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Original article

Interactions between Marek's disease virus Rispens/CVI988 vaccine strain and adenovirus field strain in chicken embryo fibroblast (CEF) cultures

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Abstract

The aim of the study was to determine the influence of adenovirus infection on the replication of Marek's disease virus vaccine strain Rispens/CVI988 during *in vitro* co-infection studies. Adenovirus field strain JN-5/10j was isolated from sick chickens. The study was conducted in chicken embryo fibroblast cultures (CEF). Monolayers of CEFs were infected with Rispens strain and field adenovirus strain JN-5/10j with different doses ($10^{1.0}$-$10^{3.0}$ $TCID_{50}$) in the following manner: a) simultaneously, b) first, infection with Rispens strain and after 24 h infection with adenovirus strain JN-5/10j and c) infection with adenovirus strain JN-5/10j 24 h before infection with Rispens strain. After 18, 24, 48, 72, and 96 h of incubation, the copy number of the pp38 gene of Rispens strain was determined using Real-time PCR. The results indicated that the Adenovirus infection before the infection with Rispens strain reduced the replication of the pp38 gene after 48 h by 2 $\log_{10}$.

Key words: Adenovirus strain, interactions, Marek's disease virus vaccine strain Rispens/CVI988, Real-time PCR

Introduction

Marek's disease virus (MDV) belongs to the *Herpesviridae* family and is the aetiological agent of Marek's disease (MD) which causes tumoral lesions in affected chickens (Tian et al. 2012). Marek's disease has been reduced by prophylactic vaccinations for over 40 years. Monovalent vaccines based on the FC126 HVT strain or Rispens/CVI988 strain and bivalent vaccines based on both of these strains have been widely applied in MD cases.

Nowadays, we observe an increase in the number of MD cases in birds. Despite the fact of vaccination, the clinical signs and lesions specific for MDV have been confirmed in birds co-infected with fowl adenoviruses (FAdV), which are ubiquitous viruses commonly existing in poultry flocks (Hess 2000, Grgić et al. 2006, McConnell and Fitzgerald 2008). The effect

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of fowl adenovirus field strain infection on the replication of turkey herpesvirus FC 126 (HVT) vaccine strain in chicken embryo fibroblast cultures has been already determined (Niczyporuk et al. 2012).

The pathogenic role of adenoviruses is not clear sufficiently. Adenovirus infections may cause the clinical form of diseases such as inclusion body hepatitis (IBH) (Singh et al. 2006), hydropericardium syndrome (HP) (Geanesh et al. 2002), haemorrhagic enteritis virus (HE) (Hess et al. 1999, Mazur-Lech et al. 2009), Egg drop syndrome (EDS) (Dhinakar et al. 2003) or gizzard erosion and ulceration (GEU) (Okuda et al. 2006). Such infections play an important role in polietiological infections of the respiratory and digestive tract (Hess 2000, McConnell and Fitzgerald 2008) or may proceed without any clinical signs of infection. They may be the reason for immunodeficiency in affected birds (Naeem et al. 1995, Shivachandra et al. 2003, Schonewille et al. 2008).

Adenoviruses are icosahedral non-enveloped double stranded DNA viruses about 70-90 nm in diameter (Chiocca et al. 1996, McConnell and Fitzgerald 2008). The virus capsid consists of 252 capsomers. A capsomer is a trimer of hexon proteins. The main protein of the virus is encoded by the hexon gene with molecular weight about 103 kDa (Roberts et al. 1986, Payet et al. 1998). This is the main protein, which determines the function and antigenic properties of the adenoviruses. The size of the protein depends on the species and serotype of the virus and is between 930-950 aa (Chiocca et al. 1996, McConnell and Fitzgerald 2008).

The aim of the study was to determine the influence of adenovirus infection on the replication of Marek's disease virus Rispens vaccine strain during *in vitro* study.

Materials and Methods

Chicken Embryo Fibroblast Cultures (CEF). CEFs were prepared from 9-11-day-old SPF chicken embryos (Lohmann, Germany) according to the standard procedure. Eagle's growth medium MEM (Gibco, Scotland) supplemented with 10% of fetal bovine serum and 0.1% of antibiotic mixture (Antibiotic – Antimycotic, Gibco, Scotland) was used as a growth medium. The maintaining medium consisted of MEM with 0.1% of antibiotic antimycotic mixture. A monolayer of CEFs was received after about 18-24 h of incubation at 37.5°C and 5% CO_2.

