The influence of maternally derived antibodies on protection against aMPV/A infection in TRT vaccinated turkeys

Marcin Śmiałek,*[,1] Joanna Kowalczyk,* Michał Gesek,† Edyta Kaczorek-Łukowska,‡ Daria Dziewulska,* Bartłomiej Tykałowski,* and Andrzej Koncicki*

*Department of Avian Disease, Faculty of Veterinary Medicine, University of Warmia and Mazury, 10-719 Olsztyn, Poland; †Department of Pathological Anatomy, Faculty of Veterinary Medicine, University of Warmia and Mazury, 10-719 Olsztyn, Poland; and ‡Department of Clinical Microbiology, Faculty of Veterinary Medicine, University of Warmia and Mazury, 10-719 Olsztyn, Poland

ABSTRACT Avian metapneumoviruses (**aMPV**) are a causative agent of turkey rhinotracheitis (TRT). Despite vaccination, cases of TRT outbreaks are frequently reported. Considering that there are aMPV-free areas, a part of turkey poults possess (**MDA+**) or do not possess (**MDA−**) maternally derived antibodies (**MDA**) in the first weeks of life, which is the time of TRT vaccination. Study was undertaken to establish the level of protection against homologue aMPV/A infection, in MDA+ and MDA− turkeys, vaccinated against TRT at the 0 or 14th d of life and infected 14 d later. With the use of ELISA test and qPCR techniques, we have established the level of immune system stimulation after the vaccination and how does it correlate with the level of protection against the aMPV infection. Vaccination of MDA+ turkeys (especially at 0 d of life) resulted in weaker IgA production in upper respiratory tract. In addition, we have demonstrated differences in both humoral and cell-mediated immunity stimulation after infection of vaccinated turkeys. Despite these differences, we have shown that all vaccinated birds were protected against the disease which was determined based on the clinical and histopathological scoring, as well as the level of aMPV/A replication and shedding. Nonvaccinated groups of turkeys displayed typical signs of TRT after infection which indicates that MDA alone are incapable of preventing the disease. Differences in TRT course were recorded between different age groups of nonvaccinated birds. Birds infected at the 28th d of life (especially MDA− birds) developed more severe signs, and the level of aMPV replication was higher than that in birds infected on the 14th d of life. Despite the minor role in alleviating TRT course, MDA seems not to interfere with the vaccination efficacy. It is hard to predict whether the observed immune system stimulation differences between MDA+ and MDA− birds after vaccination can influence the outcome of vaccination efficacy under the field conditions.

Key words: avian metapneumovirus, maternal antibody, vaccination efficacy, infection

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INTRODUCTION

Avian metapneumoviruses (**aMPV**), being members of the *Pneumoviridae* family, are severely infectious agents that colonize the upper respiratory tract, mainly in turkeys but also in chickens. The disease induced by aMPV in turkeys is called turkey rhinotracheitis (**TRT**). Infections with metapneumoviruses contribute to vast losses in the poultry industry, among other reasons due to poorer body weight gains, direct mortality, diminished laying performance, and immunosuppression. The aMPV are classified into 4 subtypes based on the surface antigens analysis with aMPV-specific antibodies or the gene sequence (especially F and G genes), namely A, B, C, and D (Collins et al., 1993; Cook et al., 1993; Juhasz and Easton 1994; Seal, 1998; Shin et al., 2002; Jones 2010).

Vaccination against TRT with live and inactivated vaccines has been found effective under both laboratory and field conditions. Unfortunately, cases of post-vaccination immunity being overcome by the field strains of aMPV are frequently reported (Smialek et al., 2012). There are a number of reasons for vaccination failure under field conditions, including, primarily, vaccination technique, the health status of birds,

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[1]Corresponding author: marcin.smialek@uwm.edu.pl

and homology of the vaccine and field antigen. In Poland, as well as other countries, a part of the turkey population includes poults derived from reproduction flocks not vaccinated against TRT (e.g., those imported from Canada). Therefore, a part of the poults possess (**MDA+**) and a part do not possess (**MDA−**) specific anti-aMPV maternally derived antibodies (**MDA**) at the time of TRT vaccination, usually taking place on the first day of a turkey's life.

Both humoral and cell-mediated immune mechanisms are stimulated in turkeys after vaccination and aMPV infection. Despite the elusive role of antibodies in the protection against aMPV, it seems that—although they are not able to protect against infection on their own—immunoglobulins may alleviate the outcomes of infection with a virulent TRT-causing virus by inhibiting its replication (Jones et al., 1992; Rubbenstroth and Rautenschlein 2009). Cha et al. (2007) demonstrated an increase in the number of IgA^{+} B cells in the upper respiratory tract and an increase of IgA in nasal secretions after turkey aMPV/C exposure; however, they did not determine the role of these antibodies in disease prevention and control. Cell-mediated immunity is becoming increasingly important in the protection against aMPV. Among the few works addressing the subject, the research by Liman and Rautenschlein (2007) is worth noting for its demonstration of a significant increase in the CD4^{+} subpopulation of T lymphocytes in the spleen that was accompanied by the enhancement of IFN-γ gene expression in splenocytes after aMPV/B vaccination. In addition, Cha (2009) reported an increased percentage of CD8^{+} cells in the upper respiratory tract but no increase in their percentage in the spleen after aMPV/C infection.

Previous works have demonstrated significant differences in the development of post-vaccine immunity against aMPV to be caused by the level of maternal immunity in poults on the day of vaccination. These differences concerned such parameters as the extent of upper respiratory tract infiltration by immunocompetent cells, the extent of humoral immunity stimulation in the upper respiratory airways (including specific IgA production), or the extent of cell-mediated immunity stimulation (including IFN-γ gene expression). In poults possessing MDA on the vaccination day, the stimulation of these parameters was significantly weaker, most likely due to the suppressed replication of vaccine aMPV by maternal antibodies (Smialek et al. 2015, 2016, 2020).

Given the aforementioned facts and because no studies have been conducted so far to determine the effect of MDA on the extent of development of protection against aMPV infections after birds' vaccination with commercial live vaccines, a study was carried out to evaluate the development of protection against experimental aMPV/A infection in MDA+ and MDA− birds vaccinated against TRT on the 0th and 14th d of life.

MATERIALS AND METHODS

Ethic Statement

The experimental procedures and animal handling procedures were conducted with the approval of the Local Ethic Committee for Animal Experiments in Olsztyn, Poland (resolution no. 48/2017). The study was carried out in accordance with EU Directive 2010/63/EU on the protection of animals used for scientific purposes.

Birds

Commercial Hybrid Converter turkeys totaling 782 were provided for the study by the Grelavi S.A. hatchery, Kętrzyn, Poland. MDA+ turkeys (n = 391) originated from a breeder turkey flock vaccinated against TRT (3 times with live aMPV/A and twice with aMPV/B-inactivated vaccine). MDA− turkeys (n = 391) originated from a Canadian breeder flock. The hatchery provided turkeys for all experiments at the same time.

The turkeys were housed in isolated units and maintained at a physical containment level 3 facility. Birds of the appropriate groups were vaccinated occulonasally with 10^{4} of TCID50/bird of commercial aMPV/A, Clone K attenuated vaccine. Sham-vaccinated birds received a vaccine diluent. Rearing conditions were consistent with the hatchery recommendations, and water and feed were given to birds *ad libitum*.

