Article

In-Vitro characterization of *chIFITMs* of Aseel and Kadaknath chicken breeds against Newcastle disease virus infection

Muthusamy Malarmathi[1*], Nagarajan Murali[1], Mani Selvaraju[1], Karuppusamy Sivakumar[2], Vasudevan Gowthaman[3], Vadivel Balasubramanian Raghavendran[1], Angamuthu Raja[1], Sunday O Peters[4] and Aranganoor Kannan Thiruvenkadan[5*]

[1] Veterinary College and Research Institute, Tamil Nadu Veterinary and Animal Sciences University, Namakkal 637 002, India.
[2] Faculty of Food and Agriculture, The University of the West Indies, St Augustine, Trinidad and Tobago
[3] Poultry Disease Diagnosis and Surveillance Laboratory, Tamil Nadu Veterinary and Animal Sciences University, Namakkal 637 002, India.
[4] Department of Animal Science, Berry College, Mount Berry, GA, United States.
[5] Veterinary College and Research Institute, Tamil Nadu Veterinary and Animal Sciences University, Salem 636 112, India.
* Correspondence: thiruvenkadan.a.k@tanuvas.ac.in (A.K.T); malarmathi.m@tanuvas.ac.in (M.M)

Simple Summary: Aseel and Kadaknath are indigenous chicken breeds of India. Due to their distinctiveness, these two breeds are becoming more important. Aseel has a reputation for cock fighting and having high-quality meat. The black flesh of Kadaknath is famous and it is mostly raised for its meat and eggs. Both breeds have strong heat and disease tolerance. The genes Chicken Interferon Inducible Transmembrane Proteins (chIFITM), which produces chicken interferon inducible transmembrane proteins, works to prevent viruses from entering their host cells, restrict the viral multiplication and limits the viral load. This research was conducted to ascertain the level of *chIFITM* gene expression against the Newcastle disease virus (NDV) in chicken embryo fibroblast cells of both breeds. There are five members of the *chIFITM* gene family: *chIFITM*1, *chIFITM*2, *chIFITM*3, *chIFITM*5, and *chIFITM10*. *chIFITM 1, 2,* and *3* have immune-related activity; as a result, these three are referred to as immune-related *IFITM* (*IR-IFITM*). All the *chIFITM* genes were shown to be highly expressed in the CEF cells of both breeds during the course of our investigation. The Kadaknath CEF cells had a significantly low viral load and a high quantity of mRNAs of *chIFITM* genes when the breeds were compared.

Abstract: Newcastle disease (ND) is a highly contagious and usually causes severe illness that affects *Aves* all over the world, including domestic poultry. Depending on the virus's virulence, it can impact the nervous, respiratory, and digestive systems and causes up to 100% mortality. The *chIFITM* genes are activated in response to viral infection. The current study was conducted to quantify the mRNA of *chIFITM* genes *in vitro* in response to ND viral infection. It also examined its ability to inhibit ND virus replication in Chicken Embryo Fibroblast (CEF) cells of Aseel and Kadaknath breeds. Results from the study showed that the expression of all *chIFITM* genes was significantly upregulated throughout the period in the infected CEF cells of both breeds compared to uninfected CEF cells. In CEF cells of the Kadaknath breed, elevated levels of expression of the *chIFITM3* gene dramatically reduced the ND viral growth and the viral load was 60% lower than in CEF cells of the Aseel breed. The expression level of the *chIFITMs* in Kadaknath ranged from 2.39 to 11.68 $\log_2$ folds higher than that of control CEFs, and was consistently ($p<0.01$) higher than Aseel CEFs. Similar to this, *IFN-γ gene* expresses strongly quickly and peaks at 13.9 $\log_2$ fold at 48 hpi. The result suggests that the Kadaknath chicken breed may have a higher level of disease tolerance compared to the Aseel chicken.

Keywords: *ISG*; *IFITM* gene; *Mx;* Interferon; CEF; Aseel; Kadaknath; Newcastle disease; viral load

 doi:10.20944/preprints202304.1180.v1

1. Introduction

Newcastle disease (ND) has been globally distributed and the causative agent, Newcastle Disease virus (NDV) belongs to the *Paramyxoviridae* family, genus *Avulavirus* and is designated as *Avian Paramyxovirus-1* (*APMV-1*). NDV is a non-segmented, negative sense, single-stranded enveloped RNA virus with approximately 15kb genome encodes namely, hemagglutinin–neuraminidase (HN), nucleoprotein (NP), fusion (F), phospho-protein (P), matrix (M), RNA-dependent RNA polymerase (L) [1]. ND virus has a wide range of hosts and infection was reported in 250 *Avian species* in the world by either natural or experimental mechanisms [2]. Depending on the viral pathotype, the incidence of ND that affects poultry manifests as gastrointestinal, respiratory and neurological conditions that can result in up to 100% mortality [3,4]. ND has a high impact on the poultry industry through heavy economic loss aroused due to heavy mortality and production loss and also due to extensive attention to the prevention and treatment of this disease such as standard vaccination protocols and biosecurity measures [5]. It was calculated that from thirteen layer farms in the Gujarat state of India suffered a total economic loss of $4,588 (₹ 37,19,223) per year [5]. Various disease preventive strategies and vaccination are not effective due to the complex genetic diversity of viruses. So, it is necessary to develop a chicken population with disease resistant naturally.

During viral infection *Type I interferon* defense mechanism are triggered to express a set of genes against viral infections known as interferon-stimulated genes (*ISGs*) [6,7]. The *IFITM* (interferon-inducible transmembrane) gene is one of these *ISGs* and has been shown to prevent the propagation of several highly virulent viral pathogens, such as the coronavirus responsible for the severe acute respiratory syndrome (*SARS*), the filoviruses Marburg and Ebola, the influenza A viruses (*IAVs*), and flaviviruses (dengue virus) [8–10]. Scientists discovered that the *chIFITM* gene expression has a negative correlation with the emergence of influenza virus and its titre in *in vitro* study, indicating that *chIFITM*s*1 ,2* and *3* had a functional role in the management of viral infections [11,12]. In chicken, *IFITM* genes were located in Chromosome 5 and found in two loci, one containing the various numbers of immune-related (*IR*)-*IFITM* (*IFITM 1, 2, 3*) genes and *IFITM*5 gene and *IFITM10* gene in another loci [13,14]. Lanz et al. found that *swIFITMs (swine IFITMs)* had a dose-dependent restriction against *IAV* after infecting porcine *HEK293*-T cells with *IAV A/WSN/33 (WSN)* for 24 or 48 hours [15]. sw*IFITM*2 and -3 were expressed at late endosomes and have most potent antiviral activity against *IAV* in porcine cells. Furthermore, no sw*IFITM*5 expression was detected in any of the tested cell lines and concludes that the *IFITM5* do not have significant role in immune function [15]. Further, the knock-down of *IFITM*3 in DF-1 cells by siRNA increased the infectivity of a vesicular stomatitis virus G protein-pseudo-typed lentiviral vector [16].

