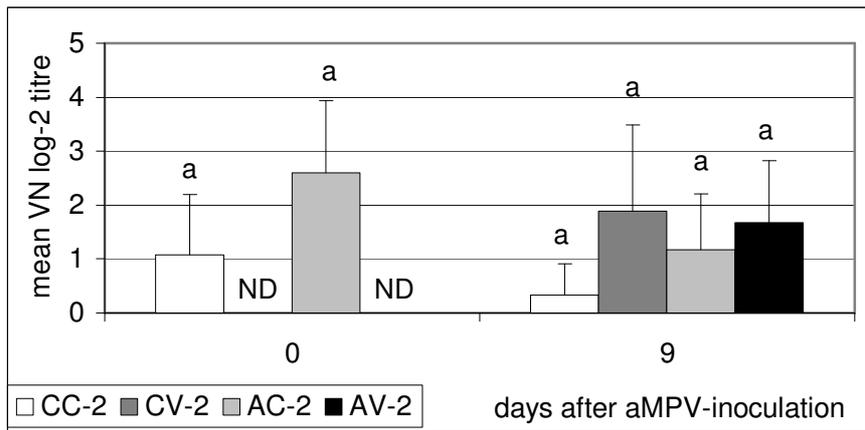


Figure 3: Detection of aMPV-specific ELISA IgG (A, B) and VN antibodies (C, D) tracheal washings (C-F) after intravenous administration of aMPV-specific antibodies and subsequent inoculation with virulent aMPV in two consecutive experiments. CC-1 and CC-2: turkeys received an aMPV-Ab- formulation and remained aMPV-free; CV-1 and CV-2: turkeys received an aMPV-Ab- formulation and were subsequently challenged with virulent aMPV subtype A; AC-1 and AC-2: turkeys received an aMPV-Ab+ formulation and remained aMPV-free; AV-1 and AV-2: turkeys received an aMPV-Ab+ formulation and were subsequently challenged with virulent aMPV subtype A. Samples at day 0 were collected 15 minutes after the second injection. Presented are mean values per group and day with standard deviation. Values marked with different superscript letters at the same experimental day are significantly different from each other (One Way ANOVA and Tukey test, $P < 0.05$); Exp. 1: $n = 6-10$; Exp. 2: $n = 3-10$; ND: not done.

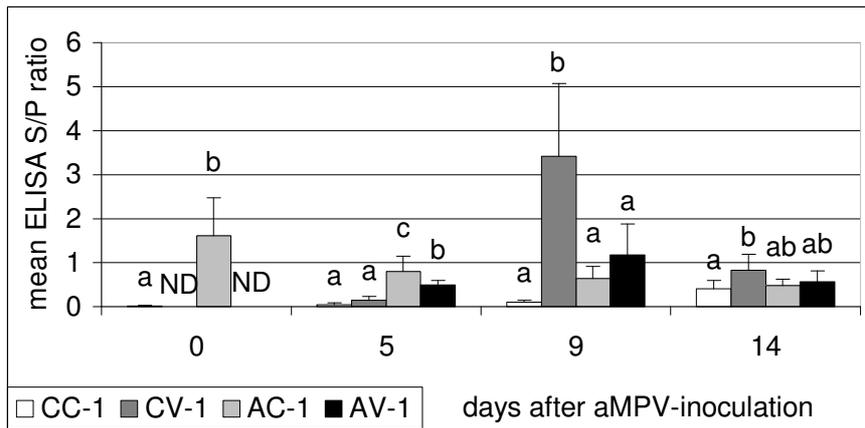


Figure 4: Detection of aMPV-specific ELISA IgG antibodies in turkey bile after intravenous administration of aMPV-specific antibodies and subsequent inoculation with virulent aMPV (Exp. 1)

CC-1: turkeys received an aMPV-Ab⁻ formulation and remained aMPV-free; CV-1: turkeys received an aMPV-Ab⁻ formulation and were subsequently challenged with virulent aMPV subtype A; AC-1: turkeys received an aMPV-Ab⁺ formulation and remained aMPV-free; AV-1: turkeys received an aMPV-Ab⁺ formulation and were subsequently challenged with virulent aMPV subtype A. Samples at day 0 were collected 15 minutes after the second antibody injection. Presented are mean values per group and day with standard deviation. Values marked with different superscript letters at the same experimental day are significantly different from each other (One Way ANOVA and Tukey test, $P < 0.05$); $n = 6-10$; ND: not done.