Experimental Layout (Experiments 1–4)

Four experiments were performed in the study.

Experiment 1 Experiment 1 was performed with 207 MDA+ turkeys (M+). On the day of delivery, blood was sampled (by decapitation) from 23 randomly selected poults to establish the MDA level. Serum samples were frozen (−20°C) until analyzed. The other birds were randomly allocated to the following experimental groups: control (M+0NV; 80 birds with 40 serving as contact birds for aMPV/A-infected groups), vaccinated (M+0V; 40 birds), infected (M+14I; 32 birds), and vaccinated and infected (M+0V-14I; 32 birds). The birds from the vaccinated groups were administered (0V) the TRT prophylactic on day 0 of life. The birds from the infected groups were inoculated with virulent aMPV/A (14I) on day 14 of life. All birds were reared until day 24 of life. On day 14, immediately before infection (0 d after infection—0 dpi), 8 birds from the control group (M+0NV) and from the vaccinated group (M+0V) were euthanized, and samples of their tracheal washings (TW) were collected to determine the level of specific anti-aMPV IgA. Washings were collected as described previously (Smialek et al., 2016) and frozen (−20°C) until analyzed. After the aMPV/A infection of the birds of the M+14I and M+0V-14I groups, contact

birds (n = 20 for each of these 2 groups) from the control group were introduced. The contact birds were sprayed with an identifying color and designated as M+14I-CB and M+0V-14I-CB, respectively. Then, at 3, 5, 7, and 10 dpi, 8 birds from the control group portion kept out of contact and from the vaccinated, infected, and vaccinated and infected groups were euthanized. Also at each of these time points, 4 control group birds in contact with each infected group were sacrificed. After euthanasia, nasal turbinate samples (n = 4) were collected from control, infected, and contact birds for qRT-PCR evaluation of aMPV/A replication. Approximately 3 × 3-mm, square sections of the rostral and middle turbinates from the nasal cavity were excised for qRT-PCR analysis. Samples of the nasal cavity and proximal part of the trachea (n = 5 for each) were taken from birds of the control and infected groups for histopathological examination and were fixed in 10% formalin. TW (n = 8) were obtained from control, vaccinated, infected and from vaccinated and infected birds for anti-aMPV IgA level determination. Also, the spleens of control, vaccinated, infected and vaccinated and infected birds (n = 4 for each) were sampled for mononuclear cell isolation and IFN-γ gene expression determination in those cells.

Experiment 2 In experiment 2, 207 MDA− turkeys (M−) were used. The experiment layout and sampling procedures were the same as those described for experiment 1. Groups examined during experiment 2 were designated as control (M−0NV), vaccinated (M−0V), infected (M−14I), control birds in contact with infected (M−14I-CB), vaccinated and infected (M−0V-14 V), and control birds in contact with vaccinated and infected (M−0V-14I-CB).

Experiment 3 Experiment 3 was carried out with 184 MDA+ turkeys. The experiment layout and sampling procedures were almost identical to those described for experiments 1 and 2, except for the birds' age. Briefly, birds were raised until 14 d of life before they were vaccinated against TRT. Blood samples were collected from the wing vein of 23 randomly selected birds on day 14 of life. After blood sampling, the birds were randomly assigned to the following groups: control (M+14NV), vaccinated (M+14V), infected (M+28I), control birds in contact with infected (M+28I-CB), vaccinated and infected (M+14V-28 V), and control birds in contact with vaccinated and infected (M+14V-28I-CB). The birds of the designated groups were vaccinated (14V) against TRT on day 14 of life. Infection with virulent aMPV/A (28I) was performed on day 28 of life (0 dpi). Further samples were collected 3, 5, 7, and 10 dpi.

Experiment 4 Experiment 4 was performed with 184 MDA− turkeys (M−). The experiment layout and sampling procedures were the same as those described for experiment 3. Groups examined in experiment 4 were designated as control (M−14NV); vaccinated (M−14V); infected (M− 28I); control birds in contact with infected (M−28I-CB), vaccinated, and infected (M−14V-28 V); and control birds in contact with vaccinated and infected (M−14V-28I-CB).

Virus Propagation and Infection

Virulent aMPV/A strain IT/Ty/A/259-01/03 (Lupini et al., 2011) was kindly provided by Catelli E. (University of Bologna, Italy). The virus was propagated and titrated in chicken embryo tracheal organ cultures (Cook et al., 1976), and median ciliostasis dose was calculated (Reed and Muench, 1938). For infection, each turkey was inoculated with 3,5 $\log_{10}$ median ciliostasis dose of aMPV/A.

Clinical Scoring

Previously described clinical scoring system (Naylor and Jones, 1994) was applied to evaluate the TRT symptoms exacerbation. In this scoring system, the following numbers are the indicators of 0 = no clinical signs, 1 = clear nasal exudate, 2 = turbid nasal exudate, 3 = swollen infraorbital sinuses and/or frothy eyes.

Histopathology

After passing the samples through intermediate liquids (increasing concentrations of alcohol and xylene), they were embedded in paraffin blocks. Sections of the examined samples 4-μm thick were stained with hematoxylin-eosin, and microscope samples were scanned with a Pannoramic MIDI scanner (3DHISTECH, Budapest, Hungary). Histopathological scoring was performed as follows; in each sample, the following changes were registered as indicators of TRT: lymphocyte infiltration, heterophils infiltration, deciliation, and exfoliation. In each group, at each dpi, the number of birds with histopathological changes was calculated, and the sum was divided by 10. Maximum histopathological score could amount for 2 (5 birds × 4 changes/10).

aMPV Identification and Quantification

Isolation of the RNA from the nasal turbinates was carried out with the use of a NucleoSpin RNA kit (Macherey-Nagel, Düren, Germany) according to the manufacturer's recommendations. The concentrations of eluted RNA were measured with a NanoDrop 2000 spectrophotometer (Thermo Scientific, Waltham, MA). Reverse transcription was performed with the use of a High-Capacity cDNA Reverse Transcription Kit (Life Technologies, Waltham, MA) following the manufacturer's protocol. The TaqMan qPCR was performed to identify and quantify the aMPV in nasal turbinates with the use of a probe and primers specific for attachment (G) protein gene, according to the method provided by Kwon et al. (2010). The reaction mixture was composed of 10 μL of TaqMan Fast Universal PCR

Master Mix (2X), No AmpErase UNG (Life Technologies, Waltham, MA), 1.8 μL of each primer of 10 μM (aMPVGAF1 5′-TGTTAGAGGAGTGCAGAAACT-3′ and aMPVGAR1 5′-CTGCTCCTGGGTGGTTGA-3′), 0.8 μL of 2.5-μM probe (FAM 5′-CAATGGAGGAGATAGAGATTGGTG-3′), 2 μL of cDNA, and 3.6 μL of ribonuclease-free water. The TaqMan qPCR was conducted under the following conditions: activation of the polymerase was at 95°C for 10 min and then followed 40 two-stage cycles of denaturation at 95°C for 30 s and primer annealing and chain elongation at 60°C for 60 s.