Hence this study was undertaken to examine the *chIFITM* gene expression pattern in two chicken breeds of India, Aseel and Kadaknath, whose levels of viral resistance are high. So, we selected the model of chicken embryo fibroblasts (CEFs) to observe chicken *IFITM* gene expression *in vitro* following infection with NDVs. We compared the expression of *IFN γ* and *Mx* gene in response to NDV infection by quantitative real-time polymerase chain reaction (qRT-PCR).

In addition to providing prospects for a deeper understanding of viral resistance, analysis of these genes in chickens offers potential strategies for preventing viruses in poultry farming. It may be possible to do a selective breeding program in poultry breeds for increased resistance against viral infections. So it needs to discover characteristics of resistance and understand how they operate. Further, The observation may have useful implications in terms of vaccine production. Many vaccines are produced in embryonated hen's eggs or continuous avian cell lines. However, it is well established that the rate-determining step in the manufacture of numerous vaccines is the induction of antiviral immune responses that prevent the replication of vaccine viruses and the high cost involved in maintain and producing specific pathogen free (SFP) eggs from chicken.

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 28 April 2023 doi:10.20944/preprints202304.1180.v1

2. Materials and Methods

2.1. Ethics statement

The experiment was conducted with the approval of the Institutional Bio-safety Committee (IBSC) of TANUVAS-Veterinary College and Research Institute, Namakkal, Tamil Nadu, India (Approval Lr. No. *1764/VCRI-NKL/IBSC/2022* dated 11.05.2022 of the Dean, VCRI, Namakkal).

2.2. Chicken embryo fibroblast Cells

CEF cells were prepared from 9-10 days old SPF chicken embryos of Aseel and Kadaknath (Department of Poultry Science, Veterinary College and Research Institute, TNUVAS, Namakkal) as previously described method [17]. Fibroblastic cells were isolated from the respective embryo by removing the head and viscera, then cut into small pieces using sterile scissors and forces. The remaining tissues were washed with PBS and trypsinized for 5 minutes with 0.25% trypsin and magnetic stir. Allow pieces to settle, collect supernatant, centrifuge at 1000 rpm for 5 min, resuspend pellet in growth medium containing *Dulbecco's modified Eagle's medium (DMEM)* (Hi-media, Cat. No.AL007S) supplemented with 10 % fetal bovine serum (FBS) ((Hi-media, Cat. No. RM112-500ML), 1% antibiotics, Antimycotic solution (100x), stabilized (Sigma, Cat.No. A5955) 37 °C with 5 % CO_2 for 24hrs.

2.3. Virus

The *velogenic genotype XIII, NDV* strain isolated from a field ND outbreak by Poultry Disease Diagnosis and Surveillance Laboratory, TANUVAS, Namakkal, India was used in this study. The ND viruse was inoculated into the allantoic cavity of 10-day-old SPF-embryonated White Leghorn chicken eggs and incubated at 37°C for 72 hrs as per the standard procedures of the Office International Des Epizooties [18]. After 72 hrs, inoculated eggs were chilled for 30 mins and then allantoic fluid was collected under sterile condition and stored at −80°C for further use. ND virus titers were quantified by Haemagglutination assay (HA) and confirmed by PCR amplification of ND viral *F-gene* using the primer to amplify 356 bp amplicon (Figure 1) using the primer pair of *NDVF* (5′-GCAGCTGCAGGGATTGTGGT – 3′) and *NDVR* (5′-TCTTTGAGCAGGAGGATGTTG – 3′) with the cycle condition of initial denaturation 95°C for 3 min followed by 35 cycle of 94°C for 45s, 52°C for 45s, 72°C for 45s and final extension 72°C for 5min [19].

2.4. Tissue Culture Infection Doses ($TCID_{50}$)

The viral infective dose was measured by $TCID_{50}$. CEF cells of SPF-embryonated White leghorn chicken eggs were cultured in 96-well plates and were incubated with the cell supernatants of different groups, which had 10-fold serially diluted viral suspension. Each dilution had five replicates. One h after NDV infection, the supernatants were replaced with DMEM containing 2% fetal bovine serum (FBS). Then incubated at 37 °C and was observed daily for CPE scoring and continued scoring daily till the control wells started dying. The $TCID_{50}$ value was determined using Spearman-Karber's method [20].

2.5. Viral infection

CEFs prepared from Aseel and Kadaknath SPF chicken embryos were seeded 24 h prior to infection in a 25 cm^2 Tissue culture Flask (T25) (Hi-media, India) at a cell density of approximately 7×10^5 cells/flask. The CEF cells in triplicate were infected with *velogenic genotype XIII, NDV strains* to circulate in Tamil Nadu, South India [21]. The ND viral suspension was diluted to 50 % tissue culture infective dose ($TCID_{50}$) of 10^6/ml and 0.5ml of viral suspension ($TCID_{50}$) was added into the flask and allowed for viral adsorption by incubating at 37°C in a humidified atmosphere containing 5 % CO_2 for 1 hr. Afterwards, the growth medium was replaced with DMEM supplemented with 2 % FBS. Uninfected cells were regarded as control samples. CEF cells were harvested from uninfected control

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 28 April 2023 doi:10.20944/preprints202304.1180.v1

and infected CEF cells at 3, 6, 12, 24 and 48 hours post-infected (hpi) and stored at −80°C for RNA extraction. Virus load in CEF cells were quantified by an absolute quantification method [22] and gene expression by relative quantification $2^{-\Delta\Delta Ct}$ method [23].

2.6. RNA extraction and cDNA synthesis

Total RNA was extracted from the infected and uninfected control group CEF cells of both breeds at each time point by Trizol method using RNAiso Plus, Takara, (Cat. # 9109)) (Total RNA extraction reagent). RNA in each sample was quantified by using Thermo Scientific's Nanodrop spectrophotometer (Thermo Scientific, USA). Approximately, 1 µg RNA from each sample was used for complementary DNA (cDNA) synthesis by using *iScript cDNA Synthes kit* (Bio-Rad, Cat # 1708891) according to the manufacturer's protocol which follows a method of Reverse Transcription (RT) random primer.

2.7. Primer Pairs Design

RT-qPCR primers for *chIFITMs* genes were designed using Primer-BlAST with a length of 20 to 23 bases and amplicon sizes ranging from 115 to 196 bp. The sequences of these genes were obtained from NCBI (https://www.ncbi.nlm.nih.gov). The primer specificity of each gene was verified using 2.5% agarose gel electrophoresis and melting curve analysis. To validate the specificity of each primer pair, it was verified by in-silico PCR with the NCBI sequence database using *NCBI PRIMER BLAST*.

Table 1. List of primer sequences for qRT-PCR.

