RESEARCH ARTICLE

Variation in the Immunoregulatory and Antiviral Cytokine mRNA Levels between Lentogenic and Velogenic Pathotypes of Avian Orthoavulavirus-1

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Abstract

Future strategies to control Avian Orthoavulavirus-1 (AOaV-1), the causative agent of Newcastle disease (ND) in poultry, would rely extensively on virus-host interactions. Cytokines, the molecular messengers of immune system, are considered a significant entity in understanding the complex interplay between virus and host immune response. In the present study, the mRNA levels of immunoregulatory (IFN-γ and IL-12) and antiviral (IFN-α and IFN-β) cytokines were studied in *in-vitro* (chicken embryo fibroblast cells - CEF) and *in-vivo* (spleen of chicken) systems in response to lentogenic (D58) and viscerotropic velogenic (D165) AOaV-1 at five time points after experimental infection (1, 2, 3, 4 and 5 days post-infection). The mRNA levels of immunoregulatory and antiviral cytokines varied significantly between lentogenic D58 and velogenic D165 across both the systems at all time points under study. It was significantly upregulated for velogenic D165 when compared to lentogenic D58. This significant difference indicated that induction of cytokinesis unique for different pathotypes of AOaV-1, thereby highlighting the importance of studying cytokine mRNA levels to understand molecular pathogenesis of AOaV-1.

Key words: Anti-viral cytokine, Cytokine mRNA levels, Immune response, Immunoregulatory cytokine, Newcastle disease.

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Introduction

Globally, poultry industry has witnessed remarkable growth in meat production in the past two decades. Food and Agricultural Organization foresees that poultry meat consumption will rise over the next decade. Hence, the health and well-being of poultry is essential to maintain global food security. The health status of poultry is threatened by various infectious diseases, of which Newcastle disease (ND) remains a constant threat to poultry production. ND is a highly contagious viral disease caused by Avian Orthoavulavirus-1 (AOaV-1), grouped under the genus *Orthoavulavirus* of *Paramyxoviridae* family of the order *Mononegavirales* (Lefkowitz *et al*., 2018). AOaV-1 is categorized into five pathotypes based on the severity of disease produced in chickens. The pathotypes include viscerotropic velogenic (VVND), neurotropic velogenic (VNND), mesogenic, lentogenic and avirulent AOaV-1 (OIE, 2012). The disease was first reported in Dutch East Indies (Java-Indonesia) and Newcastle upon Tyne in England (Alexander *et al.,* 2012)

Despite vaccination, ND occurs worldwide with varied distribution. Therefore, measures to control and prevent ND need to venture novel disease prevention strategies. These strategies could be developed only with extensive knowledge on virus-host interactions. Host immune system induces highly complex and specific immune response against each AOaV-1 pathotype in order to limit establishment of infection (Susta *et al*., 2011). Immune responses are best represented through cytokines, as they are the molecular messengers of the immune system (Ecco *et al*., 2011; Hu *et al*., 2012; Kumar *et al*., 2013; Shilpa *et al*., 2014). Moreover, incidence of ND in poultry is attributed to virulence of the virus, immune status of the host, and environment, all of which are better reflected in the cytokine response (Kapczynski *et al*., 2013). Importantly, immunoregulatory cytokines (IFN-γ and IL-12) and antiviral cytokines (IFN-α and IFN-β) are those that orchestrate immune response by facilitating communication between innate and adaptive immunity; and set up initial host defence in host cells (Kaiser, 2012).

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Therefore, the present study was conceived with the objective to elucidate variation in the mRNA levels of immunoregulatory (IFN-γ and IL-12) and antiviral cytokines (IFN-α and IFN-β) in response to a lentogenic vaccine virus (D58) and a velogenic virus (D165) in two different systems - chicken embryo fibroblast (i*n-vitro*) and 3-week-old chicken (*in-vivo*). This would provide insight towards the immune dynamics against the different pathotypes of AOaV-1 in different experimental systems, thereby providing potential implications towards enhancement of vaccination strategies to prevent and control ND.

Materials and Methods

Virus, Cells and Animals

AOaV-1 isolates, namely D58 (Genbank accession: EU330230) and D165 (Genbank accession: KX710211) belonging to lentogenic and viscerotropic velogenic pathotype, respectively, propagated and maintained in amniotic-allantoic fluid (AAF) at the laboratory were used in this study. The intracerebral pathogenicity index (ICPI) of lentogenic D58 and velogenic D165 is 0.14 (Shilpa *et al*., 2014) and 1.80 (Sarika *et al*., 2022), respectively. CEF cells prepared as described by Freshney (2000) served as the *in-vitro* system, whereas, 3-week-old specific pathogen free (SPF) chicken for uniformity obtained from Venkateshwara Hatcheries Pvt. Ltd., Pune, were used as the *in-vivo* system. The use of SPF chicken for uniformity in this study was approved by the Institutional Animal Ethical Committee (IAEC) vide No. 3028/ DFBS/B/2014.

