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Original article

Effect of infection of turkeys with haemorrhagic enteritis adenovirus isolate on the selected parameters of cellular immunity and the course of colibacillosis

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Abstract

The aim of the study was to determine the effect of a Polish low-virulence isolate of haemorrhagic enteritis adenovirus (HEV) on the immune system in turkeys and on the course of colibacillosis in birds infected under laboratory conditions. Turkeys were infected *per os* with HEV at the dose of $10^{4.3}$ EID₅₀/mL and with *E. coli* (APEC) (serotypes 078:K80:H9) at the dose of 4×10^9 CFU/mL by injection to the thoracic air sac. The birds infected with the HEV were infected with the APEC either simultaneously or after 5 days. Five days after HEV infection, the percentages of subpopulations of the CD3⁺CD4⁺ and CD3⁺CD8 α ⁺ T cells and the IgM⁺ B cells were determined in blood and spleens of the HEV-infected turkeys and in the control (uninfected) birds.

The course of colibacillosis was more severe in turkeys infected with the APEC 5 days after infection with the HEV than in those infected with the HEV and APEC simultaneously and than in those infected only with APEC. Five turkeys out of the 18 infected with the APEC 5 days after infection with HEV, died. Their body weights were statistically significantly lower with higher FCR values 41 days after the infection in comparison to turkeys in the other groups.

A considerable decrease in the percentage of the T and B cells subpopulations in the blood were found in turkeys infected with the HEV and while the percentage of CD3⁺CD4⁺ T cells subpopulation in the spleen increased significantly, the contribution of the CD3⁺CD8 α ⁺ T cells and IgM⁺ B cells subpopulations were decreased. These changes in the immune system of turkeys, occurring 5 days after infection with the HEV, made them more susceptible to infection with the APEC.

Key words: turkeys, HEV, colibacillosis, PCR, T- and B-lymphocytes subpopulations

Introduction

Infections with avian pathogenic *Escherichia coli* (APEC) are a cause of serious losses in a mass poultry breeding. According to Yogaratnam (1995) about 43% of slaughtered broilers had lesions indicating colisepticemia. The various clinical forms of APEC infections have been reported, most often in chickens and turkeys. This is caused by different virulence factors of APEC, host susceptibility to infection as well as resistance factors (environmental toxins, immunity, nutrition) (Barnes et al. 2003). Infections with the haemorrhagic enteritis virus (HEV) are a frequent factor which makes turkeys more susceptible to infections with the APEC and the development of the clinical form of colibacillosis (Newberry et al. 1993, van den Hurk et al. 1994, Pierson et al. 1996). The quoted authors conducted their studies both under laboratory conditions and in the field, on turkeys infected with the HEV of different virulence levels. The HE virus isolated in Poland in 1988 (Koncicki 1990) is a low-virulence pathogen (Koncicki 1996). Infections caused by this virus in flocks of turkeys in Poland are very common (Koncicki 1996).

HEV replicates in the B cell subpopulations, which produce multi-specific IgM antibodies and in the macrophages, causing damage to these cells and decreasing immunity (Suresh and Sharma 1995, 1996). In such cases, the susceptibility of turkeys to infections with opportunistic pathogens (which include *E. coli*) increases (Saunders et al. 1993).

In the light of the above a research study was conducted, under laboratory conditions, with the aim of determining the effect of a Polish low-virulence HEV isolate infection on the selected parameters of immune responsiveness of turkeys and on the course of colibacillosis in those birds.

Materials and Methods

Ninety-four female white BUT 9 broad-breasted turkeys were used in the experiment. From the first day after hatching they were raised in accordance with the current technology in isolated pens of the Pavilion of Avian Experimental Infections at the Department of Avian Diseases of the University of Warmia and Mazury in Olsztyn. At the age of 38 days the turkeys were randomly divided into five groups (group I, II and IV with 18 turkeys, respectively, and groups III and V with 20 birds each). The birds in each group were kept in strictly isolated pens.