To determine the viral copy number in samples and sensitivity of TaqMan qPCR, a standard curve was plotted. Standard curves were generated using 953-bp aMPV/A strain IT/Ty/A/259-01/03 amplicon which contain a nucleotide sequence of the *aMPV G* gene, complementary with the TaqMan primers and probe. The amplification of a product was conducted according to the method described by Cecchinato et al. (2010). The reaction was carried out in a Mastercycler thermal cycler (Eppendorf, Hamburg, Germany) with a HotStar TaqPlus Master Mix Kit (Qiagen, Hilden, Germany). The reaction mixture had the following composition: 10 μL of HotStar TaqPlus DNA Polymerase (Qiagen), 0.1 μL of each primer at 100 μM (aMPVaSH1 5′-GCTTTGATCTTCCTTGTTGC-3 and aMPVG6 5′-CTGACAAATTGGTCCTGATT-3′), 7.8 μL of RNase-free water, and 2 μL of cDNA. The reaction was carried out under the following conditions: The initial denaturation step was at 95°C for 5 min and was followed by 40 cycles of denaturation at 94°C for 1 min, annealing and elongation at 72°C for 1 min, and a final elongation step at 72°C for 10 min. Then, the PCR products were purified from the reaction mixture residues with the use of a Clean-Up Concentrator Kit (A&A Biotechnology, Gdynia, Poland), and amplicon concentration was measured with the NanoDrop 2000 spectrophotometer. The gene copy number was calculated based on amplicon concentration and size with a copy number calculator (Genomics and Sequencing Center, University of Rhode Island, Kingston, RI). Standard 10-fold serial dilutions of amplicons (starting at initial dilution to 10^8 and ending at final dilution to 10^3) were used as template cDNA.

Serology

Anti-aMPV IgY level evaluation was performed with the use of commercial APV ELISA kit (IDEXX) in accordance to manufacturer recommendation. Sample to positive (S/P) ratios were used to express the mean MDA level on the day of birds' vaccination.

The level of specific anti-aMPV IgA in TW was determined with in-house ELISA as described previously (Smialek et al., 2016). Briefly vaccine aMPV/A was used to coat the maxisorp ELISA plates (NUNC, Denmark). The plates were incubated (24 h, 4°C) and then washed 4 times with Wash Buffer (Invitrogen) and blocked (1h, 21°C) with Assay Buffer (Invitrogen). Undiluted TW samples were incubated (1h, 21°C, 200 RPM) on plates. Polyclonal anti-chicken IgA:HRP antibodies (1h, 21°C, 200 RPM; AbD Serotec, UK) and HRP substrate (15m, 21°C; Invitrogen) were used to detect the IgA antibodies. Mean OD ± SD were used to express the results.

ELISAs were performed with a BioTek ELx405 plate washer (BioTek), Eppendorf epMotion 5075 LH pipetting station (Eppendorf, Germany), and a BioTek ELx800 plate reader.

Isolation and Determination of Mononuclear Cell Counts

Isolation of mononuclear cells from spleens with the use of Percoll density gradient solution was performed as described previously (Śmiałek et al., 2015). Briefly, spleen samples were homogenized in a manual tissue grinder. Cell pellets, obtained after centrifugation (450 g for 10 min at 20°C), were resuspended in 40% Percoll density gradient and layered on 60% Percoll. After centrifugation (20 min, 1,900 g, 20°C, breaks off), mononuclear cells were collected from the interphase. Cells were washed twice, and finally they were resuspended in 1 mL of PBS. Lymphocyte counts were calculated with Vi-cell XR (Beckman Coulter), and 5×10^6 of isolated cells were transferred for RNA isolation and IFN-γ gene expression evaluation.

IFN-γ Gene Expression Evaluation

IFN-γ gene expression evaluation was carried out as described previously (Smialek et al., 2020). Briefly, isolation of the RNA from the mononuclear cells was again performed with the use of the NucleoSpin RNA kit, and the concentrations of eluted RNA were measured with the NanoDrop 2000 spectrophotometer. Reverse transcription was also again performed with the use of a High-Capacity cDNA Reverse Transcription Kit. Standardized RNA at a mass of 0.5 μg per sample was used for cDNA synthesis. In order to determine the IFN-γ gene expression, the real-time PCR technique was implemented. The reaction mixture for the qPCR was composed of 10 μL of Power SYBR Green PCR Master Mix (Life Technologies), 1.8 μL of each primer at 10 μM, 2 μL of cDNA, and ribonuclease-free water for completion to 20 μL. The primer sequences are given in Table 1. The expression of the IFN-γ gene was calculated using the $2^{-\Delta\Delta Ct}$ method normalized to efficiency corrections, the expression levels of the reference gene coding GAPDH (Glyceraldehyde 3-phosphate dehydrogenase), and adequate control groups.

Statistical Analysis

The significance of differences in values of the measured parameters between the investigated groups was analyzed with the Student's *t* test for independent samples. All calculations were made using Statistica

Table 1. Primers sequences used for IFN-γ gene expression evaluation with real time PCR.

Primer	Sequence 5' -> 3'	Fragment size (bp)	Gene accession no.
IFN-γ F	CTGACAAGTCAAAGCCGCAC	137	NM_001303179.1
IFN-γ R	AGTCATTCATCTGAAGCTTGGC		
GAPDH F	CCCTGAGCTCAATGGGAAGC	125	XM_003202048.3
GAPDH R	TCAGCAGCAGCCTTCACTAC		

13.1 software (StatSoft Polska, Krakow, Poland). Differences were found statistically significant at $P \leq 0.05$.

RESULTS

MDA Status

Results of serological anti-aMPV IgY level determination in MDA+ and MDA− birds on the day of vaccination are summarized in Table 2. In MDA+ birds, the mean IgY S/P ratio reached 3.43 and 1.16 on day 0 and day 14 of life, respectively. No anti–aMPV-specific IgY antibodies were detected in the serum of the MDA− birds.

Clinical Score

The mean clinical scores recorded for the birds from experiments 1–4 are summarized in Figure 1. In none of the control, vaccinated, or vaccinated and infected birds was an increase recorded in the clinical score. In contrast, the clinical score was elevated in all infected (and not vaccinated) groups of turkeys. In M+14I turkeys (experiment 1), the clinical score peaked at 5 dpi (mean score = 1.6) and started to decline from day 6 after infection. At 8 dpi, the clinical score had returned to the level of the control group. In M−14I poults (experiment 2), the clinical score was highest at 6 dpi, when it was 1.8. On day 7, the clinical score began to fall and was comparable to that of the control group at 10 dpi. In M+28I subjects (experiment 3), the mean clinical