Experimental Infection and Sample Collection

CEF cells were experimentally infected with lentogenic D58 and velogenic D165 virus separately at a multiplicity of infection (MOI) of 0.1 pfu/cell, with uninfected CEF cells serving as control, as described by Kumar *et al*. (2013). Both infected and uninfected CEF cells were harvested in duplicates at 1, 2, 3, 4 and 5 days post-infection (dpi) and were processed immediately for RNA extraction as described by Kumar *et al.* (2013).

A total of eighteen 3-week-old SPF chicken for uniformity were used, wherein they were divided into three groups namely uninfected control group (n=6), D58 (n=6) and D165 (n=6) infected groups. The uninfected control group received phosphate buffered saline solution and the other two groups received 0.1 mL (10^5EID_{50}/ 0.1 mL of AAF) of the respective viruses by subcutaneous route as described by OIE (2012). chicken for uniformity from each group were sacrificed at 1, 2, 3, 4 and 5 dpi and spleen was collected, washed in ice-cold PBS and was processed immediately for RNA extraction as described by Shilpa *et al.* (2014). Both *in-vitro* and *in-vivo* experiments were repeated twice.

Extraction of Total RNA and cDNA Synthesis

The total RNA was extracted from both CEF cells and spleen samples using TRIzol (Life Technologies, USA, Cat# 15596-026) as per manufacturer's instructions. Reverse transcription was done with 1 µg of total RNA using Verso cDNA synthesis kit (Cat# AB1453A) as per manufacturer's instructions.

Primers

The primers used in the study were designed using Primer3 (Untergasser *et al.,* 2012) and BLAST in NCBI (https://www.ncbi.nlm.nih.gov/) website. The designed primers were validated using Oligoanalyzer software v2.1. The primer sequences, annealing temperature and product size are provided in Table 1.

Quantification of Cytokine Levels and Data Analysis

Cytokine mRNA levels were quantified by qPCR in duplicates using specific primer sets as described by Shilpa *et al*. (2014). The quantitation cycle (Cq) values obtained in qPCR were substituted in the double delta Ct method (Pfaffl, 2004) and cytokine mRNA levels were analysed. Glyceraldehyde

Table. 1: Details of primers used in the study

Target gene	Primer sequences (5'-3') F –Forward; R – Reverse	Annealing temperature/ time (s)	Product size (bp)	Reference
Endogenous control genes				
GAPDH	**F**- CGG GAA CCA AAT GCA CTT CGT **R**– GGC TGC CGT AGA GGT ATG GGA	58°C/10 s	122	Li *et al*. (2007)
Antiviral cytokines				
IFN-α	**F**- CCT TCT GAA AGC TCT CGC CA **R**- TGT CCA GGA TGG TGT CGT TG	58°C/10 s	170	Designed for this study
IFN-β	**F**- CTT CGT AAA CCA AGG CAC GC **R**- ATG GTC CCA GGT ACA AGC AC	58°C/10 s	130	Designed for this study
Immuno-regulatory cytokines				
IFN-γ	**F**-GAA CGA CTT GAG AAT CCA GC **R**-GAG CAC AGG AGG TCA TAA GA	58°C/10 s	146	Designed for this study
IL12	**F**-CGA AGT GAA GGA GTT CCC AGA T **R**-GAC CGT ATC ATT TGC CCA TTG	58°C/10 s	123	Liu *et al*. (2010)

3-phosphate dehydrogenase (GAPDH) was used as endogenous control gene.

Statistical Analysis

Cytokine mRNA levels are presented as mean ± standard deviation and were statistically analyzed among groups by two-way ANOVA with Sidak post-hoc correction and t-test using SPSS software. Significance was considered when $p<0.05$ was observed.

Results and Discussion

In the present study, the cytokines IFN-γ, IL-12, IFN-α, and IFN-β were selected, as these cytokines play crucial role in both innate and adaptive immune response. The mean value of mRNA levels of immunoregulatory (IFN-γ and IL-12) and antiviral (IFN-α and IFN-β) cytokines across *in-vitro* and *in-vivo* systems in response to lentogenic D58 and velogenic D165 is depicted graphically in Figure 1 and 2, respectively. The basal mRNA level was kept as 1 fold (Li *et al*., 2007) and hence any statistically significant increase above 1 fold was considered upregulated.