At the age of 42 days the turkeys from groups I, II and III were infected *per os* (with a probe put into the crop) with the HEV at the dose of $10^{4.3}$ EID₅₀/mL. The

turkeys in group I were infected simultaneously with pathogenic *E. coli* serotype O78:K80:H9, while birds in group II were infected five days later by injecting to the thoracic air sac the volume of 1 mL of bacteria suspension at the density of 4×10^9 CFU/mL (Krasnodębska-Depta et al. 2003). Turkeys in group III constituted a control for infection with the HEV and those in group IV – for infection with the *E. coli*, while the turkeys in group V were the uninfected control.

Clinical observation of the birds in all groups was carried out for 6 weeks and during this observation clinical symptoms, mortality (anatomopathological examinations of the dead birds and bacteriological examinations of the sections of their internal organs, such as liver, heart, spleen, were conducted), fodder consumption and increase of body weight were registered. The birds were weighed at days 17, 27 and 41 of the experiment.

On day 5 of the experiment (day 47 of the turkeys' lives), 3 birds from each group I, II and IV and 5 turkeys from each group III and V were decapitated (turkeys from group II were decapitated 6 hours after *E. coli* infection) in order to determine the spleen index (SI – contribution of spleen mass (SM) in turkeys' body mass (BM) – $(SM/BM) \times 1000$), assess the anatomopathological changes typical of infections with the HEV and *E. coli* and to collect the necessary sections of the spleen in order to show the existence of the genetic material of the virus using the PCR techniques. Sections of the liver, heart and spleen were also collected for the bacteriological examinations.

During decapitation, 2 mL samples of blood were taken from five turkeys from groups III and V. The blood was collected directly to the tubes with anticoagulant (1.6 mg of EDTA/mL of the blood; Sarstedt, Germany) and then placed on a haematological stirrer. Subsequently, sections (about 1 g each) of spleen from the decapitated birds were collected into tubes with 5 mL of the RPMI-1640 medium (Sigma-Aldrich, Germany) and cooled to 4°C. The percentage of subpopulations of the CD3⁺CD4⁺ and CD3⁺CD8α⁺ T cells as well as the IgM⁺ B cells were determined in the blood and spleens of turkeys.

Bacteriological examinations of the internal organs sections of the dead and euthanized birds were carried out by routine bacteriological methods in order to check for *E. coli* infection and the growth of characteristic colonies of bacteria was evaluated. Suspension of *E. coli* infective for turkeys was prepared by culturing the bacteria in BHI medium (Difco Laboratories, USA) before transferring into the McConkey medium.

Genomic DNA was isolated with the use of the Genomic Mini kit (A&A Biotechnology, Poland), in

accordance with the procedure provided by the manufacturer. Primers HEV1F and HEV2R were constructed to be able to hybridise in the loop regions L1 and L4 of the HEV hexon gene. The precise location of primers on the HEV hexon gene is: HEV1F: 5'-TAC-TGCTGCTATTTGTTGTG-3', NT 729-748 and HEV2R: 5'-TCATTAACTCCAGCAATTGG-3', NT 2375-2356. The Dindoral[®] SPF (Merial, France) live vaccine was taken as a positive control and the spleen from a one-day-old turkey was included as a negative control.

The PCR reaction was carried out in a Mastercycler pro thermocycler (Eppendorf, Germany) according to the method described previously by Hess et al. (1999) and Mazur-Lech et al. (2009).

Of the resulting PCR mixture, 5 µL was analysed by electrophoresis on 0.5% agarose gel stained with ethidium bromide. The Gene Ruler 100 bp Ladder Plus marker (MBI Fermentas, Lithuania) was used as the molecular weight standard. The results of the amplicon electrophoresis were read out under UV light with a GelDoc XR+ visualising system (Biorad, USA) and archived as digital photographs.