Gene	Primer	Sequence (5'->3')	Reference
chIFITM1	FP	GCAGGATGTGACCACCACTA	NM_001350059.2
	RP	CTTCGCTGTCCTCCCATAGC	
chIFITM2	FP	AACAGGCGGAGGTGAGCAT	NM_001350058.2
	RP	AAGATGAGCGAGGGGAAGCA	
chIFITM3	FP	CGTGAAGTCCAGGGATCGCA	NM_001350061.2
	RP	GCAACCAGGGCGATGATGAG	
chIFITM5	FP	CCAACCCCACTTCTGGACGA	NM_001199498.1
	RP	ATCACTCCGAAGGGCACGAC	
chMx	FP	GTCCAAGAGGCTGAATAACAGAG	NM_204609
	RP	GTCGGATCTTTCTGTCATATTGG	
chIFN-γ	FP	TGAGCCAGATTGTTTCGATG	[24]
	RP	CTTGGCCAGGTCCATGATA	
chβ-Actin	FP	TATGTGCAAGGCCGGTTTC	
	RP	TGTCTTTCTGGCCCATACCAA	
NDV-NP	Pla-rt13	CAACAATAGGAGTGGAGTGTCTGA	[22]
	Pla-rt14	CAGGGTATCGGTGATGTCTTCT	

2.8. Quantitative real-time polymerase chain reaction (qRT-PCR)

A total of 4 targeted genes (*chIFITM*1, 2, 3 and 5) expression pattern in ND virus infected and uninfected control CEF cells of both breeds were studied alone with 2 Positive immune-related genes (*IFN γ* and *Mx*) and 1 housekeeping gene (*β Actin*) (Table 1). The relative expression of specific gene mRNA was quantified and the absolute quantification of viral load was done by a real-time thermal cycler (IIIumina Real-time machine, USA). All reactions were performed in a nuclease-free 48-well qRT-PCR Illumina plate with sealer. The qRT-PCR response was done with the final volume of 20 µl using 10 µl of SYBR Green PCR Master Mix Kit (Bio-rad), 10 pmol of each forward and reverse primer, and 1µl of cDNA. The cycle condition of qRT-PCR was initial denaturation at 95°C for 10 mins, followed by 40 cycles of 95 °C for 10s, and 60 °C (β Actin, *IFN γ chIFITM1* and *Mx* genes) to 62 °C (*chIFITM2, 3 and 5*) for 45s and 72 °C for 15s. Further, the target and β-actin genes'

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 28 April 2023 doi:10.20944/preprints202304.1180.v1

respective cycle threshold (Ct) values were computed. Using the $2^{-\Delta\Delta Ct}$ approach, the relative fold change of the target genes in the infected groups was calculated using the delta Ct of the uninfected control group [23].

2.9. Standard curve analysis for detection of viral load.

Standard curve was constructed by linear regression method to do absolute quantification of viral load in the samples [22]. Ten-fold serially diluted T-NP plasmid (20 ng/µl, A260/280 ratio = 1.80) with known concentration was used as a standard to construct a standard curve and to obtain a linear regression equation. A standard curve was then generated by plotting Cq values against logarithmic of plasmid copies numbers in the standard. A correlation between NDV-specific nucleoprotein (NP) gene copy numbers and Cq values as found by using Pla-rt13 and Pla-rt14 primer pairs specific for nucleoprotein (NP) region of NDV genome (Table 1). The qPCR cycle condition was initially 95°C for 5 mins, followed by 40 cycles of 95°C for 15s, 60°C for 30s and finally 72°C for 30s. Melting curve analysis was used to know the specificity of qPCR primers. A partial regression equation was obtained as $Y = -2.25x + 14.09$ (Figure 1). R^2 value ranging from 0.994 to 0.932 and it indicates strong and linear relationships between the Cq value and number of gene copies in the sample.

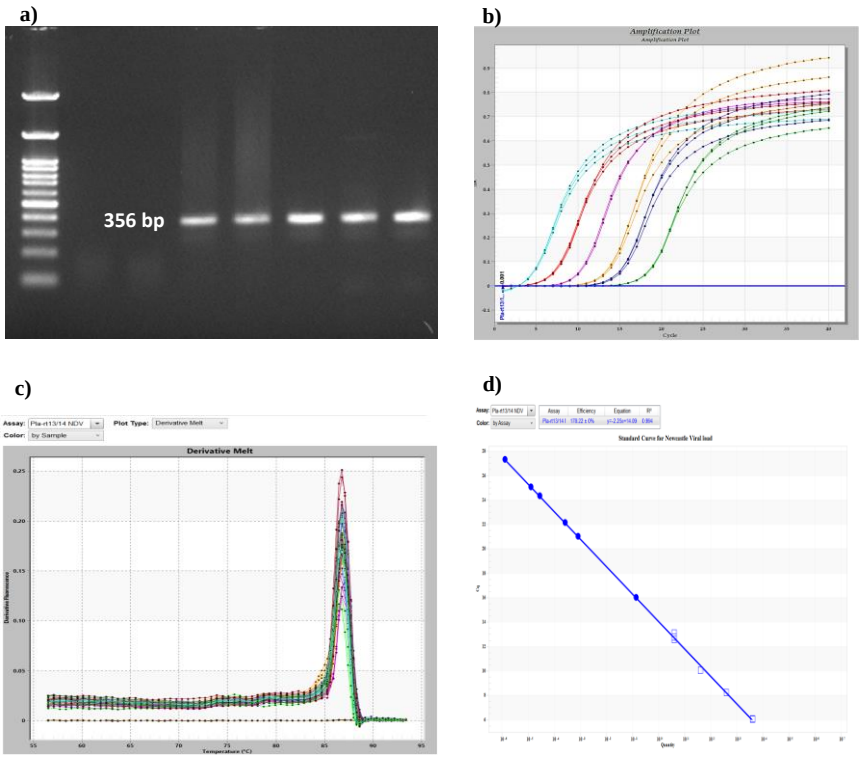

Figure 1. Newcastle disease virus detection and quantification. **(a)** PCR amplification of a 356 bp long *F-gene* amplicon specific to the ND virus. **(b, c, d)** Real-time quantitative PCR assay creation for absolute quantification. **(b)** Amplification plot of serially diluted T-NP plasmid standard. **(c)** Melting curve, **(d)** Standard curve for ND viral load detection with linear regression equation $Y = -2.25x + 14.09$ and R^2 score value 0.994.

2.10. Statistical analysis

The t-*test* and one-way *ANOVA* was conducted to analyze real time PCR data. The mean (n = 3 per time point/breed for each infected and control) and standard error of the mean are in $\log_2(2^{-\Delta\Delta Ct})$ used to express data and $\log_{10}$ (viral copies) used to express viral load. *R software* version 4.2.1 was used to analyze data and a p-value < 0.05 was used to

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 28 April 2023 doi:10.20944/preprints202304.1180.v1

determine statistical significance. Further, the R program was also used to create the graphical illustrations of the results.

