Transcriptional cytokine responses associated with pathological outcomes in chickens experimentally infected with pigeon variant of avian avulavirus type 1

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Abstract

Background: Newcastle disease (ND) caused by virulent avian avulavirus 1 (AAvV-1) within the family paramyxoviridae, affects variety of avian species around the globe as well as responsible for significant economic losses in the poultry industry.

Methods: Transcriptional response of selected cytokines associated with pathological outcomes were studied on chickens after experimental infection with three virulent isolates of pigeon variant avian avulavirus type 1 (PAvV-1). The specific pathogen free (SPF) chickens were used in the study, after virus inoculation *via* the intranasal route virus, observed daily for clinical symptoms. On day 3 of post infection, the experiment was terminated and then anatomopathogical and histopathological changes, virus load and transcriptional cytokine response were determined in the spleen.

Results: For the study, four cytokines were chosen: interferons (IFN-β, IFN-γ) and interleukins (IL-2, IL-6). The increased transcriptional levels of cytokine expression were associated with viral gene expression and marked lesions. The increase in the mRNA levels of IL-6 was most significant in chickens with clinical symptoms. The combined up-regulations of IFN-γ and IL-6 mRNA expression were detected simultaneously with infiltration of macrophages in the histopathology analysis. The RNA expression of IL-2 and slight up-regulation of IFN-β were only in chickens infected with isolate closely related to poultry isolates in the phylogenetic study.

Conclusions: The difference in the mRNA expression levels of cytokines may help to elucidate mechanisms of immune responses in birds infected with different virulent strains of avian avulavirus type 1.

Keywords: chicken; pigeon variant of avian avulavirus type 1; innate immune response; cytokine; pathogenicity; viral loads.

Introduction

Avian avulavirus type 1 (AAvV-1) cause contagious disease, that affect worldwide birds in all age. According to the world organization for animal health, it's a reportable disease in poultry when fulfilled criteria of virulence, resulting from determination of the intracerebral pathogenicity index (ICPI) or the amino acid sequence at the F protein cleavage site [1]. Infection of a variety of strains AAvV-1 initiated disease, that can have manifested differences in the severity of the symptoms. Based on virulence and clinical signs in chickens, there were distinguished pathotypes: viscetropic and neurotropic velogenic, mesogenic, lentogenic and asymptomatic forms [2]. In result of having a unique monoclonal antibody pro-

file avian avulavirus type 1 isolated from pigeons are antigenically differ-ent from those detected in domestic poultry. In general, they are clustered in lineage VIb/1 of genotype VI AAvV-1 strains, in phylogenetic study [3]. Additionally, recent studies indicated, that the some of them are classi-fied to group 4a, which indicates relatedness to the AAvV-1 isolated from poultry [4]. The AVVa-1's genetically related to pigeon variant are usually detected in their natural hosts. However, many cases of infection or isola-tion of the pathogen from other bird species including poultry, have been noted [5–7]. The ICPI value of isolates pigeon variant AVAs-1 are mostly greater than 1.0 and pathogenicity frequently characterized as from mild to intermediate virulence for domestic chickens [8]. Nevertheless, the oc-currence of PAAvV-1 in poultry with ICPI equal to or greater than 0.7 or having a multiple basic amino acid motif at the F protein cleavage site is classified as virulent and should be reported to the OIE (CEC). The most serious outbreaks of virulent pigeon variant of AAvV-1 in commercial chickens were documented in the UK during 1984, over 23 cases, where-as measures to control the spread of disease for these outbreaks resulted in the slaughter of over 800,000 chickens and expenditures of over £2 mil-lion in compensation and other costs [9–10]. However, the pathogenicity of PAAVv-1 for chickens was frequently absent, thus the role in poultry pathology still unclear [11–12].