FAdV reference strain. The reference FAdV strain, which belongs to the serotype FAdV-7 was acquired as a lyophilisate from a commercial company (Charles River Laboratory, USA), and was used as a positive control in Real-time PCR.

Adenovirus field strain. Field adenovirus strain JN-5/10j representing serotype FAdV-7 was derived from 7-week-old chickens with MDV infection. Based on the phylogenetic analyses of the L1 fragment of the hexon gene, the JN-5/10j strain was classified as serotype FAdV-7. The 3rd passage of the strain with the titre ($10^{1.0}$-$10^{3.0}$) was used for the infection of CEFs.

MDV vaccine strain. Marek's disease virus Rispens/CVI988 strain derived from commercial vaccine (Mérial, France) was used.

Experiment setup and sample collection. CEF monolayers were infected with Rispens strain at a dose of $10^{3.0}$ $TCID_{50}$ and co-infected with different doses of adenovirus strain JN-5/10j ($10^{1.0}$-$10^{3.0}$ $TCID_{50}$) in three different combinations corresponding to three different trials: 1st trial: simultaneously, 2nd trial: first, infection with Rispens strain and after 24 h infection with adenovirus field strain, and 3rd trial: adenovirus infection 24 h before infection with Rispens strain. Every combination of infected CEFs was performed in triplicate.

All CEFs used as a positive control for adenovirus JN-5/10j and Rispens strain were also performed in triplicate.

After 18, 24, 48, 72, and 96 h of incubation, infected CEFs were observed and all samples were collected.

DNA extraction. Extraction was performed using DNA Mini Kit (Qiagen, Germany) according to the standard procedure. Total cellular DNA was extracted directly from CEFs infected with adenovirus field strain and Marek's disease virus vaccine strain. DNA was also extracted from non-infected CEFs as a negative control. As a positive control DNA from adenovirus and Rispens strains were isolated from infected CEFs. DNA samples were stored at -20°C for the next tep of the experiment.

Primer and probe design. Primers for amplification of the pp38 gene of Rispens vaccine strain were designed using GenBank database and Primer 3 software. Forward and reverse primer sequences were as follows: for the pp38 gene of Rispens: pp38-F 5' CCC CAT CTG CTT CAT ACC AT 3', pp38-R 5' GTG ATG GGA AGG CGA TAG AA 3' and the *Taqman* probe – 5' CCA CCG TGA CAG CCA CTC TC 3', respectively. The fluorogenic probe was labeled with reporter: 6-carboxyfluoroscein (FAM) at the 5' end and with tetramethylcarboxyrhodamine (TAMRA) as a quencher at the 3' end of the sequence.

Specific oligonucleotide primers were designed also for the adenovirus strain using GenBank database and Primer 3 software. The primers were used to amplify the 93 bp region of the hexon loop L1

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gene of the adenovirus belonging to serotype 7 of FAdV. The primers were synthetized at the Institute of Biochemistry and Biophysics at the Polish Academy of Sciences. The sequences of nucleotide primers were: FAdV F JSN: 5'AATGTCACNACCGARAAGGC 3' and FAdV R JSN: 5'CBGCBTRCATGTACTGGTA 3'.

Real-time PCR for the identification of pp38 and hexon genes. Reaction was conducted using QuantiTect PCR Probe (Qiagen). The final volume of the master mix was 25 µL and contained: 2.5 µL of 2x QuantiTect Probe PCR Master Mix, 1 µL (0.4 µM) of each primer for the pp38 gene of Rispens vaccine strain, or 1 µL (0.4 µM) of each primer for the hexon gene of adenovirus field strain, 0.1 µL (0.02 µM) *Taqman* probe for the pp38 gene and 5 µL of deionised water. The reaction was carried out in plastic 96-well plates. As the negative control DNA isolated from non-infected CEFs was used. Two microliters of tested DNA template were added to each well. The conditions for the identification of the pp38 gene were as follows: 50°C for 2 min, 95°C for 15 min: 40 cycles – 94°C for 15 s and 60°C for 1 min. The conditions for the identification of the hexon gene were as follows: 50°C for 2 min, 95°C for 15 min, 40 cycles – 94°C for 15 s and 55°C for 1 min.