3. Results

3.1. NDV infection-induced cytopathic changes and viral load

In the current study, we have determined the IFITM gene expression against the live NDV (velogenic genotype XIII, NDV strains) in the CEF cells. Figure 2 highlights the normal morphology of the CEF cells derived from the 9–10-day-old SPF embryonated chicken eggs. The effect of NDV infection in the chicken embryo fibroblast cells was examined under an inverted microscope for their morphology to determine the cytopathic effect (CPE) at 3, 6, 12, 24 and 48 hpi and compared with uninfected control cells. The cell monolayer was intact and morphologically similar to the control group till 3 and 6 hpi in Aseel and Kadaknath respectively. However, at 6 and 12 hpi, morphological changes was observed which are typical of cytopathic effect (CPE) viz., cell rounding, a fusion of infected cells to form syncytia cells and detachment of cells from monolayer followed by cell death were noticed under the light microscope in both breeds (Figure 2).

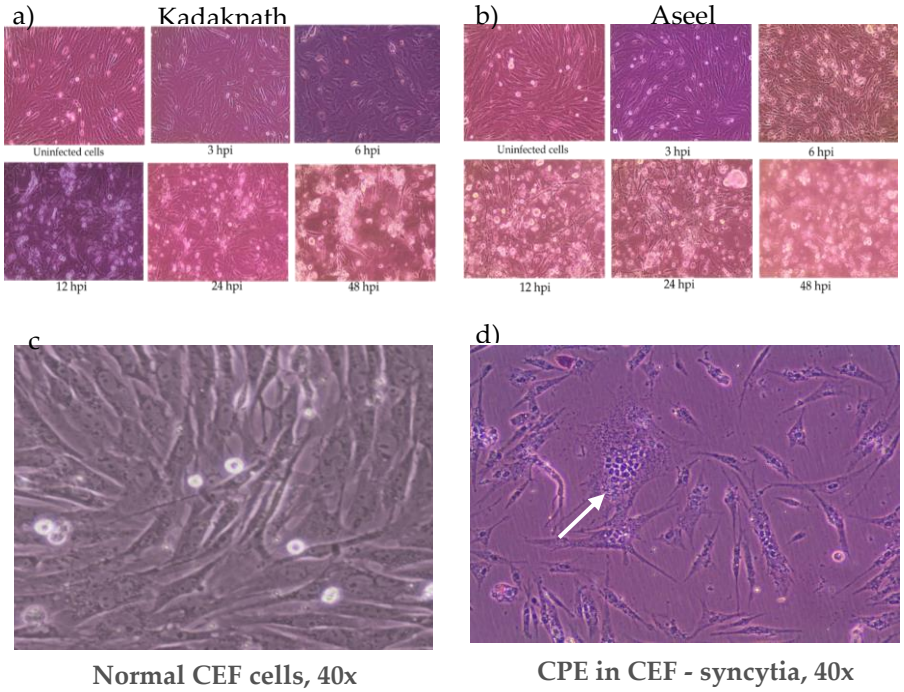

Figure 2. Observations of Chicken embryo fibroblast cell cultures under an inverted microscope exhibiting at 20X **(a, b)**, and 40X **(c, d)**, phase contrast objectives. **(a)** and **(b)**. Uninfected and Newcastle disease virus infected CEF cell at different hours post infection (hpi). **(a)** Kadaknath CEF showing delayed cytopathic changes, cell rounding noticed at 12hrs and **(b)** Aseel CEF started showing cytopathic changes such us cell rounding noticed at 6hrs. **(c)** Normal CEF cells and **(d)** Cytopathic effect of ND virus in CEF showing multinucleated cells (Syncytia).

Further, NDV infected CEF cells also showed morphological alterations typical of apoptosis, rounding of a cell and cytoplasm vacuolation was noticed in both breeds. But cell rounding starts from 6h and progressed and at 48h, complete cell detachment was observed in Aseel CEF cells. However, it was delayed in the case of Kadaknath CEFs, cell rounding starts from 12 h and progressed, and at 48h cell detachment progressed. This revealed that CPE advancement related to virus load and time of infected cells, in addition to confirming virus infection and its replication in infected cells.

Viral load was calculated by standard curve analysis using the T-NP plasmid as a standard and we observed a steady increase in viral load till 12h and 24h respectively in

 doi:10.20944/preprints202304.1180.v1

Aseel and Kadaknath CEF cells (Figure 3). In the case of Aseel CEF, viral load was significantly ($p<0.01$) higher than Kadaknath at 3 hpi (2.01 $\log_{10}$), 6 hpi (2.75 $\log_{10}$) and at 12 hpi (5.38 $\log_{10}$). In contrast, in Kadaknath CEF, the viral load was significantly ($p<0.01$) lower when compared to Aseel at each point 3 hpi (1.01 $\log_{10}$), 6 hpi (1.59 $\log_{10}$) and 12 hpi (2.15 $\log_{10}$). But there is no significant difference found at 24 hpi (4.54 and 4.63 $\log_{10}$) and 48 hpi (2.98 and 2.95 $\log_{10}$) between Aseel and Kadaknath CEF respectively. In Aseel, the viral load peaked at 12 hours and the largest load was observed at approximately 5.3 $\log_{10}$ viral copies in Aseel, while it was significantly ($p<0.001$) low as 2.15 $\log_{10}$ viral copies as in Kadaknath CEF cells at 12 hpi. Whereas, in Kadaknath highest load was recorded at 24 hours and found as 4.63 $\log_{10}$ viral copies (Figure 3d). In the later hours, the viral production gradually decreased. However, the viral load in the Aseel CEF was still higher than in Kadaknath CEF cells at any time, confirming that Kadaknath cells potentially restrict the multiplication of ND viruses compared to Aseel CEF cells.

3.2. Expression analysis of chIFITM gene in Newcastle disease virus infected CEF cells

The relative expression of chIFITM genes in the control (uninfected) and infected cells were quantified by qRT-PCR. Our results showed that the expression of the chIFITM 1, 2, 3, 5, IFN-γ and Mx genes are in a time-dependent fashion (Table 2 and Figure 3a and 3b). After ND viral infection of the CEF cells, the mRNA levels of all four selected genes (chIFITM 1, 2, 3, 5) and positive immune-related genes (IFN-γ and Mx) were gradually increased reaching a peak at different hours post-infection (hpi) in both breeds. In comparison to the calibrator (uninfected-control CEF cells), the relative expression of IFN- γ reaches peck at 48 hpi as 2.59 $\log_2$ fold and 13.9 $\log_2$ fold higher in Aseel and Kadaknath, respectively. The results show that the chIFITMs are expressed at basal levels in CEF cells of both breeds. It also demonstrates that the breeds that were studied exhibit various patterns of expression. Compared to Kadaknath CEF, expression of the chIFITMs gene is lower and more variable in Aseel.