Considering the fact of widespread of PAAvV-1 in pigeons and yearly low number of reported occurrence in poultry in EU countries, interest-ing a molecular mechanism underlying of PAAvV-1 path infection in dif-ferent species of birds. This study was conducted to extend knowledge about chicken response to infection of virulent strains of pigeon variant avian Avulavirus type 1. The transcriptional levels of mRNA of four cyto-kines, that play an important role in host antiviral response by stimulating the T- lymphocyte dependent system and being potent macrophage acti-vation factors: IFN-β, IFN-γ, IL-2 and IL-6, were investigated.

Materials and Methods

Viruses: For the experimental study three isolates were chosen, marked as: PPMV-1/PL/PW166-175/02, PPMV-1/PL/332/05, and PPMV-1/PL/H2/10. All isolates were isolated from sick or dead feral pigeons from different parts of Poland over a 10-year period and had been characterized, details are listed in Table 1 [**13–14**].

Table 1. Background information of PPMV-1 isolates used in the study

Investigated parameters		Virus		
		PPMV-1/PL/ PW166-175/02	**PPMV-1/PL/ 332/05**	**PPMV-1/PL/ H2/10**
Mab 161/617 reactivity		1:64	1:2048	1:32
ICPI		0,61	1,05	1,29
Genotype		4b	4b	4a
F1/F2 cleavage site, aa motif		^{112}R-R-Q-K-R*F^{117}	^{112}R-R-Q-R-K*F^{117}	^{112}K-R-Q-K-R*F^{117}
L protein aa at position	-1564:	N	N	T
	-1694:	V	V	V

Briefly, two of the isolates, PPMV-1/PL/332/05 and PPMV-1/PL/PW166-175/02, were identified as virulent, based on an ICPI value of greater than 0.7 and an amino acid sequence at the Fusion protein (F) cleavage site. PPMV-1/PL/PW166-175/02 had a slightly lower ICPI index (ICPI= 0.61), but the amino acid sequence at the cleavage site of F1/F2 typical of virulent strains. The phylogenetic analysis of nucleotide sequences of F gene each isolates indicated, that PPMV-1/PL/332/05 and PPMV-1/PL/PW166-175/02 belong to the 4b sublineage as typical pigeon variants and closely related to other PAAVv-1 isolated in Poland. Additionally, PPMV-1/PL/H2/10 is atypical in phylogenetic analyses, belong to the genetic sublineage 4a closely related to PAAvV-1 isolates from poultry.

The sequence analysis of L proteins of the viruses revealed a lack of molecular markers associated with an increased virulence in chickens, but a unique amino acid profile was found in PPMV-1/PL/H2/10 [13]. Prior to the experiment, stocks of the viruses were prepared in 10-day-old SPF embryonated eggs (VALO BioMedia, Germany) according to standard procedure. Virus titers were calculated from the results of inoculation of 10-day-old embryonated eggs with a serial 10-fold dilutions of the virus in phosphate-buffered saline (PBS).

Experimental design: SPF layer chickens were hatched from embryonated eggs purchased from VALO-BioMedia (VALO BioMedia, Germany). Twenty 3-week-old chickens were used in the study. Birds were divided into 4 groups. Three groups of five chickens were inoculated bilaterally both intranasal and *via* the conjunctival route with a 10^6 median embryo infectious dose (EID_{50}) at 0,1 ml/bird with different isolates of PPMV-1. The mock group received PBS. The birds were observed daily for clinical signs. The experiment was terminated at day 3 post infection (dpi), all birds were humanely sacrificed and their spleens were removed aseptically to determine histopathological changes, viral load and cytokine gene expression. The animal experiments performed in this study were approved by the II Local Ethics Committee of Animal Experimentation in Lublin (Permit number: 95/2015) in accordance with European and national regulations on animal experiments and welfare.

Histopathological examination: Spleens from infected and mock groups were immediately fixed after postmortem examination in 10% buffered formalin, embedded in paraffin, sectioned at 4μm and stained with haematoxylin and eosin (H&E).