In order to determine the percentage of the CD3⁺CD4⁺ and CD3⁺CD8α⁺ T cells and the IgM⁺ B cell subpopulations, 1.5 mL samples of the blood were transferred to the tubes containing 1.5 mL of PBS buffer with 1% FCS. Such prepared blood samples (3 mL volume) were then gently layered over 3 mL of the Histopaque-1077 gradient (Sigma-Aldrich, Germany) and centrifuged at 400 g at room temperature for 30 min. After centrifuging, the mononuclear cells were aspirated gently and washed twice with PBS buffer with 1% FCS. Thereafter, they were suspended in 1 mL of PBS and counted with an automatic cell counter (Vi-Cell, Beckman Coulter, USA). 1x10⁶ of suspended cells were transferred to cytometric tubes (Becton Dickinson; BD, USA). The monoclonal antibodies (in the volume recommended by the manufacturer), specific for the surface receptors of the T (CD4 and CD8α) or B (IgM) cells, were added to each of the tubes. Mouse Anti Chicken CD4-FITC (clone 2-35), Mouse Anti Chicken CD8A-PE (clone 11-39) and Rat Anti Human CD3 Epsilon-APC (clone CD3-12) monoclonal antibodies, as well as Goat Anti Chicken IgM-FITC polyclonal antibodies (Abd Serotec, UK) were used in the experiment.

The samples were incubated for 30 minutes on ice. The cells were subsequently washed twice with PBS (centrifuged at 250 g for 7 minutes at 4°C) and the resulting pellets were treated with 100 µL of fixing medium (Reagent A, Leucoperm, Abd Serotec, UK) and incubated for 15 minutes at the ambient temperature. After incubation, 3 mL of PBS buffer was added

to each tube and centrifuged for 5 minutes at 300 g. The supernatants were decanted, cell pellets were suspended in 100 µL of permeabilizing medium (Reagent B, Leucoperm, Abd Serotec, UK) and 10 µL of monoclonal antibodies (Rat Anti Human CD3 Epsilon) stained with APC were added. The samples were shaken vigorously on a vortex and incubated for 30 minutes at the ambient temperature. After incubation, 3 mL of PBS buffer was added to each tube and tubes were centrifuged for 5 minutes at 300 g. The supernatants were decanted and the cell pellets were suspended in 0.5 mL of PBS and examined with the FACSCanto II (BD, USA) flow cytometer. Data were acquired during the flow cytometry determination with specialist FACSDiva Software 6.1.3 (BD, USA), recording 30,000 events for each sample. Analysis and immunophenotyping of the cells were determined with the FlowJo 7.5 program (Tree Star, USA).

In order to determine the CD3⁺CD4⁺ and CD3⁺CD8α⁺ T cells and IgM⁺ B cells subpopulations in the spleen, approximately 0.35-0.4 g fragments were taken (in the laminar chamber) from spleen sections, placed in 1 mL of RPMI-1640 medium with 5% FCS and homogenised individually in automatic homogeniser Tissue Lyser II (Qiagen, Germany). After uniform suspensions were obtained, they were filtered through a 70 µm mesh, sterile nylon cellular filters (BD, USA). The isolated cells were counted and adjusted to a concentration of 2x10⁷/mL in RPMI-1640 medium with 5% FCS. The remaining procedure was identical with the procedure of determination of subpopulations of T and B cells in blood.

Statistic analyses were performed using the StatisticaPL and the WINSTAT software packages and calculations were conducted with the use of analysis of variance at the level of significance of α=0.05. Statistical differences were calculated using the Duncan's test.

Results

The results of infections of turkeys with HEV and APEC are shown in Table 1. Data provided in the table show that the turkeys used in the experiment were susceptible to infection with HEV which is indicated by increased SI and the marble-like appearance of the spleen, typical for infection with this HEV virus. Moreover, the presence of the HEV genetic material in the spleens of turkeys infected with the virus was shown by the PCR techniques (Fig. 1). The turkeys infected with the HEV virus and the APEC, as well as those infected only with the APEC, became apathetic and listless 12 hours after experimental infection. This condition lasted for 72 hours and then birds'

Table 1. The results of infection of turkeys with HEV and *E. coli* (n=18-20; $\bar{x} \pm SD$).

Group of turkeys	SI 5 days after infection with HEV	Detection of genetic material of HEV in the spleen of turkeys by PCR/number of birds examined	Clinical symptoms	Deaths	Isolation of <i>E. coli</i>
I Infected simultaneously with HEV and <i>E. coli</i>	2.67 ± 0.12 ^A	3/3	+	2/18	+
II Infected with HEV and <i>E. coli</i> 5 days later	2.81 ± 0.48 ^A	3/3	+	5/18	+
III Infected with HEV	2.76 ± 0.26 ^A	3/3	-	0/20	-
IV Infected with <i>E. coli</i>	1.62 ± 0.32 ^B	0/3	+	2/18	+
V Uninfected	1.58 ± 0.16 ^B	0/3	-	0/20	-

AB - p ≤ 0.01

Table 2. Production results of turkeys infected with HEV and *E. coli*.