The quantity of pp38 gene copy number was analysed using computer software (Applied Biosystems, Version 2.0.1) on the basis of standard curves.

The presence of the FAdV hexon gene was analysed using Applied Biosystems 7500 system software.

Plasmid standards. pPP38 plasmids were constructed by cloning the 188 bp fragment of the pp38 gene into the pGEM-T Easy Vector (Promega, US) and Blue/White X-Gal/IPTG selection. pPP38 was then amplified in *E.coli* (DH5α) (Invitrogen, US) in liquid LB medium with the addition of 100 µg/ml ampicillin in 37°C Max 4000Q apparatus (Barnstead/Lab-line). Extraction of plasmid DNA was performed from 10 ml of liquid 18 h culture of *E.coli* (DH5α) cells using Plasmid Maxi Kit (Qiagen, Germany).

Quantification of the pp38 gene copy number. This method was evaluated for the quantity of pp38 gene exact copy number in examined samples using standard curve in Applied Biosystems software (Version 2.0.1). Quantification of the viral copy number was performed according to the previously described procedure (Baigent et al. 2005). On the basis of serial ten-fold dilutions of pPP38 from $10^{2.0}$ to $10^{5.0}$ DNA copies/1 µL, 4 point standard curves were prepared and used for pp38 MDV gene copy number calculation. The fluorescent curve indicated the amplification of specific fragments for examined genes.

Statistical analysis. Statistical differences between the average copy number of the pp38 gene in coinfected CEFs and the control – single infection of CEFs were calculated by Student's t- test. The significance value was set as $P<0.05$.

Results

During the first trial with simultaneous infection the copy number of the pp38 gene of Rispens strain was determined between $10^{9.5}$ at 18 h to above $10^{11.0}$ copies at 96 h of incubation during infection with FAdV strain with $10^{1.0}$ $TCID_{50}$ dose. From 18 h untill 96 h of incubation, the copy number of the pp38 gene was determined to be from $10^{10.0}$ to $10^{11.0}$ in CEFs infected with $10^{2.0}TCID_{50}$ of FAdV field strain, and in the case of $10^{3.0}TCID_{50}$ dose of FadV the copy number of pp38 ranged from $10^{9.0}$ to $10^{10.5}$. The replication of Rispens strain alone in the control cultures showed a slightly lower copy number from $10^{7.0}$ after 18 h p.i. to $10^{10.0}$ after 96 h of incubation (Fig. 1). The results indicated a lack of significant difference between the copy number of the pp38 gene of Rispens strain in examined samples and the control samples ($P>0.05$).

Similar results were obtained in the second trial when CEFs were infected with Rispens strain, then after 24 h with FAdV strain. The copy number of the pp38 gene of Rispens ranged from $10^{9.2}$ at 18 h of incubation to $10^{11.5}$ copies at 96 h of incubation with the FAdV strain at a dose of $10^{1.0}TCID_{50}$. Infection with FAdV strain at a dose of $10^{2.0}TCID_{50}$ resulted in the pp38 copy number ranging from $10^{8.5}$ to $10^{11.0}$. During FAdV infection at the $10^{3.0}TCID_{50}$ dose, the copy number of the pp38 gene was between $10^{8.5}$ and $10^{9.5}$ copies. Similarly to the previous experiment, the replication rate of Rispens strain in control CEFs without FAdV was slightly lower than in coinfected cultures. The results are presented in Fig. 2. During these trials statistical analysis was done. The mean copy number of the pp38 gene in the examined samples and control samples was not significantly different ($P>0.05$). Adenovirus infection after herpesvirus infection had a small influence on the replication of the pp38 gene in the examined samples.

During the third experiment, in contrast to the previous trials, a negative influence of the adenovirus strain on the replication of Rispens strain was observed. The copy number of the pp38 gene was from $10^{5.5}$ to $10^{6.0}$ during the replication of this strain with different FAdV doses. The replication was inhibited by FAdV strain. The copy number of Rispens strain within the control culture reached from $10^{6.0}$ to $10^{7.5}$ and was higher by 0.5 to 2 log_{10} in 96 h. The results are presented in Fig. 3. There were statistically significant differences between the pp38 copy number in groups after dual infection and the control group ($P<0.05$).