Immunohistochemistry (IHC): To detect PPMV-1 antigen in spleens, immunohistochemistry was performed on samples from infected birds. After deparaffinization, tissue sections were subjected to heat induced antigen retrieval by incubation in citrate buffer at pH 7.6 for 20 min in a preheated pressure cooker to expose antigenic sites. Endogenous peroxidase

blocking was done with 3% hydrogen peroxide diluted in methanol. The primary antibody (rabbit monoclonal, provided by Steven Essen, APHA, GB) was used at 1:500 dilutions for 60 min at room temperature. Staining was performed using labelled streptavidin-biotin visualization system kit, Dako REAL™ Detection Systems (Dako, USA). For visualization, 3,3'-diaminobenzidine-4HCl (Dako, USA) was used. Sections were counterstained with Mayer's haematoxylin, dehydrated and mounted. Sections incubated without the primary antibody and the use of negative tissues from non-infected chickens confirmed specificity of staining.

Quantification of viral RNA: Collected spleens from all birds (100 mg) were homogenized separately in 1 ml of phosphate buffered saline (PBS) and centrifuged (2000 x g 15 min). The RNA was extracted from 200 μl of supernatant using commercial kit according to the manufacturer's instructions (RNasin, Qiagen, Germany). A quantitative one-step real-time RT-PCR (qRT-PCR) was performed with primers and probe specific to the matrix gene, designed by Wise *et al.* [15] using a QuantiTect Probe RT-PCR kit (Qiagen, Germany) in a 25-μl reaction volume on an ABI 7500 Fast Real-Time PCR System (Applied Biosystems, USA). The cycle conditions of the RT and PCR steps were as follows: 50°C for 30 min, 95°C for 15 min and 40 cycles at 95°C for 10 sec, 56°C for 30 sec and 72°C for 10 sec. The results of the qRT-PCR were converted from Ct values and expressed as $eqEID_{50}$ per gram of tissue. Samples with a threshold cycle (Ct) <36.0 were considered positive.

Quantification of cytokine gene expression: The mRNA expression levels of IFN-β, IFN-γ, IL-6 and IL-2 in spleens from infected and control chickens were analyzed using a two-step qRT-PCR with *β-actin* as a reference gene. Total RNA was isolated from 50 mg of each spleen, following disruption in liquid nitrogen, using Total RNA Mini (A&A Biotechnology, Poland) according to the manufacturer's instructions. Resulting RNA concentration was measured by determination of A260/A280 ratio in NanoDrop Spectrophotometer (Thermo Fischer Scientific, Germany) and ranged between 1005-1880 ng/μl. To prevent genomic DNA

contamination, 1 µg of total RNA per sample was treated with 1U of DNase I (Fermentas, USA) and used for reverse transcription reaction using 40 U of M-MuLV reverse transcriptase (Invitrogen, USA) with 50 ng random primers (Invitrogen, USA) in the following conditions: 25°C for 10min, 42°C for 60min and 70°C for 15min. Relative changes in mRNA expression were measured using QuantiTect SYBR Green PCR Kit (Qiagen, Germany) and primers. The primers used in study were selected from previously published sequence [16–17], listed in Table 2.

Table 2. Primer sequences used in real-time PCR

Target RNA	Primer sequence (5´-3´)	Product size (bp)
β-actin	F:AGAGGCTCCCCTGAACCCCAAAGC R:CTGGATGGCTACATACATACATGGCTGG	94
IFN-β	F:GCCCACACACTCCAAAACACTG R: TTGATGCTGAGGTGAGCGTTG	150
IFN-γ	Forward:GTGAAGAAGGTGAAAGATATCATGGA Reverse:GCTTTGCGCTGGATTCTCA	71
IL-6	Forward:GCTCGCCGGCTTCGA Reverse:GGTAGGTCTGAAAGGCGAACAG	71
IL-2	Forward:TTGGAAAATATCAAGAACAAGATTCATC Reverse:TCCCAGGTAACACTGCAGAGTTT	93
Virus matrix (M) gene	Forward:AGTGATGTGCTCGGACCTTC Reverse:CCTGAGGAGGCATTTGCTA	121

Reactions were done in triplicate in 25-µl volume containing 7.5 pmol of each primer and 60 ng of total RNA. Amplification and detection of the specific products were performed using ABI 7500 Fast Real-Time PCR System (Applied Biosystems, USA) with the following thermal profile: one cycle at 95°C for 15 min, 45 cycles of 94°C for 15s, 58°C for 30s and 72°C for 30s. At the end of each run a dissociation curve step was added and melting curve analyzed.