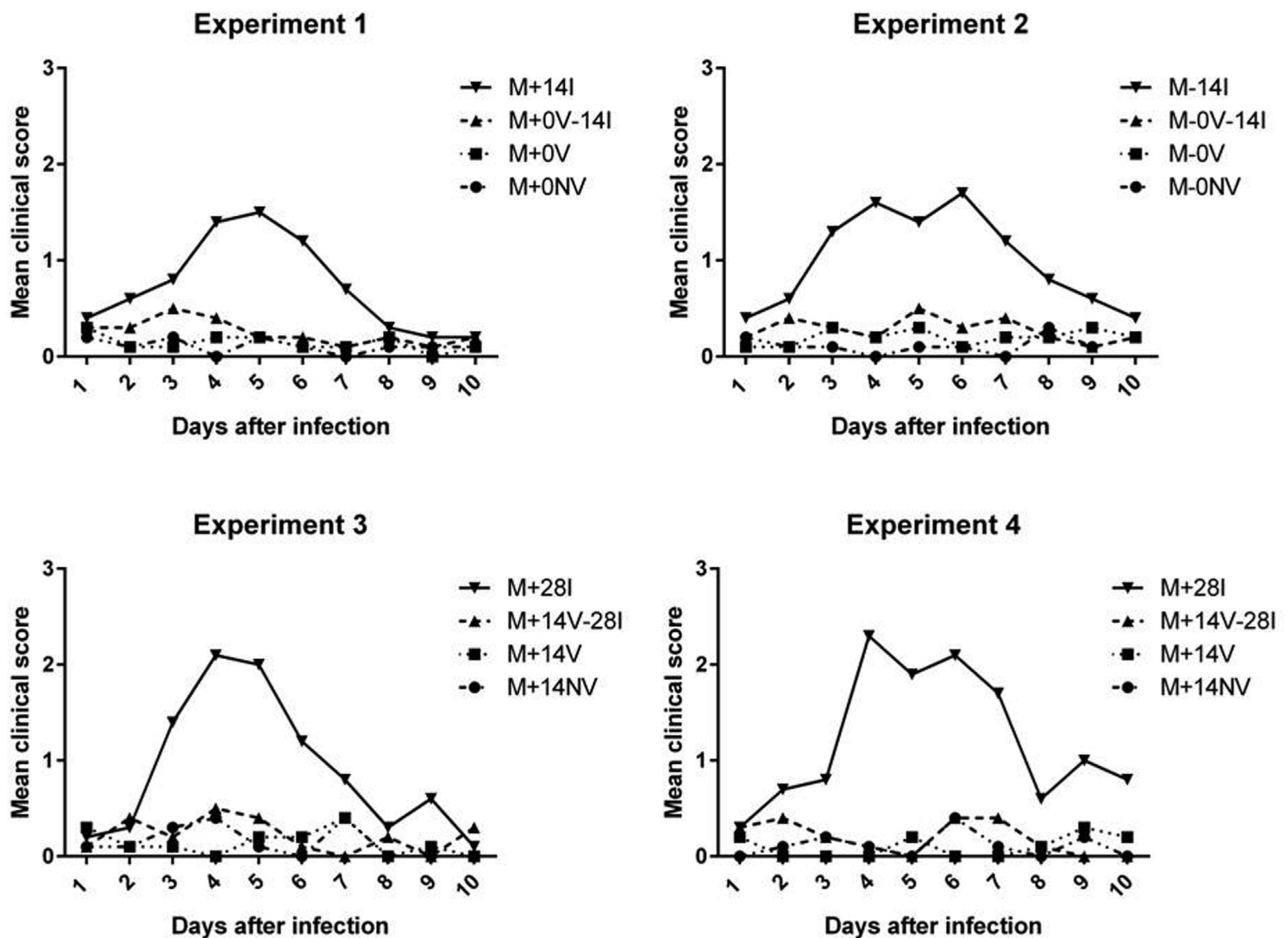

Figure 1. Mean daily clinical scores after aMPV/A infection of turkeys in experiments 1–4. Birds of experiments 1 and 2 were infected (at 14 d of life) 14 d after aMPV/A vaccination on the first day of life. Turkeys of experiments 3 and 4 were infected (at 28 d of life) 14 d after aMPV/A vaccination of the 14th d of life.

score was higher until at 4 dpi (2.1) and had diminished to the level of the control group at 8 dpi. In the MDA-28I group of birds, the mean clinical score apex also occurred at 4 dpi when 2.3 was seen. In this group, the mean clinical score remained elevated until the end of the experiment.

Histopathology

The mean histopathological scores recorded in nasal cavity samples are shown in Figure 2. As demonstrated, elevated mean histopathological scores were recorded in infected and not vaccinated groups of birds in each experiment. In experiments 1–3, the mean histopathological score peaked at 5 dpi, reaching 0.6, 0.7, and 0.8 in the M+14I, M−14I, and M+28I groups, respectively. In those groups, the mean score started to decline from day 7 after infection, dropping to the levels of the respective control groups at 10 dpi. In the M−28I group (experiment 4), the mean histopathological score increased from 5 dpi and achieved the highest value of 0.8 at 7 dpi. At 10 dpi, the score of M−28I birds was no higher than that of the control group (M−14NV). Elevated histopathological scores were also recorded in the vaccinated and infected groups in experiments 1, 3, and 4. In the M+0V-14I group (experiment 1), the score rose to 0.5 at 3 dpi. In the M+14V-28I group (experiment 3), it reached 0.3 at 3 dpi and 0.5 at 5 dpi, while in the M−14V-28I group (experiment 4), it reached 0.6 at 7 dpi. No increased histopathological scores were recorded in any of the experiments in the trachea of turkeys after infection (data not shown).

aMPV/A Replication

The mean numbers of aMPV/A viral copies in nasal turbinate samples of the birds from experiments 1–4 are presented in Table 3. In experiment 1, a significant increase in aMPV/A copy number was recorded at 7 dpi in the M+14I (P = 0.047) and M+14I-CB (P = 0.048) groups in respective comparison to M+0V-14I and M+0V-14I-CB birds. In experiment 2, a significantly higher number of aMPV/A viral copies was noted in the M−14I (P = 0.049) group than in its M−0V14I counterpart and in the M−14I-CB (P = 0.042) group than in its M−0V14I-CB counterpart. In experiment 3, a greater total of aMPV/A copies was counted in the M+28I group than in the M+14V-28I turkeys at 7 (P = 0.049) and 10 dpi (P = 0.044) and in M+28I-CB turkeys than in the M+14V-28I-CB group at 7 dpi