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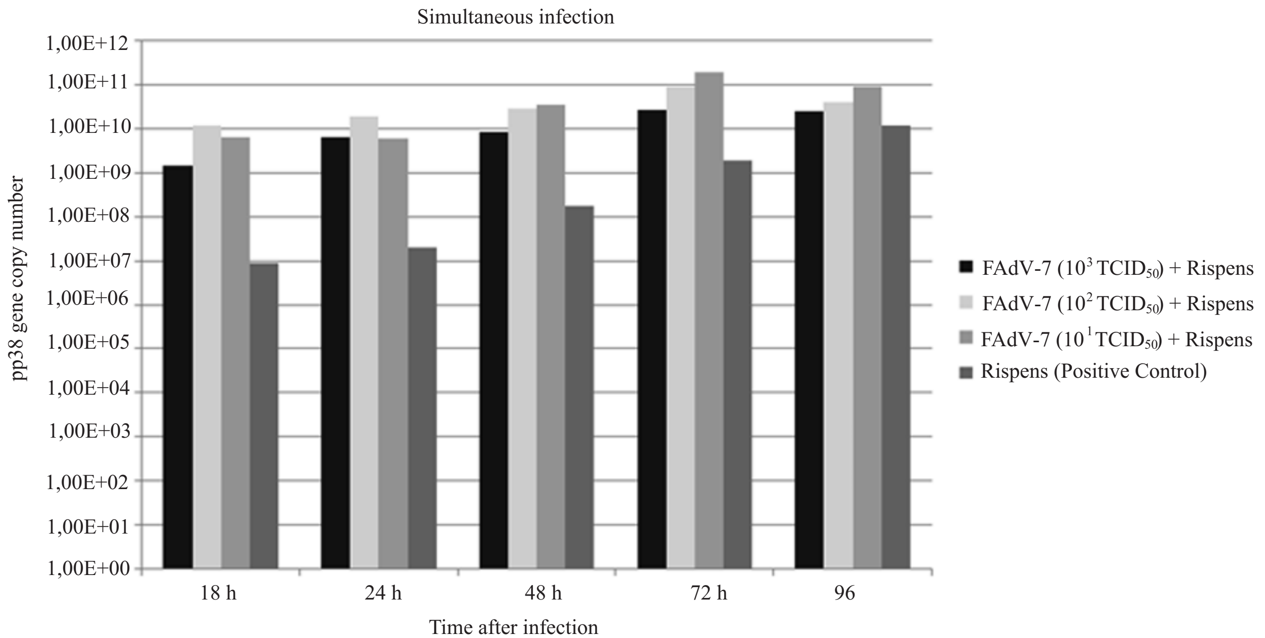

Fig. 1. Simultaneous infection. Average copy number of the pp38 gene of Rispens vaccine strain propagated in CEF with adenovirus field strain JN-5/10j ($10^{1.0}$-$10^{3.0}$$TCID_{50}$) (lack of significant difference P>0.05).