Statistical Analysis. The relative expression of the target genes in the infected and mock group was calculated with the $2^{-\Delta\Delta Ct}$ method. The housekeeping gene encoding β-*actin* was used as the reference against which to normalize the expression levels of the target genes. Statistical significance was calculated with the Mann-Whitney-Wilcoxon test. A probability (p) value <0.05 was considered statistically significant. All statistical calculations were computed in *STATISTICA* version 6.0 (StatSoft, Inc., USA).

Results

Clinical disease and pathology: At 3 dpi depression and slightly swollen tissues around the eyes were observed in chickens infected with PPMV--1/PL/PW166-175/02. In the same group, we noticed enlarged and friable spleen with many multifocal white lesions in 3/5 birds (Figure 1). No clinical signs or pathological changes were observed in either of the two remaining groups.

Figure 1. Images of 3 dpi spleens (A) spleen from chicken infected with PPMV-1/ PL/PW166-175/02, enlarged, mottled indicating multifocal necrosis, (B) spleen from chicken from mock group.

Histopathology and Immuno-histochemical labelling: Microscopic lesions were observed in all infected birds, but these did not differ substantially irrespective of the isolate of PAAvV-1 used in the experiment. We observed only differences of the intensity of the histopathological changes (Table 3).

Table 3. Differences in intensity of histopathological lesions (HE) and immune-histochemical staining (IHC) of spleen sections between the infected groups of chicken. HE grading: (++) –multifocal to coalescing areas of hyperplasia of EAC (ellipsoid-associated cells), (+) – multifocal hyperplasia of EAC, (-) – no observed changes. IHC (immunohistochemistry): (+) – positive IHC labelling visible in over 50% of the tissue section, (+/-) – positive IHC labelling visible in less than 50% of the tissue section

	PPMV-1/PL/ PW 166-175/02	PPMV-1/PL/ 332/05	PPMV-1/PL/ H2/10
HE EAC hyperplasia	++	+	+
Mild lymphoid depletion	+	–	–
IHC	+	+/–	+

Histopathological examination revealed multifocal hyperplasia of ellipsoid-associated cells morphologically resembling macrophages in each of infected spleen. In the group PPMV-1/PL/PW166-175/02 the accumulation of macrophages and surrounding mononuclear cells was most prominent, the lesions were coalescing and accompanied by mild depletion of peri-arterial lymphoid sheaths (Figure 2).

The spleen tissue samples from a chicken infected with PPMV-1/PL/PW166-175/02 showing coalescing accumulations of ellipsoid-associated macrophages and moderate depletion of lymphocytes (B, D). The spleen tissue samples from a chicken in the mock group (A, C). Bar scale = 50 µm
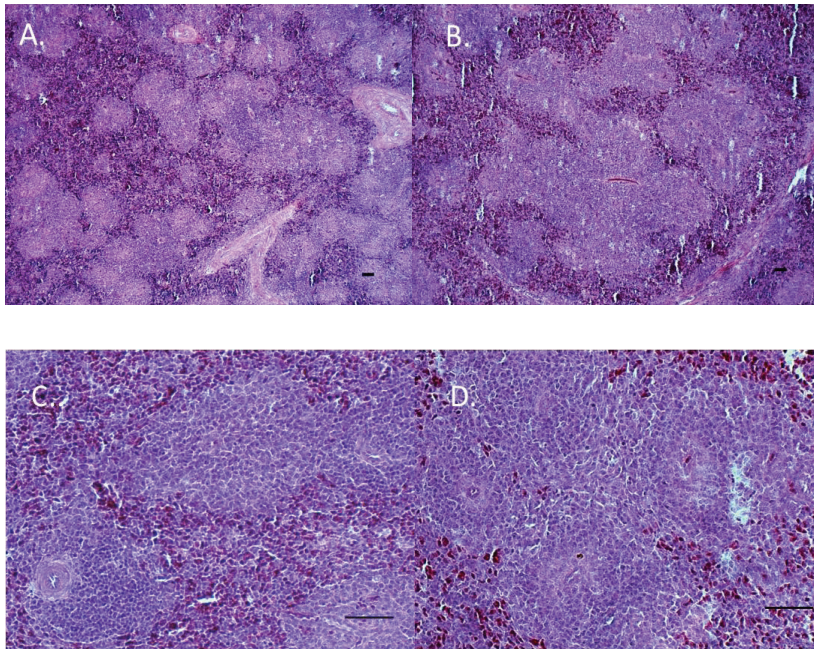
Figure 2. The histopathological changes in spleen