Group of turkeys	Body weight (kg) on day after infection			FCR kg/kg of b.w.
	17	27	41	
I Infected simultaneously with HEV and <i>E. coli</i>	3.53 ± 0.16 ^A	4.01 ± 0.26 ^A	5.19 ± 0.35 ^A	2.39
II Infected with HEV and <i>E. coli</i> 5 days later	3.36 ± 0.20 ^A	3.88 ± 0.17 ^A	4.88 ± 0.25 ^B	2.49
III Infected with HEV	3.67 ± 0.11 ^A	4.76 ± 0.27 ^{Ba}	6.26 ± 0.08 ^C	2.11
IV Infected with <i>E. coli</i>	3.64 ± 0.11 ^A	4.46 ± 0.20 ^{Bb}	5.31 ± 0.37 ^A	2.31
V Uninfected	4.00 ± 0.30 ^B	4.86 ± 0.36 ^{Ba}	6.36 ± 0.14 ^C	2.09

ABC - p ≤ 0.01; ab - p ≤ 0.05

Table 3. Percentage of CD3⁺CD4⁺ and CD3⁺CD8α⁺ T-lymphocyte and IgM⁺ B-lymphocyte subpopulations in blood and spleens of turkeys 5 days after infection with hemorrhagic enteritis virus.

Group of turkeys	Percentage of T- and B- lymphocyte subpopulations in turkeys					
	Blood			Spleen		
	CD3 ⁺ CD4 ⁺	CD3 ⁺ CD8α ⁺	IgM ⁺	CD3 ⁺ CD4 ⁺	CD3 ⁺ CD8α ⁺	IgM ⁺
Noninfected	21.38 ± 0.94 ^a	2.87 ± 0.24 ^A	2.86 ± 0.80 ^a	12.93 ± 2.59 ^A	49.11 ± 5.51 ^A	24.67 ± 2.51 ^A
Infected	17.34 ± 1.25 ^b	1.76 ± 0.32 ^B	2.34 ± 0.69 ^b	40.51 ± 4.42 ^B	30.59 ± 4.7 ^B	11.67 ± 0.88 ^B

AB - p ≤ 0.01; ab - p ≤ 0.05

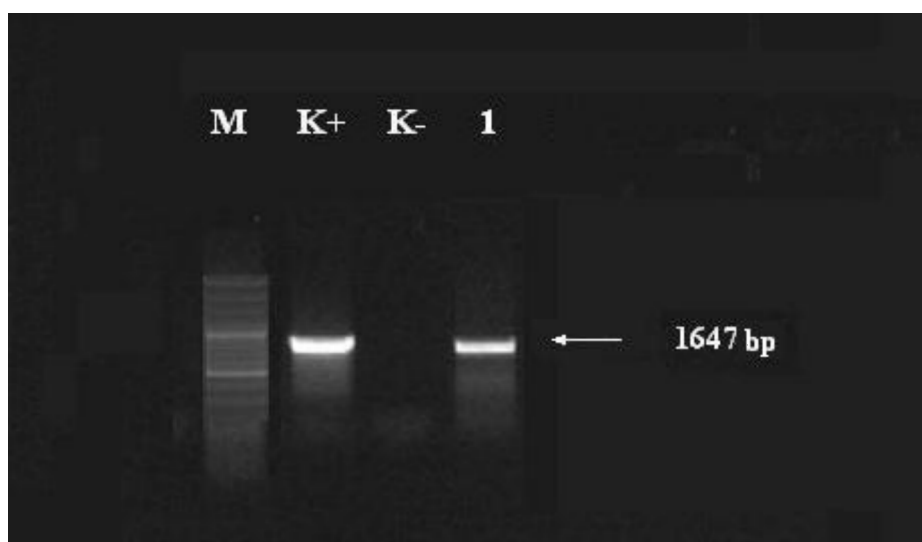


Fig. 1. PCR detection of HEV genetic material in the spleen of turkey 5 days after infection. The size of the PCR product is 1647 bp.

behaviour gradually returned to normal. The turkeys in group II (birds infected with the APEC 5 days after infection with the HEV) had more severe clinical symptoms of colibacillosis, and some of the birds started to limp 96 hours after infection. One bird in this group died after 12 hours from infection and 4 others died after 36 hours. Two turkeys died in group I and IV approximately 48 and 72 hours after infection. Anatomopathological examinations of the dead turkeys revealed typical colibacillosis lesions and *E. coli* bacilli were isolated during the bacteriological examinations (Table 1).