In Aseel, only at 12 hpi expression of the chIFITM2 gene was dramatically increased, whereas chIFITM5 upregulation begins at 6 hpi and continues until 24 hpi. In addition, the highest expression in chIFITM2 and 5 genes were recorded as 3.25 $\log_2$ fold and 3.82 $\log_2$ fold respectively at 6 hpi and 24 hpi. The chIFITM3 gene was strongly expressed from 3 to 12 hpi, with the maximum level being 3.46 $\log_2$ fold at 6 hpi. Like chIFITM3, chIFITM1 upregulation starts at 3 hpi, but it continues strongly until 48 hpi and reaches a maximum of 6 $\log_2$ fold at 6 hpi. Mx and chIFITM3 exhibit comparable patterns of expression, Mx gene reaching its highest level of expression at 3 hpi as a 4.18 $\log_2$ fold increase.

As opposed to Aseel, Kadaknath CEF cells express all chIFITMs genes strongly and severalfold significantly higher from 3 to 48 hours after ND virus infection. At 6 hpi, the highest expression of chIFITM2 was detected. chIFITM1, 5 and Mx were at 24 hpi, but chIFITM2 and IFN-γ at 48 hpi. In Kadaknath, the expression level among the chIFITMs ranged from 2.39 to 11.68 $\log_2$ folds more than that of control CEFs, which was over the entire time span, significantly ($p<0.01$) higher than Aseel CEFs (Figure. 3c). Similarly, IFN-γ expresses strongly from the beginning and reaches maximum as 13.9 $\log_2$ fold at 48 hpi. The expression levels of chIFITM1 and 3 were found to be higher than the other chIFITMs in Aseel and Kadaknath respectively.

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 28 April 2023 doi:10.20944/preprints202304.1180.v1

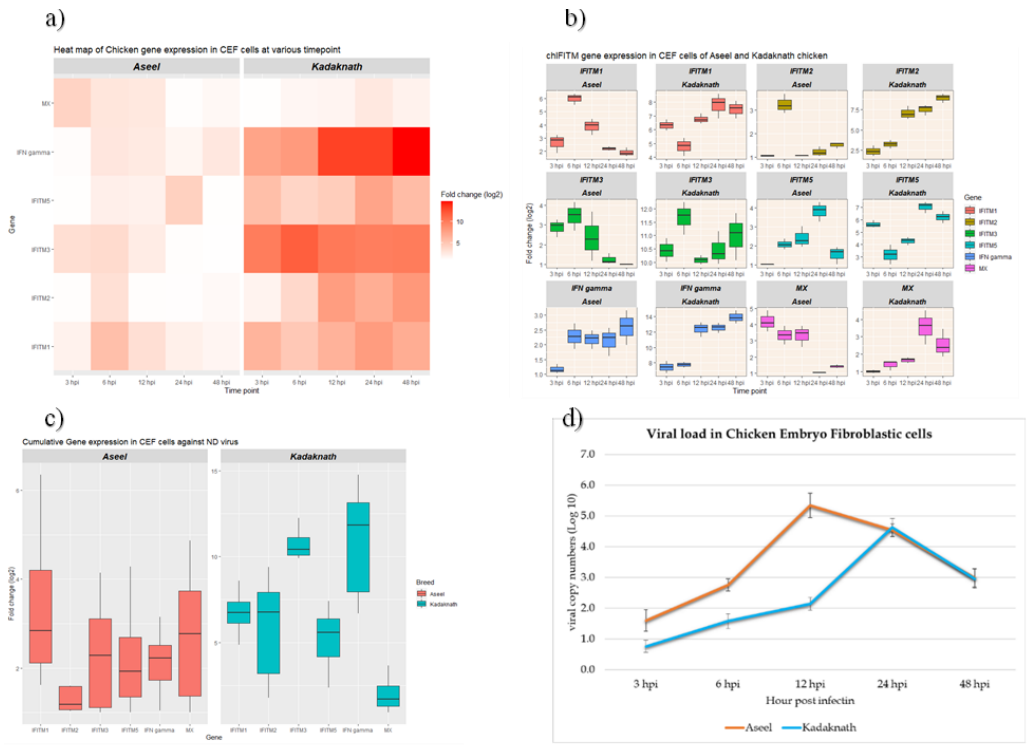

Figure 3. Gene expression analysis and viral load in CEF of Aseel and Kadaknath. **(a, and b)** Heatmap and Boxplot respectively, illustrating the various levels of gene expression at different hours post-infection (hpi) of ND virus infection. **(c)** Boxplot showing the cumulative gene expression of each gene in the Kadaknath and Aseel throughout time. **(d)** A line graph depicts the absolute quantity of viral load based on T-NP plasmid standards at various time point in CEF cells.

Table 2. Chicken embryo fibroblastic cells' gene expression fold changes ($\log_2 (2^{-\Delta\Delta Ct})$) in response to the Newcastle disease virus infection.

Breed	Time point	*IFITM1*	*IFITM2*	*IFITM3*	*IFITM5*	*IFN-γ*	*MX*
Aseel	**3 hpi**	2.64±0.40^{c}	1.06±0.01^{c}	2.87±0.26^{b}	1.01±0.00^{b}	1.17±0.09^{c}	4.18±0.38^{a}
	6 hpi	6.00±0.25^{a}	3.25±0.24^{a}	3.46±0.42^{a}	2.07±0.16^{b}	2.28±0.24^{ab}	3.35±0.33^{b}
	12 hpi	3.88±0.35^{b}	1.06±0.00^{c}	2.38±0.71^{b}	2.41±0.32^{b}	2.18±0.18^{b}	3.34±0.38^{b}
	24 hpi	2.19±0.09^{c}	1.22±0.12^{bc}	1.26±0.16^{c}	3.82±0.30^{a}	2.13±0.28^{b}	1.03±0.01^{c}
	48 hpi	1.91±0.20^{cd}	1.51±0.08^{b}	1.00±0.00^{c}	1.53±0.27^{c}	2.59±0.34^{a}	1.43±0.06^{c}
Kadaknath	**3 hpi**	6.33±0.24^{b}	2.39±0.38^{d}	10.44±0.26^{b}	5.63±0.17^{b}	7.46±0.45^{c}	1.01±0.05^{c}
	6 hpi	4.77±0.38^{c}	3.25±0.29^{c}	11.68±0.36^{a}	3.18±0.46^{c}	7.77±0.21^{c}	1.38±0.16^{c}
	12 hpi	6.77±0.21^{b}	7.02±0.47^{b}	10.08±0.10^{b}	4.28±0.19^{bc}	12.38±0.54^{b}	1.66±0.10^{c}
	24 hpi	7.79±0.52^{a}	7.48±0.36^{b}	10.47±0.36^{ab}	7.04±0.27^{a}	12.56±0.39^{b}	3.57±0.56^{a}
	48 hpi	7.48±0.36^{a}	8.89±0.33^{a}	11.00±0.51^{a}	6.22±0.27^{a}	13.9±0.49^{a}	2.56±0.46^{b}

Mean value with different superscript shows significant different at $p<0.05$.