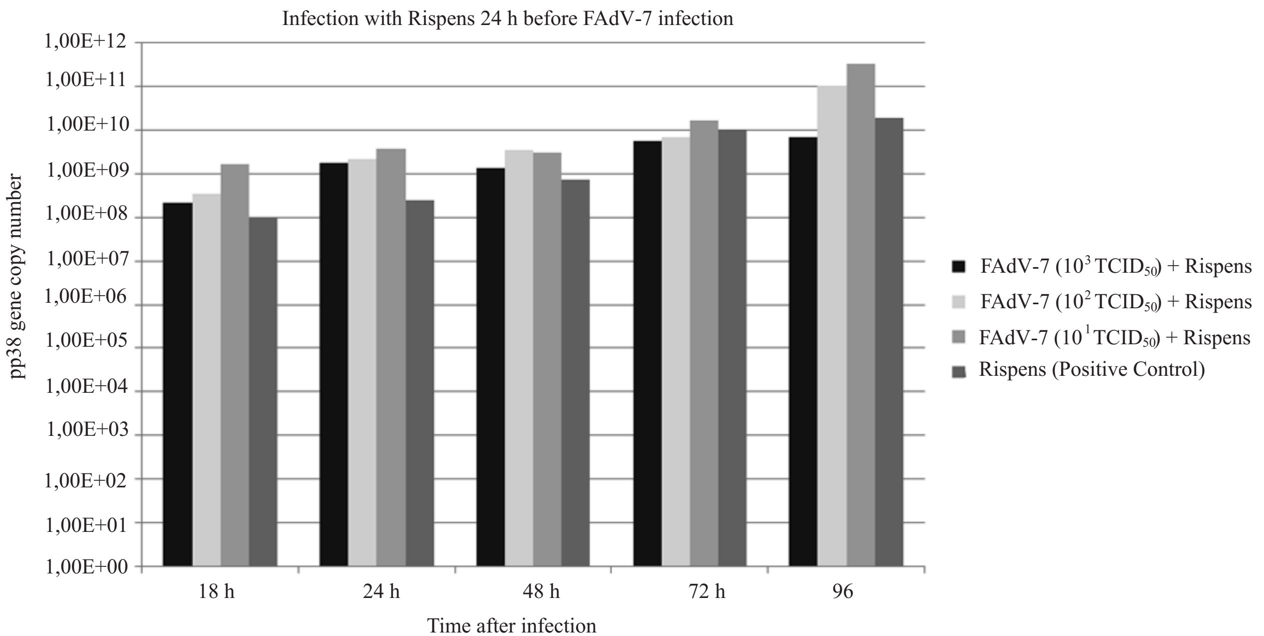

Fig. 2. Average copy number of the pp38 gene of Rispens vaccine strain propagated in CEF 24 h before infection with adenovirus strain JN-5/10j ($10^{1.0}$-$10^{3.0}$$TCID_{50}$) (not significantly different P>0.05).

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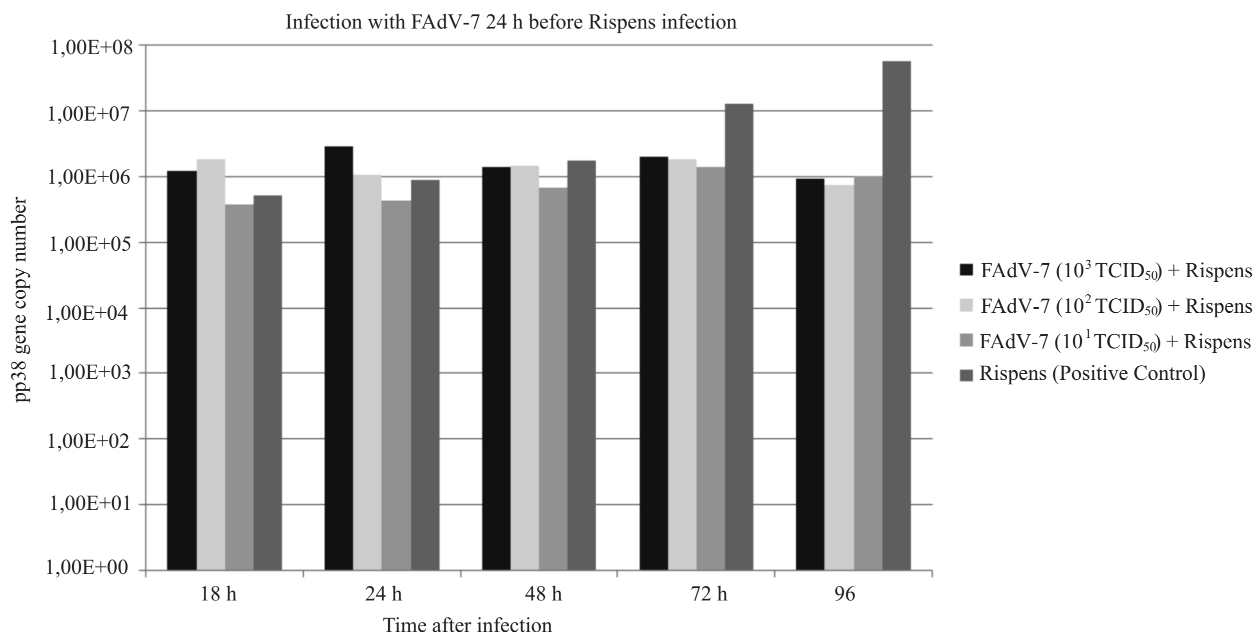

Fig. 3. Average copy number of the pp38 gene of Rispens vaccine strain propagated in CEF with adenovirus JN-5/10j strain ($10^{1.0}$-$10^{3.0}$TCID$_{50}$) 24h before infection with CVI988/Rispens. Statistically significant differences $P<0.05$.