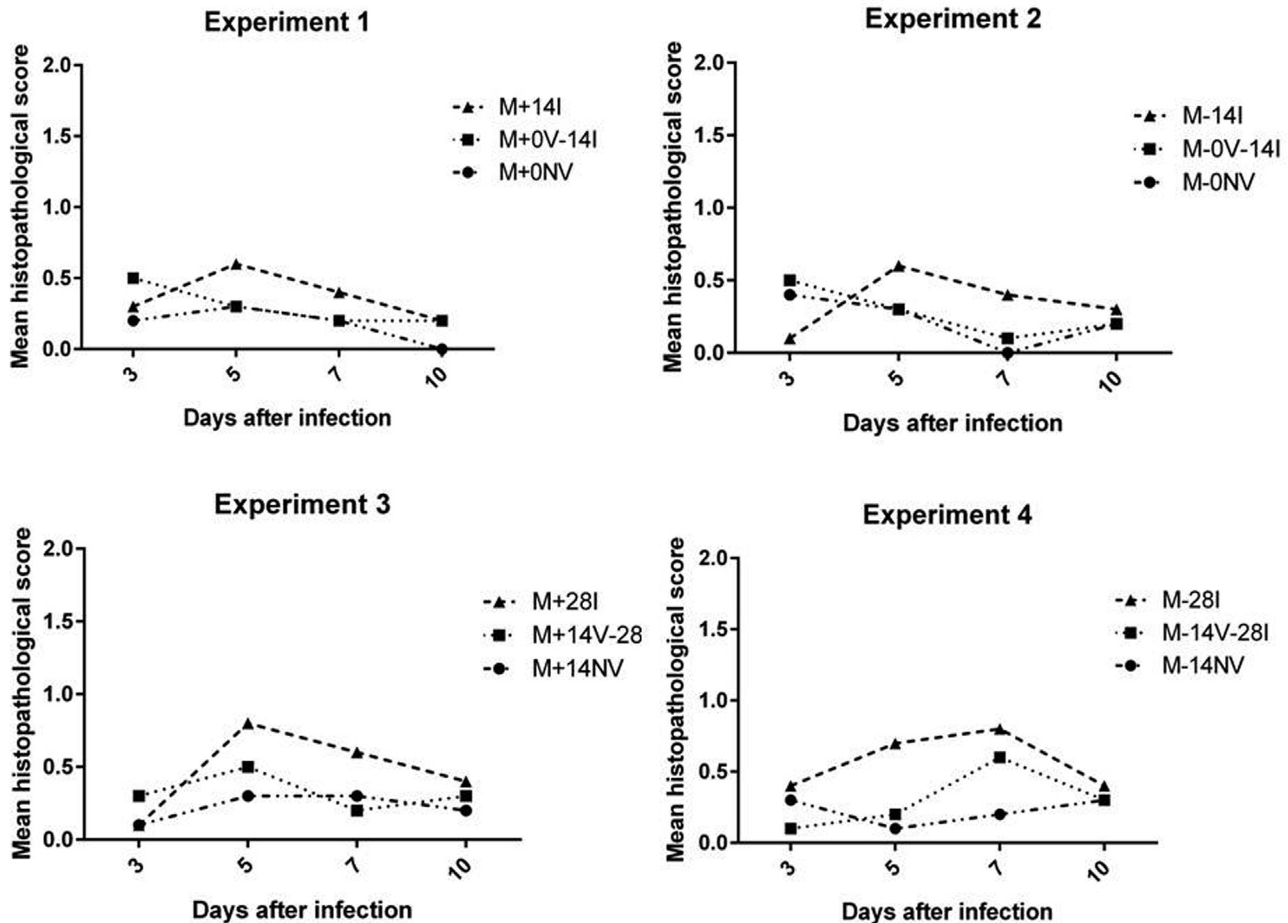

Figure 2. Mean daily histopathological scores in nasal cavity samples after aMPV/A infection of turkeys in experiments 1–4. Birds of experiments 1 and 2 were infected (at 14 DOL) 14 d after aMPV/A vaccination on the first day of life. Turkeys of experiments 3 and 4 were infected (at 28 DOL) 14 d after aMPV/A vaccination of the 14th d of life.

Table 2. Serum anti–aMPV IgY maternally derived antibody levels at the day of aMPV/A vaccination of turkeys in experiments 1–4.

Birds' MDA status	Mean MDA S/P ratio ± SD[1] at the time of vaccination	
	0 DOL	14 DOL
MDA+	3.43 ± 1.72 (Experiment 1)	1.16 ± 1.03 (Experiment 3)
MDA−	0.00 ± 0.00 (Experiment 2)	0.00 ± 0.00 (Experiment 4)

Abbreviations: aMPV, avian metapneumoviruses; DOL, days of life; MDA, maternally derived antibodies.

[1]Twenty-three blood samples per group were analyzed.

(P = 0.038). In experiment 4, the number of aMPV/A copies in M−14V-28I-CB (P = 0.013) turkeys significantly exceeded that in M−28I-CB birds at 3 dpi. In addition, in this experiment, significantly more aMPV/A copies were present in the M−28I-CB group than in M−14V-28I-CB turkeys at 5 (P = 0.046) and 7 dpi (P = 0.017) and in the M−28I group than in M−14V-28I poults at 5 dpi (P = 0.041).

IgA profiles

TW anti-aMPV IgA antibody levels recorded in experiments 1–4 are summarized in Table 4. In experiment 1, an increased IgA antibody level was recorded in the M+0V-14I group at 3, 5, and 7 dpi (P = 0.024, P = 0.001, and P = 0.049, respectively; compared with the M+0NV group) and in the M+14I group at 5 dpi (P = 0.003; in comparison to M+0NV turkeys). In experiment 2, significantly higher IgA antibody levels were recorded in M-0V birds at 0, 3, and 5 dpi (P = 0.039, P = 0.015, and P = 0.015, respectively; against the M-0NV group), in the M-0V-14I group at 5, 7, and 10 dpi (P = 0.049, P = 0.015, and P = 0.008, respectively; against the M-0NV group), and in the M-14 group at 5 dpi (P = 0.48; in comparison to the M-0NV group). In experiment 3, increased levels of IgA were recorded in M+14V, M+14V-28I, and M+28I groups at 3 dpi (P = 0.001, P = 0.001, and P = 0.001, respectively; against M+14NV turkeys) and 5 dpi (P = 0.002, P = 0.009, and P = 0.009, respectively; against M+14NV turkeys) and in the M+28I group at 7 dpi (P = 0.001; in comparison to the M+14NV group). In experiment 4, significantly higher IgA levels were recorded in the M-14V group at 0, 3, 5, and 10 dpi (P = 0.032, P = 0.031, P = 0.001, and P = 0.010, respectively; in comparison to the M-14NV group). In addition, in this experiment, higher antibody levels were registered in the M-14V-28I group at 3, 5, and 7 dpi (P = 0.007, P = 0.013, and P = 0.028, respectively; against the M-14NV group), as well as in the M-28I group at 3 and 5 dpi (P = 0.001 and P = 0.004, respectively; against M-14NV turkeys).

IFN-γ Gene Expression

Results of relative IFN-γ gene expression measurement in splenocytes of the birds from experiments 1–4 are set down in Table 5. In experiment 1, significantly increased expression of the gene was recorded in the

Table 3. aMPV/A replication in nasal turbinates at different days after aMPV/A infection of experimental turkeys in experiments 1–4.

Experiment	Group	Mean number of viral copies ± SD[4] in nasal turbinates at days after aMPV/A infection			
		3	5	7	10
1	M+14I[1]	2,13 ± 8,89[a]	474,23 ± 937,18[a]	21222,75 ± 17035,58[a]	5900,40 ± 10067,13[a]
	M+14I-CB[2]	4,58 ± 8,88[a]	62,93 ± 62,31[a]	16299,75 ± 13171,70[a]	5369,75 ± 6346,70[a]
	M+0V-14I[3]	0,12 ± 0,26[a]	0,53 ± 0,45[a]	3,53 ± 2,56[b]	2,63 ± 1,78[a]
	M+0V-14I-CB	0,12 ± 0,24[a]	1,46 ± 0,30[a]	1,45 ± 2,65[b]	7,52 ± 14,77[a]
2	M-14I	0,00 ± 0,00[a]	0,13 ± 0,27[a]	12790,23 ± 14460,96[a]	25440,74 ± 19276,97[a]
	M-14I-CB	0,00 ± 0,00[a]	0,59 ± 1,18[a]	4157,54 ± 8288,31[a]	15195,04 ± 11045,82[a]
	M-0V-14I	0,22 ± 0,25[a]	0,00 ± 0,00[a]	3,67 ± 1,75[a]	42,70 ± 70,56[b]
	M-0V-14I-CB	0,14 ± 0,27[a]	0,78 ± 0,76[a]	3,73 ± 6,42[a]	1,38 ± 2,10[b]
3	M+28I	19,02 ± 37,71[a]	13063,40 ± 19045,03[a]	2293,33 ± 1027,43[b]	510,78 ± 845,71[b]
	M+28I-CB	0,14 ± 0,27[a]	13432,44 ± 26096,55[a]	13177,75 ± 8786,21[a]	13078,25 ± 9878,94[a]
	M+14V-28I	172,55 ± 206,21[a]	5,82 ± 8,22[a]	0,78 ± 0,91[c]	2,05 ± 1,56[b]
	M+14V-28I-CB	7,66 ± 13,10[a]	6261,80 ± 9151,97[a]	827,63 ± 1044,92[b,c]	1993,86 ± 2410,65[a,b]
4	M-28I	571,58 ± 1126,96[a,b]	20400,86 ± 16417,31[a]	5349,00 ± 4568,30[b]	471,23 ± 702,87[a,b]
	M-28I-CB	0,00 ± 0,00[b]	40294,00 ± 29560,19[a]	21076,00 ± 11644,57[a]	3756,33 ± 2881,40[a]
	M-14V-28I	1372,00 ± 811,87[a]	43,25 ± 34,81[b]	14,77 ± 12,72[b]	30,65 ± 54,53[b]
	M-14V-28I-CB	1144,75 ± 663,76[a]	3246,75 ± 1959,38[b]	1884,59 ± 2177,73[b]	252,80 ± 362,82[a,b]

Different superscript letters indicate statistical difference within the experiment at different dpi.