4. Discussion

To measure the level of *chIFITM* gene expression against NDV in the current work, we employed the *velogenic genotype XIII* of NDV strain that was used to infect CEF cells.

The ND viral load increases, prompting the chicken embryo fibroblast (*CEFs*) cells to express more *chIFN-γ* significantly [25]. In Aseel's CEF cells, there was noticeable upregulation starting at 6 hpi and continuing until 48 hpi. In contrast, in Kadaknath CEF cells, *chIFN-γ* expression began to increase significantly at 3 hpi and peaked (13.9±0.49 $\log_2$ fold) like Aseel CEF at 48 hpi. Further, it was significantly ($p<0.001$) several folds higher than Aseel. However, interferons (IFNs), a vital component of innate immune signaling, serve as the first line of defense against invading viruses [26]. Therefore, it consistently and strongly expressed against the ND virus [27]. *chIFITM*s and *Mx* are members of the interferon-stimulating gene (*ISG*) group (Mycovirus resistant gene) [28,29]. Chicken has five members of the *IFITM* family: *chIFITM*1, *chIFITM*2, *chIFITM*3, *chIFITM*5, and *chIFITM10*. These protein genes are activated and made to express themselves by type I and *type II IFNs*, signifying the start of the innate host response [13,30]. Recent studies confirm that *STAT/IRF* signaling pathways activate *IFITM* gene expression together with other *ISGs* during infection and inflammation [31]. This study examines the relative mRNA expression profile of chicken *IFITM*s after Newcastle disease virus (NDV) infection *in vitro* with a focus on how the cells react during the early stages of NDV infection.

We observed significant upregulation of *chIFITM*s, *chIFN-γ* and *Mx* in the CEF cells of both breeds. A significant viral load indicated the presence of a replicating virus in the CEF cells. *chIFITM1, 2, 3*, and *5* are noticeably and gradually upregulated in both breeds of CEFs after NDV infection. In Kadaknath CEF cells, *chIFITM*s and *IFN-γ* expressions were relatively high with statistical significance ($p<0.001$) from 3 to 48 hours post-infection compared to control uninfected. Researchers also found that high quantities of *chIFITM1, 2*, and *3* are expressed in CEFs from 4 to 24 hours after H9N2 infection.[32]. While CEF cells from Aseel took longer to exhibit strong mRNA expression of *chIFN-γ* and *chIFITM* genes strongly expressed at 6 hours post-infection. In Kadaknath CEF, among the *IR-IFITM* family members, *chIFITM*3 (11.68 $\log_2$ folds) has the greatest expression observed at 6 hours post-infection, followed by *chIFITM*2 (8.89 $\log_2$ folds) at 48 hours post-infection, *chIFITM1* (7.79 $\log_2$ folds) and *chIFITM*5 (7.04 $\log_2$ folds) at 24 hours post-infection [13,15]. But in Aseel, *IFITM*1 (6.00 $\log_2$ folds) came first, then *chIFITM*3 (3.46 $\log_2$ folds), and then *chIFITM*2 (3.25 $\log_2$ folds) at 6hpi, lastly *chIFITM5* (3.82 $\log_2$ folds) at 24hpi. In our finding that Kadaknath expressed large levels of *IFN-γ* and stimulated high levels of *chIFITMs* compared to Aseel is supported by studies from other scientists that *IFN* treated CEFs expressed high levels of *chIFITMs* [13,30,32]. It is known that *chIFITM 1, 2*, and *3* prevent the replication of a variety of RNA viruses that enter the host cell through endocytosis [8]. Infected CEF of Kadaknath showed a significant ($p<0.01$) and robust overexpression of all *chIFITM*s started from 3hpi when compared to the control. Whereas similarly in Aseel *chIFITM* genes upregulation starts from at 3 hours post-infection, compared to the control. A Significant and high level *Mx* gene response has been observed at 3 hours post-infection (4.18 $\log_2$ folds) and then decreased level of expression similar to H9N2 infected CEFs [32] and it was delayed in Kadaknath at 24 hours post-infection (3.57 $\log_2$ folds) [31]. *Mx* gene was used as positive control gene because it is a one of the well-known *IFN*-stimulating and it highly expressed as restriction factor of influenza A viral infection [33].

Results from other publications' findings support in a similar way that the expression of *chIFITM*-2, 3, and *Mx* significantly increased after H3N8 infection, and this increase started at 6 hours after infection. Although there was a reduction in *Mx* expression at 12 hours after infection and both *chIFITM*2 and 3 were significantly elevated. At 6 hours after infection, *chIFITM1 2, 3*, and *5* and *Mx* expression significantly increased and persisted for 24 hours in H5N3-infected CEF cells [32]. Like this, *IFITM*s were constantly and significantly upregulated in Kadaknath CEF cells throughout the study, which may be related to the increased level of *IFN-γ* gene expression. In contrast, Aseel CEFs expressed low levels of *IFN-γ* and *chIFITM* genes investigated. This is supported by the findings of other papers. Thus, it was hypothesized that interferons would activate and upregulate the expression of *chIFITM* in CEFs based on evidence from researchers Whitehead and

 doi:10.20944/preprints202304.1180.v1

Smith et al. [14,32]. It was further demonstrated that it is possible to inhibit virus replication by simply preventing access into a cell as evidenced by the production of the *IFITM*s following *IFN* treatment [26,30].

We measured the $\log_{10}$ viral copies of NDV at 3-, 6-, 12-, 24- and 48-hours post-infection in CEF cells and contrasted both breeds. The viral load steadily increased from 3 hours post-infection itself in both breeds and the viral load was significantly ($p<0.01$) lower in Kadaknath when compared to Aseel from 3- to 12- hours post-infection and also overall load through the infected period. The outcomes showed that the ND viral load at 12 hours post-infection in Kadaknath CEF (2.15 $\log_{10}$) cells was reduced by 60% in comparison to Aseel CEF (5.38 $\log_{10}$) cells. Inversely proportional to viral load, Kadaknath showed significantly ($p<0.01$) high expression of all the *chIFITM* genes at all the periods than Aseel. Like this, Blyth et al. found that overexpressing *chIFITM*3 reduces influenza H6N2 and H1N9 strain infection in DF-1 cells by 30 to 40% [34]. Similarly to this, *in vitro* overexpression of *chIFITM*3 limits the multiplication of the influenza virus by 55% [14]. Infectious Bursal viral (IBV) strains of QX, M41-CK and Beaudette infection significantly upregulates all *IR-chIFITM* genes at 24 hpi [13]. Scientist concluded that *chIFITM*2 and 3 greatly decrease the lyssavirus infection [13,14].