Furthermore, in all the groups, hyperaemia and occasional presence of fibrinous exudate in the sinusoids of spleen, as well as necrosis of ellipsoid-associated cells were observed. Immuno-histochemical labelling revealed positive reaction for viral antigen in all the infected spleens. The reaction was visible in the cytoplasm of mononuclear cells and sheaths of large macrophage-type cells (Figure 3). The diffuse labelling was most intense in PPMV-1/PL/PW166-175/02 and PPMV-1/PL/H2/10 groups while tissues of PPMV-1/PL/332/05 group shown weaker, multifocal labelling.

The chicken infected with PPMV-1/PL/H2/10 isolate, 3dpi; the diffuse immune-histochemical reaction is visible in a cytoplasm of cells morphologically resembling macrophages and lymphoid cells. immune-histochemical avidin-biotin-peroxidase, hematoksylin counterstain. Bar scale = 50 μm
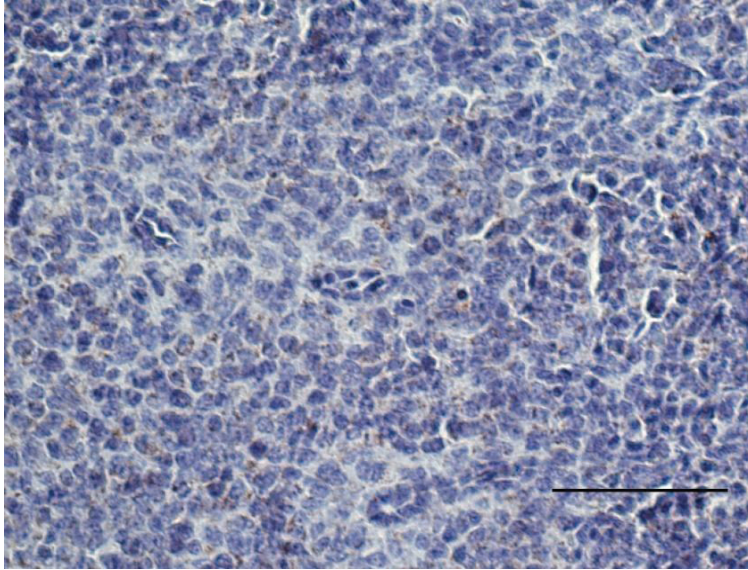
Figure 3. The immune-histochemical changes in spleen