Abbreviations: aMPV, avian metapneumoviruses.

[1]In all experiments, birds of infected (I) groups were inoculated with 3,5 $\log_{10}$ median ciliostasis dose of virulent aMPV/A.

[2]In each group of aMPV/A-infected birds, 16 contact birds (CB; the same origin, not vaccinated and not infected) were placed after aMPV/A infection.

[3]In all experiments, birds of vaccinated (V) groups were inoculated oculonassaly with 10^4 $TCID_{50}$ of live attenuated aMPV/A vaccine.

[4]Four nasal turbinates samples per group were analyzed with qRT-PCR technique.

Table 4. Tracheal washings anti–aMPV IgA antibody level at different dpi after aMPV/A infection of turkeys.

Experiment	Group	Mean OD ± SD[3] at days after aMPV/A infection				
		0	3	5	7	10
1	M+0NV	0.081 ± 0,013[a]	0.081 ± 0,013[b]	0.079 ± 0,015[b]	0.083 ± 0,019[b]	0.075 ± 0,022[a]
	M+0V[1]	0.079 ± 0,035[a]	0.093 ± 0,037[b]	0.097 ± 0,047[b]	0.080 ± 0,017[b]	0.082 ± 0,016[a]
	M+0V-14I[2]	nd	0.134 ± 0,051[a]	0.166 ± 0,097[a]	0.151 ± 0,098[a]	0.086 ± 0,018[a]
	M+14I	nd	0.126 ± 0,064[a,b]	0.160 ± 0,044[a]	0.103 ± 0,049[a,b]	0.109 ± 0,046[a]
2	M-0NV	0.091 ± 0,017[b]	0.086 ± 0,011[b]	0.079 ± 0,023[b]	0.086 ± 0,014[b]	0.082 ± 0,017[b]
	M-0V	0.153 ± 0,036[a]	0.214 ± 0,130[a]	0.215 ± 0,121[a]	0.096 ± 0,021[b]	0.139 ± 0,103[a,b]
	M-0V-14I	nd	0.103 ± 0,029[b]	0.222 ± 0,105[a]	0.214 ± 0,130[a]	0.195 ± 0,100[a]
	M-14I	nd	0.088 ± 0,013[b]	0.163 ± 0,074[a]	0.138 ± 0,103[a,b]	0.088 ± 0,013[b]
3	M+14NV	0.074 ± 0,027[a]	0.083 ± 0,017[b]	0.077 ± 0,019[b]	0.072 ± 0,029[b]	0.084 ± 0,021[a]
	M+14V	0.101 ± 0,032[a]	0.136 ± 0,023[a]	0.143 ± 0,043[a]	0.093 ± 0,022[b]	0.073 ± 0,018[a]
	M+14V-28I	nd	0.169 ± 0,054[a]	0.140 ± 0,050[a]	0.096 ± 0,027[b]	0.092 ± 0,024[a]
	M+28I	nd	0.162 ± 0,053[a]	0.159 ± 0,049[a]	0.134 ± 0,022[a]	0.101 ± 0,029[a]
4	M-14NV	0.081 ± 0,019[b]	0.090 ± 0,008[c]	0.087 ± 0,017[b]	0.072 ± 0,010[b]	0.088 ± 0,012[b]
	M-14V	0.120 ± 0,032[a]	0.116 ± 0,029[b]	0.154 ± 0,030[a]	0.137 ± 0,109[a,b]	0.134 ± 0,041[a]
	M-14V-28I	nd	0.166 ± 0,067[a]	0.250 ± 0,159[a]	0.173 ± 0,095[a]	0.099 ± 0,017[b]
	M-28I	nd	0.165 ± 0,036[a,b]	0.168 ± 0,063[a]	0.117 ± 0,074[a,b]	0.117 ± 0,059[a,b]

Different superscript letters indicate statistical difference within the experiment at different dpi.
Abbreviations: aMPV, avian metapneumoviruses; nd, not determined.
[1]In all experiments, birds of vaccinated (V) groups were inoculated oculonassaly with $10^{4\cdot}$ $TCID_{50}$ of live attenuated aMPV/A vaccine.
[2]In all experiments, birds of infected (I) groups were inoculated with 3,5 log_{10} median ciliostasis dose of virulent aMPV/A.
[3]Eight samples/group of undiluted TW were analyzed.

M+0V-14I group at 3 dpi ($P = 0.023$; in comparison to the M+0NV group) and in M+14I turkeys at 5 dpi ($P = 0.042$; greater than in M+0NV). In experiment 2, enhanced IFN-γ gene expression was recorded in M−14I turkeys at 3 dpi ($P = 0.002$; against the M-0NV group). In experiment 3, significantly more intensive expression of the IFN-γ gene was recorded in M+14V-28I turkeys at 3 dpi ($P = 0.049$; than in the M+14NV group). In addition, in this experiment, upregulated gene expression was recorded in the M+28I group at 5 and 10 dpi ($P = 0.016$ and $P = 0.041$, respectively; when compared to the M+14NV group). Finally, in experiment 4, the IFN-γ gene was significantly more expressed in the M−14V28I and M−28I groups at 3 dpi ($P = 0.001$ and $P = 0.027$, respectively; in comparison to the M-14NV group).

DISCUSSION

aMPV infections of poultry are associated with serious economic and animal welfare problems, particularly in commercial turkey flocks. Cecchinato et al. (2013)

Table 5. IFN-γ gene expression in spleen samples at different days after aMPV/A infection of experimental turkeys in experiments 1–4.