However, by altering the characteristics of cellular membranes and blocking the cell surface receptors to restrict viral entry, the Interferon-inducible transmembrane proteins (*IFITM*s) prevent many harmful viruses from infecting cells and causing infection [35–37]. This ultimately prevents viral fusion [36]. Several reports confirm that the *IFITM*s effectively control the RNA viruses such as Avian influenza A virus (IAV), Lyssaviruses [14], Infectious Bronchitis virus (IBV) [13] and Avian Reovirus multiplication (ARV) [30], which follows the endosomal pathway to enter the host cell membrane for multiplication.

Microscopic analysis of the infected cells showed that the monolayer remained intact, much like in mock-infected cells, and that no CPE occurred until 3 h post-infection (Aseel) to 6 h post-infection (Kadaknath). However, after 6 to 12 hpi, the light-microscopy analysis revealed morphological changes indicative of CPE, including rounding, the fusing of infected cells to form syncytia, and the detachment of cells from the monolayer followed by cell death. Cellular rounding, membrane blebbing, cytoplasm vacuolation, nuclear condensation, and nuclear envelope collapse are among the morphological changes brought on by NDV infection. This result is congruent with what has been documented in other publications [38]. The CPE in the current investigation, however, showed that CEF cells of both breeds began exhibiting CPE sooner than had been previously reported. According to Li *et al*., overexpression of *IFITM*3 inhibited the inflammatory response of PF15 cells and is crucial to the *TLR4-NF-B* signalling pathway, which is implicated in the inflammatory response [39]. In Kadaknath, *chIFITM*3 expression levels are consistently high ($p<0.01$), with a $\log_2$ fold range of 10.08 to 11.68 in a contrast to 1.00 to 3.46 in Aseel. As a result, compared to Aseel, the Kadaknath CEF cells had a delayed cytopathic effect and cell death. Elevated *IFITM* gene expression inhibits the spread of infections by restricting host cell proliferation. It is also involved in inhibiting cell adhesion and controlling cell growth [40]. Additionally, Anjum et al. noticed a decrease in cytopathic effects in *chIFN*-treated CEF cells when they were infected with ND and AIV [41]. Similarly, in Kadaknath CEF cells expressed high level of *IFN-γ* and delayed cytopathic changes was observed.

5. Conclusions

In this study: we have shown the variability in the magnitude of *chIFITM*s mRNA expression between breeds during the course of Newcastle disease Viral-infection. Such variation suggests that the *chIFITM* response may be breed-dependent and intragenic factor-dependent. Our data suggest CEF cells start expressing all *chIFITM* genes significantly in the early stage of infection, regardless of the breed of the chicken. Elevated levels of expression of *chIFITM*s in Kadaknath CEF cells restrict the viral multiplication compared to the Aseel CEFs. Together the result shows *chIFITM*s plays a critical role in restricting the ND virus multiplication. In addition, it has been shown that the basal level of *IFN-γ*

expression will impact the *chIFITM* gene expression. Therefore, we have revealed that viral entry is restricted depending on the level of *chIFITM*s expression and the expression depends on other factors. This study was conducted *in vitro* and more experiments are necessary to clarify the underlying mechanism in controlling the viral diseases in chicken. In future, *In-Ovo and In vivo* studies will be required to the better understand the role of this gene in immune system.

Author Contributions: Conceptualization, M.M., A.K.T. and A.R; methodology, M.M., and A.K.T.; software, M.M., and V.B.R.; validation, N.M., K.S., and M.S.; formal analysis, A.K.T.; investigation, M.M.; resources, V.G.; data curation, M.M. and A.K.T.; writing—original draft preparation, M.M., and A.K.T.; writing—review and editing, N.M., M.S., A.R., S.O.P and K.S.; supervision, A.K.T., N.M., M.S., and K.S; project administration, N.M., K.S., and M.S.; funding acquisition, N.M., and M.S.: All authors have read and agreed to the published version of the manuscript.

Funding: Please add: This research was funded by TANUVAS, India. (USO. No. 20652/A1/2020 and No.273/A1/2021, dated 26.02.2021 of Registrar, TANUVAS, Chennai – 51, India.)

Institutional Review Board Statement: The experiment was conducted with the approval of the Institutional Bio-safety Committee (IBSC) of TANUVAS-Veterinary College and Research Institute, Namakkal, Tamil Nadu, India (Approval Lr. No. 1764/VCRI-NKL/IBSC/2022 dated 11.05.2022 of the Dean, VCRI, Namakkal).

Informed Consent Statement: Not applicable.

Data Availability Statement: The data set created and analysed in the current study will be made available on reasonable request.