Adenovirus infection before herpesvirus infection had an influence on the Rispens strain pp38 gene copy number.

Discussion

Coinfection with two viruses from the same and different strain or species occurs on all levels of evolutionary organisation, starting from Archea infected with several phages, to plants, and animals (for example Adeno-Associated Virus which needs a coinfection with adenovirus or herpesvirus) (Buller et al. 1981, Goncalves 2005). Organism infection with only one virus is an obvious approach during laboratory study, but this rarely occurs in nature. The effect of viral infection such as a change in the cell's genome, modification of cell receptors, and interference with the organisms' immunological system, may affect the course of another virus infection. In addition, asymptomatic infections play a role as a background for all future infections.

The previous study on the interactions between fowl adenovirus representing serotype 7 of FAdV and turkey herpesvirus FC 126 conducted in CEFs indicated that infection with adenovirus lowered replication of FC 126 (HVT) in CEFs (Niczyporuk et al. 2012). The study of these interactions allowed us to deduce the influence of adenovirus infection in birds vaccinated against MD. The results may indicate the inhibition of the replication of Marek's disease virus vaccine, Rispens strain, which may occur in cases of adenovirus infections in birds, thus influencing the effectiveness of immunoprophylaxis.

Other studies indicated that virulent strains of adenoviruses FAdV-4 have a predilection for the lymphoid tissues (Dhinakar et al. 2003, Tian et al. 2012). The immunosuppressive influence of adenoviruses on the immune response of birds has been observed with different virulent adenovirus strains (Samorek--Salamonowicz 1986, Saifuddin and Wilks 1992).

In contrast, a pathogenic course of adenovirus infection can be induced by co-infection with another virus or previous immunosuppression, as presented in studies on FAdV and chicken anemia virus (CAV) coinfection (Toro et al. 2000). Furthermore, previous studies showed that the infection of adenovirus-infected cells by herpesvirus may lead to inhibition of adenovirus-RNA synthesis decreasing the rate of adenovirus protein synthesis (Spector and Pizer 1978).

Our results showed the significance of simultaneous adenovirus and herpesvirus infection on the copy number of the pp38 gene specific for Rispens strain vaccine. As shown by Lappalainen (2012) the risk of intestinal intussusceptions of young children caused by HHV-6 occurs in the presence of other viruses.

The aim of this study was to clarify if the observed Marek's disease virus infections in poultry flocks vaccinated against MD, might be related to adenovirus infections, which commonly coexist in the environment and may decrease the effectiveness of vaccination. The *in vitro* results were useful for the analysis of the influence of adenovirus field strain on the replication of Marek's disease virus Rispens strain vaccine.

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Conclusion

The results indicate that CEF cultures which have been infected by adenovirus strain and then 24 h later by a vaccine strain reveal that the adenovirus strain lowered the replication of Rispens vaccine strain by about 2 log_{10}. In other trials the inhibition was not observed.

The influence of Rispens vaccine strain on the replication of adenovirus field strain JN-5/10j was not observed. The obtained results will be confirmed during *in vivo* studies on chickens.

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References

Baigent SJ, Petherbridge LJ, Howes K, Smith LP, Currie RJ, Nair VK (**2005**) Absolute quantitation of Marek's disease virus genome copy number in chicken feather and lymphocyte samples using real-time PCR. J Virol Methods 123: 53-64.

Buller RM, Janik JE, Sebring ED, Rose JA (**1981**) Herpes simplex virus types 1 and 2 completely help adenovirus-associated virus replication. J Virol 40: 241-247.

Chiocca S, Kurzbauer R, Schaffner G, Baker A, Mautner V, Cotten M (**1996**) The complete DNA sequence and genomic organization of the avian adenovirus CELO. J Virol 70: 2939-2949.

Dhinakar RG, Sivakumar S, Matheswaran K, Chandrasekhar M, Thiagarajan V, Nachimuthu K (**2003**) Detection of egg drop syndrome virus antigen or genome by enzyme-linked immunosorbent assay or polymerase chain reaction. Avian Pathol 32: 545-550.

Ganesh K, Suryanarayana VV, Raqhavan R (**2002**) Detection of fowl adenovirus associated with hydropericardium hepatitis syndrome by polymerase chain reaction. Vet Res Commun 26: 73-80.

Goncalves MA (**2005**) Adeno-associated virus: from defective virus to effective vector. Virol J 6: 2-43.