Experiment	Group	Relative IFN-γ gene expression[3] at days after aMPV/A infection				
		0	3	5	7	10
1	M+0NV	1,00[a]	1,00[b]	1,00[b]	1,00[a]	1,00[a]
	M+0V[1]	1,13 ± 0,61[a]	0,61 ± 0,30[b]	1,73 ± 1,76[a,b]	0,75 ± 0,25[a]	0,93 ± 0,80[a]
	M+0V-14I[2]	nd	4,49 ± 1,17[a]	0,93 ± 0,26[b]	0,96 ± 0,36[a]	0,72 ± 0,25[a]
	M+14I	nd	1,64 ± 1,10[a,b]	2,17 ± 0,78[a]	1,39 ± 0,33[a]	2,04 ± 0,86[a]
2	M-0NV	1,00[a]	1,00[b]	1,00[a]	1,00[a]	1,00[a]
	M-0V	2,30 ± 3,18[a]	0,79 ± 0,18[b]	1,22 ± 0,83[a]	0,97 ± 0,18[a]	1,63 ± 1,11[a]
	M-0V-14I	nd	0,91 ± 0,04[b]	0,85 ± 0,21[a]	2,04 ± 1,15[a]	1,61 ± 0,44[a]
	M-14I	nd	2,61 ± 0,39[a]	1,46 ± 1,27[a]	1,78 ± 0,58[a]	0,93 ± 0,30[a]
3	M+14NV	1,00[a]	1,00[b]	1,00[b]	1,00[a]	1,00[b]
	M+14V	1,01 ± 0,82[a]	0,74 ± 0,47[b]	0,81 ± 0,15[b]	0,58 ± 0,44[a]	1,70 ± 2,69[a,b]
	M+14V-28I	nd	1,59 ± 0,31[a]	1,50 ± 1,30[a,b]	0,95 ± 0,33[a]	1,14 ± 0,42[a,b]
	M+28I	nd	0,78 ± 1,09[b]	5,56 ± 3,26[a]	1,86 ± 0,71[a]	2,69 ± 0,98[a]
4	M-14NV	1,00[a]	1,00[b]	1,00[a]	1,00[a]	1,00[a]
	M-14V	1,74 ± 1,07[a]	1,60 ± 0,57[a,b]	1,01 ± 0,18[a]	1,18 ± 0,54[a]	0,55 ± 0,51[a]
	M-14V-28I	nd	1,63 ± 0,13[a]	0,89 ± 0,06[a]	1,53 ± 0,64[a]	1,57 ± 0,88[a]
	M-28I	nd	1,81 ± 0,38[a]	1,04 ± 0,06[a]	1,44 ± 1,81[a]	1,42 ± 0,40[a]

Different superscript letters indicate statistical difference within the experiment at different dpi.
Abbreviations: aMPV, avian metapneumoviruses; nd, not determined.
[1]In all experiments, birds of vaccinated (V) groups were inoculated oculonassaly with 10^4 $TCID_{50}$ of live attenuated aMPV/A vaccine.
[2]In all experiments, birds of infected (I) groups were inoculated with 3,5 log_{10} median ciliostasis dose of virulent aMPV/A.
[3]Four spleen samples per group were analyzed with qRT-PCR technique. The relative expression of IFN-γ gene was calculated using the $2^{-\Delta\Delta Ct}$ method normalized to efficiency corrections, expression levels of reference gene coding glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and adequate control (NV) groups.

demonstrated that most aMPV-positive results, as assayed by RT-PCR in commercial turkey flocks, were obtained in turkeys aged from 9 to 12 wk. The results obtained for the vaccinated and nonvaccinated turkey groups indicate that we were only successful in inducing TRT after experimental infection with aMPV/A in the nonvaccinated groups. Birds from these groups manifested typical symptoms and histopathological lesions. In turn, the infected and contact birds showed significantly higher aMPV replication than the analogous vaccinated and infected turkey groups in the individual schemes of experiments 1–4. Regardless of MDA status, the nonvaccinated turkeys were more susceptible to the clinical form of TRT when infected in week 4 of life than in week 2 of life. This was reflected in the clinical and histopathological scoring results, which indicate that the intensification of symptoms and pathological lesions typical of TRT was more pronounced in the group of older birds (infected in week 4 of life). In addition, the levels of pathogenic aMPV replication and aMPV shedding, established based on the extent of virus replication in the contact birds, were higher in the older birds than in the birds infected in week 2 of life. These differences were most likely due to the increasing pool of cells sensitive to infection and the development of the respiratory and immune systems in older birds, which both increase the replication potential of aMPV. This would also explain why the frequency of aMPV field isolations rises in turkeys from 3 to 4 wk of age and reaches its peak in 9- to 12-wk-old turkeys (Cecchinato et al., 2013).

Jones et al. (1992) demonstrated that in turkeys subjected to chemical B lymphocyte depletion, the clinical course of TRT was more severe and persisted for a longer time after experimental infection of birds. It was concluded that despite the lack of direct correlation between the level of specific anti-aMPV antibodies and degree of protection against the disease, the presence of antibodies alleviates the course of TRT (Cook et al., 1989a,b; Jones et al. 1988, 1992; Rubbenstroth and Rautenschlein 2009). This was corroborated in our study which demonstrated that the clinical course of TRT after experimental infection was more severe and persisted for a longer time in turkeys from MDA− groups than in MDA+ birds. Jones et al. (1992) claimed that antibodies could inhibit virulent aMPV replication, contributing to the alleviation of TRT's clinical symptoms. Again, this was confirmed by the results of our study showing more intensive and longer aMPV replication in the nonvaccinated MDA− birds. Interestingly, in all our experiments, a comparable increase in IgA level in TW was observed after infection in the nonvaccinated birds. Except for in a few cases, this increase was long-term and occurred at 5 dpi. In the M+28I birds, the increased IgA level was also maintained at 7 dpi. Nonetheless, this does not change the fact that aMPV replication was more enhanced in MDA− groups. However, a higher and longer-lasting IFN-γ gene expression in splenocytes was determined after infection in the nonvaccinated birds from the MDA+ groups than in those from the MDA− groups, which may explain why the clinical course of TRT was less severe in these birds. The IFN-γ gene is an antiviral cytokine that has previously been shown to contribute to fighting aMPV infection (Liman and Rautenschlein, 2007). In the infected MDA− birds, the enhanced IFN-γ expression was noted only at 3 dpi, whereas in the MDA+ birds it was additionally at 5 dpi, and even at 10 dpi in the birds infected in wk 4 of life (M+28I). Therefore, it is likely that MDA residues (which can be in exudate on the surface of upper respiratory tract mucous membranes) present on the day of infection can directly strengthen the possibility of virus neutralization and be involved in the mechanisms stimulating cell-mediated immunity tending to eliminate the virus. The observed dependencies can be confirmed by the higher and longer-lasting aMPV replication noted in the MDA− contact birds, which points to a higher level of virus shedding by the nonvaccinated and experimentally infected turkeys or—indirectly—to a greater potential for aMPV replication in these birds.

On the other hand, it has to be concluded that maternal antibodies were unable to protect the nonvaccinated birds against TRT. Irrespective of MDA status, all nonvaccinated birds showed symptoms and histopathological lesions typical of aMPV infection. These results are partly consistent with findings reported by Rubbenstroth and Rautenschlein (2009). They showed no differences in the clinical course and replication degree of virulent aMPV between birds without specific antibodies and poults that received specific anti-aMPV antibodies by intravenous injection. MDA+ animals' lack of protection against infection has been demonstrated for other members of the *Pneumoviridae* subfamily, such as bovine respiratory syncytial virus (Kimman et al., 1988). However, in this study, the incidence and severity of disease were inversely related to the level of specific maternal antibodies. This phenomenon indicates possible differences in the course of the disease in antibody-positive animals which result from the mechanism of antibody transfer (natural or artificial).