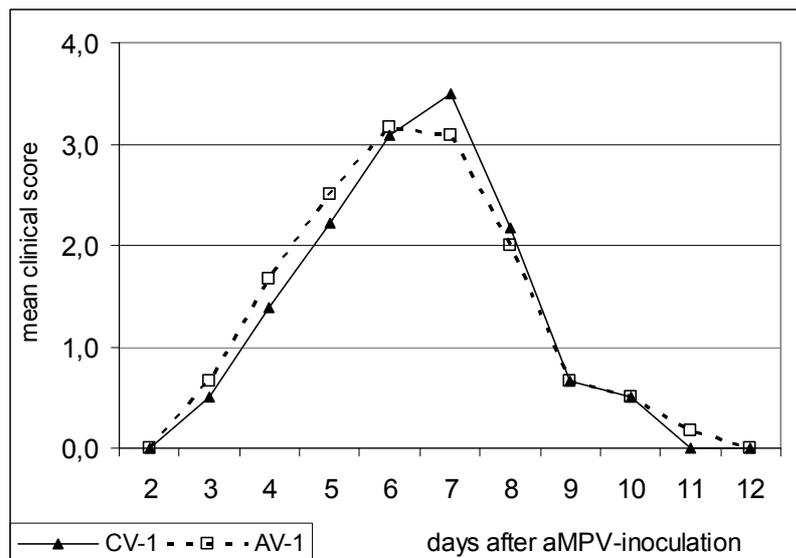
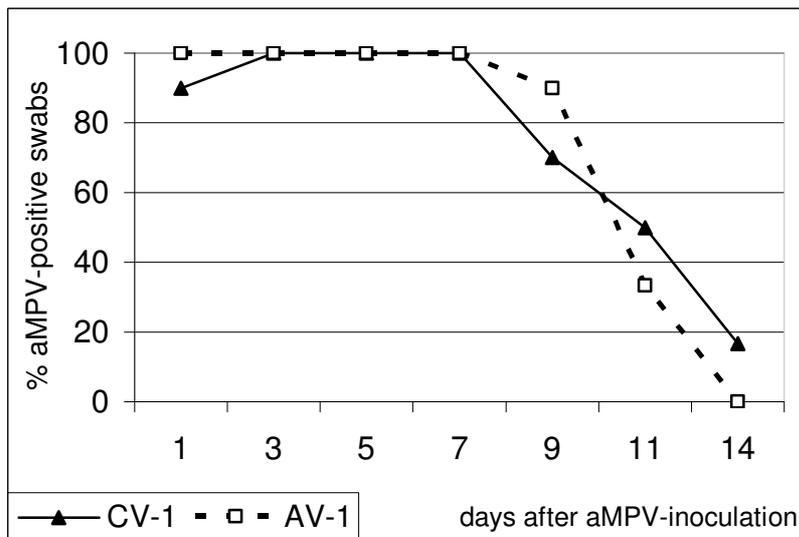


Figure 5: Development of clinical signs after passive transfer of aMPV-antibodies and subsequent inoculation with virulent aMPV (Exp. 1).

Turkeys were individually examined for clinical signs on a daily base. Results are presented as mean clinical scores; observed symptoms were nasal and ocular discharge and swollen infraorbital sinus. CV-1: turkeys received an aMPV-Ab⁻ formulation and were subsequently challenged with virulent aMPV subtype A; AV-1: turkeys received an aMPV-Ab⁺ formulation and were subsequently challenged with the same strain. Unchallenged birds of groups CC-1 and AC-1 remained free of respiratory symptoms throughout the experiment. n = 6-24

(A) Experiment 1



(B) Experiment 2

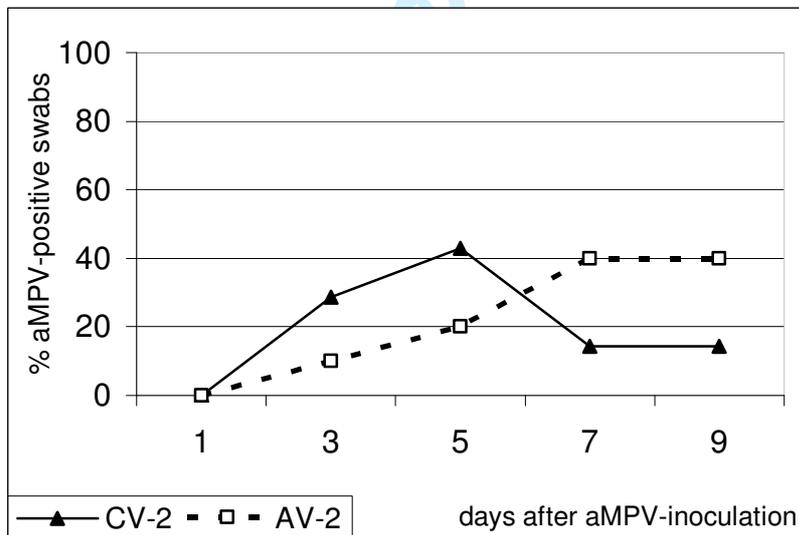


Figure 6. Detection of aMPV subtype A by RT-PCR from choanal swabs taken after intravenous administration of aMPV-antibodies and subsequent inoculation with virulent aMPV in Exp. 1 (A) and Exp. 2 (B).

Concentrated aMPV-specific antibodies were injected intravenously at two consecutive days (day -1; 0) and turkeys were subsequently inoculated with a virulent aMPV subtype A strain at day 0 by oculonasal route. CV-1 and CV-2: turkeys received an aMPV-Ab⁻ formulation and were subsequently challenged; AV-1 and AV-2: turkeys received an aMPV-Ab⁺ formulation and were subsequently challenged. Exp. 1: n = 6-10; Exp. 2: n = 7-10. In the unchallenged groups CC-1, AC-1 (Exp. 1) and CC-2, AC-2 (Exp. 2) aMPV subtype A was not detected.