IFN-γ is produced by Th1 cells, which are associated with innate immune and inflammatory cytolytic responses (Rue *et al*., 2011); and IL-12 promotes T cell differentiation and has stimulatory effect on IFN-γ (Liu *et al*., 2010). Further, both these cytokines are essential for combating intracellular AOaV-1. In the present study, the IFN-γ and IL-12 mRNA levels in response to velogenic velogenic D165 were significantly upregulated when compared to lentogenic D58 at all time points of study in both *in-vitro* and *in-vivo* systems. This indicates the highly virulent nature of D165 and underscores the necessity for a strong innate and adaptive immune response to combat this virulent strain (Rue *et al*., 2011). In addition, the upregulated mRNA levels of IFN-γ coincided with the increased levels of IL-12 in response to both lentogenic D58 and velogenic D165 across both systems, justifying the stimulatory action of IL-12 over IFN-γ (Rahman and Eo, 2012).

The leukocyte derived IFN-α and fibroblast derived IFN-β are key antiviral cytokines in the innate immune response, responsible for inhibition of viral replication and activation of immune cells early in infection (Rahman and Eo, 2012). Increase in the level of IFN-α and IFN-β depends on the type of cells employed and the pathotype of AOaV-1 (Ginting *et al*., 2019). The mRNA levels of IFN-α and IFN-β *in-vivo* were significantly higher when compared to *in-vitro*. By contrast, the mRNA level of IFN-β in response to both lentogenic D58 and velogenic D165 *in-vitro* was earlier and significantly higher compared to the *in-vivo* system. This could be due the origin of IFN-β from fibroblasts (Rahman and Eo, 2012). The mRNA levels of IFN-α and IFN-β were higher in both *in-vitro* and *in-vivo* systems in response to velogenic D165 than lentogenic D58. This represents that the induction of IFN-α and IFN-β against virulent AOaV-1 is unaffected, irrespective of the cell type employed (López de Padilla and Niewold, 2016). The lesser level of IFNs against D58 could be due to its lentogenic nature (Kumar *et al*., 2013).

In conclusion, the present study underscores the significant roles of cytokines, IFN-γ, IL-12, IFN-α, and IFN-β, in the immune response to AOaV-1 in chickens. The significant upregulation of IFN-γ and IL-12 in response to the velogenic D165 strain across both *in-vitro* and *in-vivo* systems highlights the necessity of a robust innate and adaptive immune response to combat highly virulent AOaV-1 pathotypes. Similarly, the elevated levels of IFN-α and IFN-β, particularly in response to velogenic D165, reflect the importance of these antiviral cytokines in the early stages of infection. These findings provide insights into the molecular mechanisms of immune response against AOaV-1, thereby offering valuable data to the scientific community to address the challenges of ND in India.

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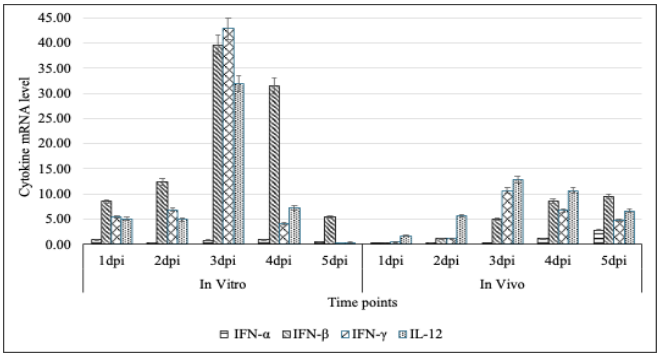

Fig. 1: Mean bar diagram of cytokine mRNA levels in response to lentogenic D58

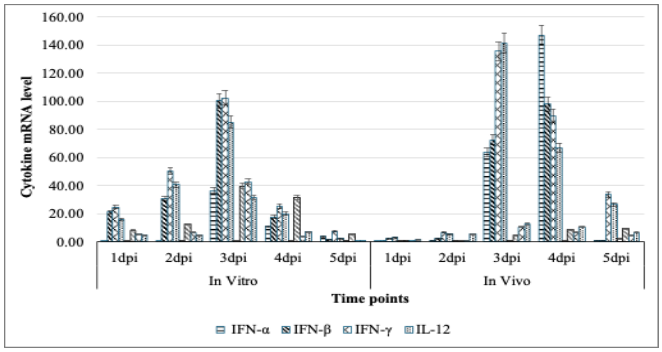

Fig. 2: Mean bar diagram of cytokine mRNA levels in response to velogenic D165

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