Turkey humoral immunity is strongly stimulated by infection and vaccination against TRT (Jones et al., 1988; Cook et al., 1989a,b; Cha et al., 2007; Liman and Rautenschlein 2007). An increase in the number of IgA^+ B cells in the upper respiratory tract of turkeys exposed to aMPV/C with a concurrent increase of IgA in the nasal secretion after aMPV/C re-exposure emerged in the findings of Cha et al. (2007). On the other hand, Liman and Rautenschlein (2007) demonstrated a significant increase in the $CD4^+$ subpopulation of T lymphocytes in the spleen that was accompanied by enhanced IFN-γ gene expression in splenocytes after aMPV/B vaccination. Cha (2009) reported an increased percentage of $CD8^+$ cells in the upper respiratory tract but no increase in their percentage in the spleen after aMPV/C infection. Despite differences suggested in the stimulation of immune mechanisms against aMPV depending on the virus subtype, it should be explicitly stated that in this study, the infecting subtype was immaterial to the specificity of immune mechanisms stimulated, and these were both humoral and cell-mediated and those most likely

involved in the process of resistance to infection or secondary infection. In our previous articles, differences were demonstrated in postvaccination cell-mediated and humoral immunity stimulation after TRT vaccination between MDA+ and MDA− birds (Smialek et al. 2015, 2016, 2020). The MDA+ poults showed abnormalities in acquiring IgA^+ B lymphocyte specificity after vaccination, which resulted in the lack of specific IgA antibodies in TW, as well as IgY in TW and serum of MDA+ birds. In contrast, the MDA− birds produced those antibodies after aMPV/A vaccination (Smialek et al. 2015, 2016). The vaccination of MDA− birds caused stronger stimulation of $CD8^+$ T cells in the upper respiratory tract, whereas mainly $CD4^+$ T cells were stimulated in MDA+ birds (Smialek et al., 2015). In addition, IFN-γ gene expression and the number of anti-aMPV IFN-γ–secreting cells were increased mainly in MDA− birds or birds with relatively low levels of MDA (birds vaccinated on d 14 of life) on the day of TRT vaccination (Smialek et al., 2020). It was concluded that the observed differences were due to the inhibition of vaccine aMPV/A replication by MDA, which has already been reported (Jones et al., 1992; Smialek et al., 2015), and that these differences may influence the efficacy of TRT vaccination.

To our surprise, the results of the present study demonstrate that birds from all groups vaccinated with a live aMPV/A preparation, regardless of whether the vaccination was performed on day 1 or 14 of life and regardless of MDA status, were effectively immunized against the homologous experimental infection. In the vaccinated birds, no increases were observed after infection in the clinical or histopathological scores, or in aMPV replication and shedding, with few exceptions. In experiments 1, 3, and 4, a short-term increase was noted in the histopathological scores of the vaccinated and infected birds on the third, third and fifth, and seventh dpi, respectively, but the mean histopathological score in those groups of turkeys was below the maximum level determined for the infected and not vaccinated turkeys. In addition, in the MDA− birds vaccinated on day 14 of life, a short-term increase in the aMPV population was recorded at 3 dpi (on day 28 of life), in both the infected birds (M−14V-28I) and contact birds (M−14V-28I-CB). This increase could be because these birds were older and had no maternal antibodies, which promoted aMPV replication. However, the increase was short-term, and no differences were noted at 5 dpi. Although these differences were statistically insignificant, it is worth noticing that the postinfection level of aMPV replication in birds from the M+14V-28I and M−14V-28I groups and in respective contact birds was higher than that in the M+0V-14I and M−0V-14I groups, which again suggests the birds' higher susceptibility to infection in week 4 of life. The general findings from our research are in agreement with previous articles (Cook et al., 1989a,b) reporting a lack of differences in the development of postvaccination immunity between MDA+ and MDA− turkeys administered an attenuated vaccine against TRT.

It has been previously reported that turkeys subjected to specific chemical depletion of T or B lymphocytes by intravenous injection of either cyclosporin or cyclophosphamide and vaccinated against TRT were protected against experimental aMPV challenge (Jones et al., 1992; Rubbenstroth and Rautenschlein 2010). These findings question the correlation between immunity, either cell-mediated or humoral, and protection against TRT infection. On the other hand, they could explain why the MDA+ birds were still protected against experimental infection with aMPV after weaker stimulation of their immune system subsequent to vaccination against TRT, as reported in our previous research (Smialek et al. 2015, 2016, 2020). Interestingly, in the present study, differences emerged in immune system stimulation between MDA+ and MDA− birds vaccinated against TRT after aMPV/A infection. In all groups of vaccinated birds except for the M+0V poults, we showed a higher level of IgA antibodies in TW, which is in agreement with our previous findings (Smialek et al., 2016). The vaccinated and infected MDA− birds (M−0V-14I and M−14V-28I) had a higher level of specific anti-aMPV IgA in TW than the analogous MDA+ birds. In addition, the increased level of these antibodies was maintained longer after infection in the vaccinated MDA− birds (up to 7–10 dpi) than in MDA+ birds (5–7 dpi). These results confirm the higher reactivity of IgA^+ B cells against infectious aMPV/A in vaccinated MDA− birds, which has also been previously suggested (Smialek et al., 2016).

Enhanced IFN-γ gene expression was not confirmed in any of the vaccinated groups of turkeys. In all the experiments, the vaccinated and infected birds revealed a short-term increase in IFN-γ gene expression in splenocytes at 3 dpi, and the highest level of this expression was determined in the M+0V-14I group. In our previous study (Smialek et al., 2020), we demonstrated that after aMPV/A vaccination increased, IFN-γ gene expression was recorded 3 d after vaccination in MDA− birds and MDA+ birds vaccinated on d 14 of life but was not in MDA+ birds vaccinated on day 0 of life. In addition, we previously demonstrated an increased number of anti-aMPV IFN-γ–producing cells within splenocytes of MDA−, aMPV/A vaccinated birds 7 d after vaccination. We may speculate that the vaccination of MDA+ birds on d 0 of life was incapable of inducing IFN-γ gene expression (probably due to a high MDA level and suppressed vaccine aMPV replication), which, in turn, resulted in high upregulation of the gene after infection with fully pathogenic aMPV/A. This would also explain why the level of IFN-γ gene expression was relatively lower after aMPV/A infection in the other groups of vaccinated and infected birds.

CONCLUSIONS

Even though the birds from groups vaccinated against TRT were effectively immunized against experimental aMPV/A infection in all experiments (regardless of

MDA status), we found differences in the stimulation of the basic immune parameters after infection. They were probably due to dissimilarity in the mechanisms of their development after vaccination, where the mechanism depended on the level of maternal antibodies (on the day of vaccination). We demonstrated that the nonvaccinated birds infected in week 4 of life, including particularly the MDA− birds, were more susceptible to TRT development than the week-2 birds possessing maternal antibodies. Even though under laboratory conditions, all vaccinated birds were protected against homologous aMPV/A infection, and it is difficult to predict whether and how the noted differences can affect the course of TRT in flocks vaccinated under field conditions. This refers in particular to the likely mistakes associated with vaccination technique, which may negatively influence the level of postvaccination immune stimulation and, consequently, raise the percentage of birds susceptible to infection with a field virus and heighten the possibility of heterologous infections with other aMPV subtypes.

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Ethic statement: The experimental procedures and animal handling procedures were conducted with the approval of the Local Ethic Committee for Animal Experiments in Olsztyn, Poland (resolution no. 48/2017). The study was carried out in accordance with EU Directive 2010/63/EU on the protection of animals used for scientific purposes.

DISCLOSURES

The authors declare that there is no conflict of interest.

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