Real-time RT-PCR: As illustrated in Figure 4, the viral RNA was detected in all spleens from infected chickens, but the number copies were not identical in spleens within the group. The amount of viral RNA reached its highest level in spleens from chickens infected with PPMV-1/PL/H2/10, as the estimated median of equivalent titers in this group ranged from $2.96 \times 10^5 eqEID_{50}$/mg to $2.07 \times 10^6 eqEID_{50}$/mg. The lower viral RNA load, ranging from $4.9 \times 10^4 eqEID_{50}$/mL to $5.4 \times 10^5 eqEID_{50}$/mg was observed in spleens from the group of chickens infected with PPMV-1/PL/PW166-175/02 and the lowest was found in spleens infected with PPMV-1/PL/332/05 and this ranged from 1.1 to $4.8 \times 10^4 eqEID_{50}$/mg.
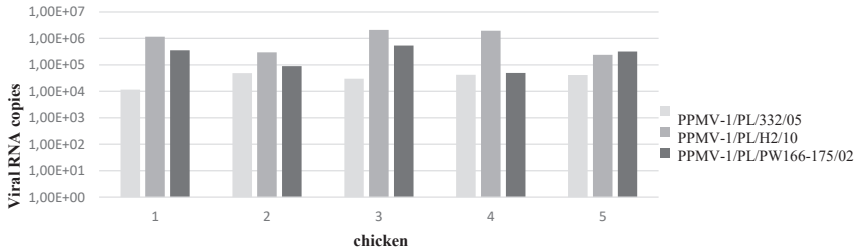
Figure 4. The eqEID$_{50}$ in spleen at 3 dpi from chickens infected with different PPMV-1 isolates

IL-6, IL-2, IFN-γ, IFN-β mRNA relative expression: Relative changes in cytokine expression are presented graphically in Figure 5.
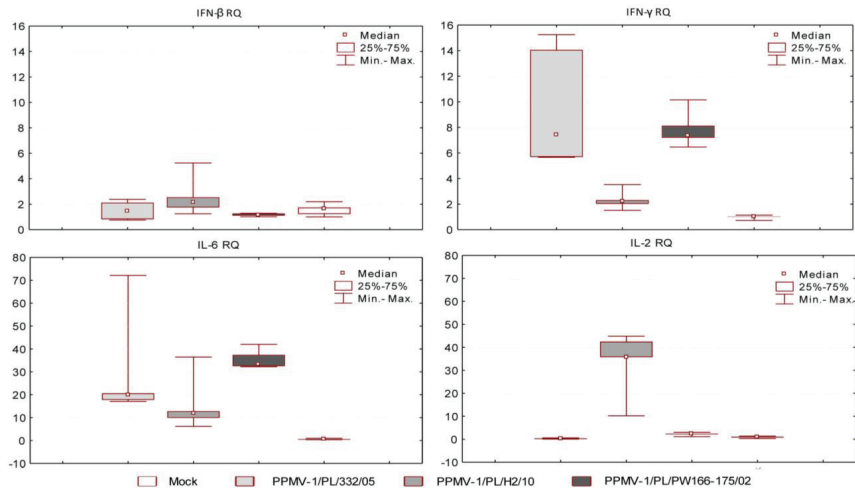

Figure 5. Determination of mRNA relative expression of IFN- ?, IFN-?, IL-6, IL-2 genes in the spleen of chickens infected with different isolates of PPMV-1 calculated with the 2$^{-\Delta\Delta Ct}$ method

The changes in gene expression were compared to the mock group with the Student T test. The results showed that IL-6 mRNA relative expression (RQ) was upregulated and this increase appeared to be the most significant compared with other tested cytokines. The most marked upregulation

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of IL-6 expression was observed in chickens infected with PPMV-1/PL/PW166-175/02 compared to the mock group (33.38 vs. 0.48) (p=0.00005). IL-6 transcriptional expression in birds infected with PPMV-1/PL/332/05 and PPMV-1/PL/H2/10 was also increased but to a lesser extent (20.07 vs. 0.48). This difference was statistically significant (p=0.000007). The analysis of IL-2 showed several higher RQ (35.83 vs. 1.00), but only in chickens infected with PPMV-1/PL/H2/10 (p=0.0000005). Relative IL-2 RNA expression did not appear to change in chickens infected with the two other isolates. Upregulated levels of IFN-γ gene expression were observed in each spleen from the infected groups. Similar levels were observed in both cases: PPMV-1/PL/332/05 and PPMV-1/PL/PW166-175/02 (7.42; 7.35 vs. 1.01) p=0.000003. In comparison, the IFN-β RNA relative expression levels were less remarkable and only in chickens infected with PPMV-1/PL/H2/10 (2.15 vs. 1.6) was there any statistical significance (p=0.03).