Grgić H, Philippe C, Ojkić D, Nagy E (**2006**) Study of vertical transmission of fowl adenoviruses. Can J Vet Res 70: 230-233.

Hess M (**2000**) Detection and differentiation of avian adenoviruses: a review. Avian Pathol 29: 195-206.

Hess M, Raue R, Hafez HM (**1999**) PCR for specific detection of hemorrhagic enteritis virus of turkeys an avian adenovirus. J Virol Methods 81: 199-203.

Lappalainen S, Ylitalo S, Arola A, Halkosalo A, Rasanen S, Vesikari T (**2012**) Simultaneous presence of human herpesvirus 6 and adenovirus infections in intestinal intussusception of young children. Acta Pediatr 101: 663-670.

Mazur-Lech B, Koncicki A, Janta M (**2009**) Investigations on using a polymerase chain reaction (PCR) to detect and evaluate the distribution of haemorrhagic enteritis virus in turkeys. Med Weter 65: 413-415.

McConnell BA, Fitzgerald AS (**2008**) Group I Adenovirus Infections. In: Saif YM (ed) Disease of Poultry, 12[td] ed., Blackwell Publishing Company, pp 252-266

Naeem K, Niazi T, Malik SA, Cheema AH (**1995**) Immunosuppressive potential and pathogenicity of an avian adenovirus isolate involved in hydropericardium syndrome in broilers. Avian Dis 39: 723-728.

Niczyporuk JS, Woźniakowski G, Samorek-Salamonowicz E, Czekaj H (**2012**) Effect of fowl adenovirus (FAdV-7) infection on the replication of turkey herpesvirus FC126 in chicken embryo fibroblast cultures. Bull Vet Inst Pulawy 56: 441-446.

Okuda Y, Ono M, Shibata I, Sato S, Akashi H (**2006**) Comparison of the polymerase chain reaction-restriction fragment length polymorphism pattern of the fiber gene and pathogenicity of serotype-1 fowl adenovirus isolates from gizzard erosions and from feces of clinically healthy chickens in Japan. J Vet Diagn Invest 18: 162-167.

Payet V, Arnauld C, Picault JP, Jestin A, Langlois P (**1998**) Transcriptional organization of the avian adenovirus CELO. J Virol 72: 9278-9285.

Roberts MM, White JL, Grutter MG, Burnett RM (**1986**) Three – dimensional structure of the adenovirus major coat protein hexon. Science 232: 1148-1151.

Saifuddin M, Wilks CR (**1992**) Effects of fowl adenovirus infection on the immune system of chickens. J Comp Pathol 107: 285-294.

Samorek-Salamonowicz E (**1986**) Characteristic of adenovirus strains isolated from birds in Poland with the consideration on their influence on the replication of herpes virus turkey strain. Thesis, UMCS Lublin.

Schonewille E, Singh A, Gobel TW, Gerner W, Saalmuller A, Hess M (**2008**) Fowl adenovirus (FAdV) serotype 4 causes depletion of B and T cells in lymphoid organs in specific pathogen-free chickens following experimental infection. Vet Immunol Immunopathol 121: 130-139.

Shivachandra SB, Sah RL, Singh SD, Kataria JM, Manimaran K (**2003**) Immunosupression in broiler chicks fed aflatoxin and inoculated with fowl adenovirus serotype-4 (FAdV-4) associated with hydropericardium syndrome. Vet Res Commun 27: 39-51.

Singh A, Grewal GS, Maiti NK, Oberoi MS (**2006**) Effect of fowl adenovirus-1 (IBH isolate) on humoral and cellular immune competency of broiler chicks. Com Immnunol Micobiol Infect Dis 29: 315-321.

Spector D, Pizer LI (**1978**) Herpesvirus infection modifies adenovirus RNA metabolism in adenovirus type 5-trnsformed cells. J Virol 27: 1-12.

Tian F, Luo J, Zhang H, Chang S, Song J (**2012**) MiRNA expression signatures induced by Marek's disease virus infection in chickens. Genomics 99: 152-159.

Toro H, Gonzalez C, Cerda L, Hess M, Reyes E, Geissea C (**2000**) Chicken anemia virus and fowl adenoviruses: association to induce the inclusion body hepatitis/hydropericardium syndrome. Avian Dis 44: 51-8.

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