The production results for turkeys infected with HEV and APEC are shown in Table 2. The data provided in the table indicate that the growth of the turkeys infected with HEV and with APEC 5 days later (group II) was inhibited. The average body weight of turkeys in this group amounted to 4.88 kg 41 days after the infection, with the FCR value of 2.49. The body weight of uninfected turkeys (group V) was equal to 6.36 at the time and the FCR was 2.09 while these values for group IV of turkeys, infected only with the APEC, were 5.31 kg and 2.31, respectively.

The percentage of subpopulations of the T and B cells in turkeys infected with the HEV and in the uninfected ones are shown in Table 3. The data provided in the table show that the percentage of all the examined lymphocyte subpopulations in the blood decreased significantly on day 5 after infection with the HEV. However, the percentage of the CD3⁺CD4⁺ T cells increased significantly and that of the CD3⁺CD8^α⁺ T cells and the IgM⁺ B cells decreased, at the same time, in the spleens of turkeys infected with the HEV.

Discussion

Although HEV was first isolated in Poland in 1988 (Koncicki 1990), no detailed studies have been conducted so far to determine the effect of the virus on the immune system of turkeys or on the susceptibility of birds infected with the HE virus to infection with opportunistic pathogens, such as *E. coli*. The clinical observation (Koncicki 1996, 2004) and assessment of immune response to immunisation of turkeys against ND (Guirou and Koncicki 2004) indicated a decrease of the immunity in turkeys infected with HEV and consequently worsened production results. The current study determined the effect of infection of turkeys with the Polish HEV isolate on the functioning of their immune system and related susceptibility to infection with a pathogenic strain of *E. coli* (APEC). The flow cytometry method was used to determine the functionality of the turkeys' immune sys-

tems. The CD4 and CD8 T lymphocytes receptors were marked out with monoclonal antibodies intended for chickens, with a cross-reactivity with turkey lymphocytes, which has been certified in multiple studies (Li et al. 1999, 2000, Powell et al. 2009). The amino acid sequence in the endoplasmatic domain of the Σ chain of CD3 receptor of the T cells is highly conservative (Göbel and Fluri 1997, Kothlow et al. 2005) and it is very similar in many animal species and humans, therefore the CD3⁺ cells could be detected in turkeys with the use of Rat Anti Human CD3-APC (clone CD3-12) monoclonal antibodies. The IgM⁺ B cells were marked out with the Goat Anti Chicken IgM-FITC polyclonal antibodies which show cross-reactivity to the turkeys' IgM (van Nerom et al. 1997). In the light of the above, the results of cytometric analysis can be considered as fully credible and they indicate that the percentage of examined subpopulations of the T and B lymphocytes decreased in the blood of turkeys infected with the HEV, while the percentage of the CD3⁺CD4⁺ T cells in the spleens increased, whereas that of CD3⁺CD8^α⁺ T cells and the IgM⁺ B cells decreased in the spleens of such turkeys. Disturbance of the function of the immune system of turkeys infected with the HEV resulted in their higher susceptibility to infection with the APEC and the occurrence of more severe clinical form of colibacillosis. These findings are significant because of the low-virulence of the HE virus used in the experiment and that the infections with this virus in the field caused no clinical signs while post-mortem examination revealed only the enlargement and marble-like appearance of the spleen which is typical for the infection with the HE virus. The findings of this study are similar to those of other authors (Sponenberg et al. 1985, Newberry et al. 1993, van den Hurk et al. 1994, Pierson et al. 1996).

The present study indicates that the Polish HEV isolate, despite its low virulence, causes immunosuppression in infected turkeys, which makes the birds more susceptible to infections with other pathogens, including *E. coli*.

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