Discussion

Newcastle disease is one of the most important infectious diseases in poultry, and it causes significant economy impact to the poultry industry [18]. Virulent pigeon type 1 paramyxoviruses are dangerous for pigeons, but can also cause infections in other domestic birds. The pathogenicity of Newcastle disease viruses (NDV) is determined by the amino acid sequence of the precursor fusion protein protease cleavage site and cleavability at this site by the cellular protease [2]. However, NDV isolates that share the same F cleavage site have been recovered from many different species of birds and these isolates may produce mild or severe pathogenesis or even death [10]. Interestingly, some pigeon variants of NDVs, where the F protein is typical for virulent NDV strains, cause only minimal disease and show a low virulence in chickens [19]. Moreover, replacement of the *F* gene of a virulent NDV strain with that of a non-virulent PPMV-1 strain does not affect the virulence of the host, thus indicating that the non-virulent PPMV-1 strain must be determined by other factors [12, 20]. Consequently, it is highly desirable to conduct more research to

explain the mechanism which contributes to the virulence of NDVs and its ability to cause differing pathogeneses in the host [4, 21]. Although many studies have been performed to evaluate virus pathogenicity, evidence focusing on differences in virus-host interplay with different virulent isolates of NDV is limited [22].

Immediately following a viral infection, a host pro-inflammatory response is initiated. The first line of defense against a virus is innate immune response, which is the primary mechanism for resisting and clearing viruses during the early stage of infection [16]. Avian cytokines, like their mammalian equivalents, are influential in innate immune response to pathogenic infection [23]. Their appearance contributes to the reduction of viral replication, leads to the limitation of viral spread, and thus is associated with viral pathogenesis and pathological outcomes in the host. Interestingly, the induction of some cytokines may play a major role in the nature of the response to genetic variants of the pathogen, like in the case of serovars of *Salmonella enterica* in chickens [16]. Invasion of chicken cells by serovars *S. Typhimurium* or *S. Enteritidis* results in an increase of IL-6 mRNA determined by quantitative RT-PCR. In contrast, invasion by the avian serovar *S. Gallinarum* does not lead to an increase of IL-6 mRNA [16]. The role of cytokines in the pathogenesis of NDV infections has not yet been extensively studied. There have been very few studies concentrating on immune response in chickens infected with NDV. Research has focused mostly on response to virulent NDV infection. In the study carried out by Ecco *et al.* [17], transcriptional IFN-β, IFN-γ, IL-2, IL-6 cytokine responses were studied in archived fixed, paraffin-embedded (FFPE) spleens from 4-week-old White Leghorn chickens from different pathogenesis experiments. They observed marked upregulation of INF-γ and IL-6 relative expression at 2 and 3 dpi in spleens from chickens infected with traditional velogenic and atypical velogenic viscetropic, and mesogenic of strains of Newcastle Disease. The birds inoculated with either of the two velogenic visectropic viruses at 3 dpi had evident clinical disease: histologically the spleen had evidence of necrosis in the lymphoid area. Interestingly, most significant upregulation of IL-6 was observed at 3 dpi in spleens of chickens infected with mesogenic strains of NDV, but

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clinical signs were not observed in those birds during experimental infection. Histologically, there was mild follicular hyperplasia and depletion in the spleen at 3 dpi. Relative IL-2 and IFN-β mRNA expression did not appear to change for either velogenic or mesogenic strains. Similar findings have been reported by other researchers [24] in relation to innate response in chickens infected with highly virulent NDV strains from the 2002 outbreak in California. Measurement of early host response to NDV *in vivo* in microarray experiments showed induced multiple gene signals at 48h post infection in the Toll-like receptor which initiated IFN-α and IFN-β expression. In an *in vitro* study, Rue *et al.* [24] characterized the early host response of splenocytes infected with virulent CA-NDV and lentogenic LaSota strains, showed that the lentogenic strains of NDV is not capable of rapidly and strongly inducing IFN-α, IFN-γ IL-6 and IL-1β, genes integral to pro-inflammatory response in contrast to virulent CA-NDV. The understanding of chicken innate response to Newcastle disease viruses is limited and, to our knowledge, there is no data regarding immunological response during infection with PPMV-1. In the present study, we investigated transcriptional expression of four cytokines – IFN-α, IFN-γ, IL-2 and IL-6 – in spleens of chickens at 3 dpi after infection with three different isolates of PPMV-1. The spleen was chosen for analysis of the early innate host response, because it is a lymphoid organ that the virus reaches early in Newcastle disease [22, 25]. It has been shown in multiple studies that macrophages and also lymphocytes play a crucial role during NDV infection [26]. Several major cell types present in the spleen (macrophages, heterophils, B-cells and T-cells) which means that this appears to be a promising organ for studies into immune response after NDV infection [27]. Macrophages have a distinct role in host response against viral infection. They promote clearance of virus-infected cells, and act as antigen presenting cells expressing pattern recognition receptors (PRRs) that recognize foreign molecules (pathogen associated molecule patterns-PAMPs). The activation of PRR by PAMP triggers the activation of transcription factors and the expression of innate antiviral genes such as interferon, which is the most significant factor for host antiviral defenses. The Newcastle disease virus can induce alpha (INF-α) and beta (IFN-β)

interferons in macrophages and gamma (IFN-γ) interferon mRNA in peripheral blood monoclear cells. Our data are consistent with some previous findings. The histopathology studies provide a good visual correlation to PCR assays of IFN-γ expression levels. Interferon-γ produced by T helper cells is a potent activator of macrophages, enhancing destruction of cell-associated pathogens [**28**]. We showed statistically significant differences in relative upregulation of IFN-γ genes in all spleens of infected chickens. The highest level of expression corresponded with the higher intensity of the histopathological changes, but was not connected with the highest viral load in the spleen tissue. We did not observe this correlation in the case of relative gene expression of IFN-β in any chickens from infected groups. IFN-β is produced mainly by macrophages, which helps to protect neighboring cells from viral infection. We demonstrated an increase only in chickens infected with atypical strains of PPMV-1, where we detected the highest viral load. For our study, we chose IL-6 comes from activated macrophages, fibroblasts, and endothelial cells and serves to enhance vascular permeability and stimulates recruitment of inflammatory cells to the affected site. In this study, IL-6 RNA levels were notably increased in all the viruses of PPMV-1, but most especially in the case of typical strains of PPMV-1 which cause clinical signs of pathological changes in the spleen of infected chickens. The results suggest that increases in IL-6 expression play an important role in promoting inflammation and tissue damage in chickens infected with PPMV-1. The most significant differences of expression of the tested cytokines was demonstrated in IL-2 in each group. IL-2 is produced by T cells and is critical for the proliferation of a number of immune cells, including T helper lymphocytes, NK cells and B lymphocytes. However, IL-2 can lead to apoptosis as well as proliferation, making it unclear whether IL-2 has a predominantly positive or negative effect. However, we did not observe clinical signs or severe changes in chickens infected with the atypical strain of PPMV-1.

Conclusions: In order to further our studies and to demonstrate the role of PPMV-1 in chicken pathology, more research focused on the determination of changes in the percentage of T and B lymphocytes and

macrophages isolated from the spleen of chickens infected with PPMV-1 should be performed, in particular for atypical strains. To summarize, we demonstrated increase IL-6 and IFN-γ cytokines with accumulation of numerous cells morphologically resembling macrophages, but not with the quantity of the virus. The atypical strain of PPMV-1 showed a clearly different profile for the tested cytokine compared to typical strains of pigeon variant Newcastle disease virus. The difference in the mRNA expression levels of cytokines may help to elucidate the mechanisms of immune responses in birds infected with different virulent strains of APMV-1 that cause distinguishable clinical courses and pathologic changes.

Conflict of interests' statement: The authors declare that there is no conflict of interests regarding the publication of this article.

Animal rights ethical statement: The experiment was approved by Local Ethics Commission (University of Life Sciences in Lublin, Poland).

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