Acknowledgments: The authors gratefully acknowledge the faculties of the Poultry Disease Diagnosis and Surveillance Laboratory (PDDSL), TANUVAS, Namakkal, India, for their support and for laboratory facilities.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Chambers, P.; Millar, N.S.; Bingham, R.W.; Emmerson, P.T. Molecular Cloning of Complementary DNA to Newcastle Disease Virus, and Nucleotide Sequence Analysis of the Junction between the Genes Encoding the Haemagglutinin-Neuraminidase and the Large Protein. *J. Gen. Virol.* **1986**, *67 (Pt 3)*, 475–486, doi:10.1099/0022-1317-67-3-475.
2. O.I.E. *Newcastle Disease. Chapter 2.3.14*; In, Ed.; World Organisation for Animal Health: Paris, 2012;
3. Qosimah, D.; Murwani, S.; Sudjarwo, E.; Lesmana, M.A. Effect of Newcastle Disease Virus Level of Infection on Embryonic Length, Embryonic Death, and Protein Profile Changes. *Vet. World* **2018**, *11*, 1316–1320, doi:10.14202/vetworld.2018.1316-1320.
4. Lamb, R.A.; Parks, G.D. Paramyxoviridae: The Viruses and Their Replication. In *Fields virology*; Fields, B.N., Knipe, D.N., Howley, P.M., Eds.; Lippincott, Williams, and Wilkins, 2007; pp. 1449–1496.
5. Kalaria, V.A.; Prajapati, K.S.; Javia, B.B.; Bhadaniya, A.R.; Fefar, D.T.; Vagh, A.A.; Trangadiya, B.J.; Padodara, R.J.; Mokaria, K.N.; Kumbhani, T.R. An Economical Impact of Newcastle Disease Outbreaks in Various Commercial Broiler Chicken Farms During 2020- 21 in Gujarat, India. *Int J Curr Microbiol App Sci* **2021**, *10*, 411–420, doi:doi:
6. Aristizábal, B.; González, Á. *Innate Immune System*; Anaya, J.M., Shoenfeld, Y., Rojas-Villarraga, A., Eds.; Autoimmunity: Bogota (Colombia, 2013;
7. Dai, M.; Xie, T.; Liao, M. Systematic Identification of Chicken Type I, II and III Interferon-Stimulated Genes. *Vet Res* **2020**, *51*, 70, doi:10.1186/s13567-020-00793-x.
8. Brass, A.L.; Huang, I.-C.; Benita, Y.; John, S.P.; Krishnan, M.N.; Feeley, E.M.; Ryan, B.J.; Weyer, J.L.; van der Weyden, L.; Fikrig, E.; et al. The IFITM Proteins Mediate Cellular Resistance to Influenza A H1N1 Virus, West Nile Virus, and Dengue Virus. *Cell* **2009**, *139*, 1243–1254, doi:10.1016/j.cell.2009.12.017.
9. Jiang, D.; Weidner, J.M.; Qing, M.; Pan, X.-B.; Guo, H.; Xu, C.; Zhang, X.; Birk, A.; Chang, J.; Shi, P.-Y.; et al. Identification of Five Interferon-Induced Cellular Proteins That Inhibit West Nile Virus and Dengue Virus Infections. *J. Virol.* **2010**, *84*, 8332–8341, doi:10.1128/JVI.02199-09.
10. Huang, I.-C.; Bailey, C.C.; Weyer, J.L.; Radoshitzky, S.R.; Becker, M.M.; Chiang, J.J.; Brass, A.L.; Ahmed, A.A.; Chi, X.; Dong, L.; et al. Distinct Patterns of IFITM-Mediated Restriction of Filoviruses, SARS Coronavirus, and Influenza A Virus. *PLOS Pathog.* **2011**, *7*, e1001258, doi:10.1371/journal.ppat.1001258.
11. Feeley, E.M.; Sims, J.S.; John, S.P.; Chin, C.R.; Pertel, T.; Chen, L.-M.; Gaiha, G.D.; Ryan, B.J.; Donis, R.O.; Elledge, S.J.; et al. IFITM3 Inhibits Influenza A Virus Infection by Preventing Cytosolic Entry. *PLoS Pathog.* **2011**, *7*, e1002337, doi:10.1371/journal.ppat.1002337.
12. Fife, M.; Moore, J. IFITM Knockdown/Knockout Technology for Vaccine Production.

13. Steyn, A.; Keep, S.; Bickerton, E.; Fife, M. The Characterization of ChIFITMs in Avian Coronavirus Infection In Vivo, Ex Vivo and In Vitro. *Charact. ChIFITMs Avian Coronavirus Infect. Vivo Ex Vivo Vitro Genes* **2020**, *11*, 918, doi:10.3390/genes11080918.
14. Smith, S.E.; Gibson, M.S.; Wash, R.S.; Ferrara, F.; Wright, E.; Temperton, N.; Kellam, P.; Fife, M. Chicken Interferon-Inducible Transmembrane Protein 3 Restricts Influenza Viruses and Lyssaviruses In Vitro. *J. Virol.* **2013**, *87*, 12957–12966, doi:10.1128/JVI.01443-13.
15. Lanz, C.; Yángüez, E.; Andenmatten, D.; Stertz, S. Correction for Lanz et al., Swine Interferon-Inducible Transmembrane Proteins Potently Inhibit Influenza A Virus Replication. *J. Virol.* **2015**, *89*, 2988, doi:10.1128/JVI.03533-14.
16. Kidani, S.; Okuzaki, Y.; Kaneoka, H.; Asai, S.; Murakami, S.; Murase, Y.; Iijima, S.; Nishijima, K. Expression of Interferon-Inducible Transmembrane Proteins in the Chicken and Possible Role in Prevention of Viral Infections. *Cytotechnology* **2017**, *69*, 477–484, doi:10.1007/s10616-016-9958-1.
17. Hitchner, B.; Donermuch, H.; Graham Purchas, H.; William, E. *Isolation and Identification of Pathogens*; 2nd edn.; American Association of Avian Pathologists, Inc, 1980;
18. Office International des Epizooties (OIE) OIE Terrestrial Manual 2021 2021.
19. Toyoda, T.; Sakaguchi, T.; Hirota, H.; Gotoh, B.; Kuma, K.; Miyataj, T.; Nagai, Y. Newcastle Disease Virus Evolution: II. Lack of Gene Recombination in Generating Virulent and Avirulent Strains. *Virology* **1989**, *169*, 273–282, doi:10.1016/0042-6822(89)90152-9.
20. Ogbole, O.O.; Akinleye, T.E.; Segun, P.A.; Faleye, T.C.; Adeniji, A.J. In Vitro Antiviral Activity of Twenty-Seven Medicinal Plant Extracts from Southwest Nigeria against Three Serotypes of Echoviruses. *Virol. J.* **2018**, *15*, 110, doi:10.1186/s12985-018-1022-7.
21. Gowthaman, V.; Ganesan, V.; Gopala Krishna Murthy, T.R.; Nair, S.; Yegavinti, N.; Saraswathy, P.V.; Suresh Kumar, G.; Udhayavel, S.; Senthilvel, K.; Subbiah, M. Molecular Phylogenetics of Newcastle Disease Viruses Isolated from Vaccinated Flocks during Outbreaks in Southern India Reveals Circulation of a Novel Sub-Genotype. *Transbound. Emerg. Dis.* **2019**, *66*, 363–372, doi:10.1111/tbed.13030.
22. Qiu, X.; Yu, Y.; Yu, S.; Zhan, Y.; Wei, N.; Song, C.; Sun, Y.; Tan, L.; Ding, C. Development of Strand-Specific Real-Time RT-PCR to Distinguish Viral RNAs during Newcastle Disease Virus Infection. *Sci. World J.* **2014**, 934851, doi:10.1155/2014/934851.
23. Pfaf, M.W. A New Mathematical Model for Relative Quantification in Real-Time RT–PCR. *Nucleic Acids Res* **2001**, *29*, 45.
24. Ramakrishnan, S.; Annamalai, A.; Sachan, S.; Kumar, A.; Sharma, B.K.; Govindaraj, E.; Chellappa, M.M.; Dey, S.; Krishnaswamy, N. Synergy of Lipopolysaccharide and Resiquimod on Type I Interferon, pro-Inflammatory Cytokine, Th1 and Th2 Response in Chicken Peripheral Blood Mononuclear Cells. *Mol. Immunol.* **2015**, *64*, 177–182, doi:10.1016/j.molimm.2014.11.013.
25. Susta, L.; Cornax, I.; Diel, D.G.; Garcia, S.C.; Miller, P.J.; Liu, X.; Hu, S.; Brown, C.C.; Afonso, C.L. Expression of Interferon Gamma by a Highly Virulent Strain of Newcastle Disease Virus Decreases Its Pathogenicity in Chickens. *Microb Pathog* **2013**, *61–62*, 73-83 , doi:10.1016/j.micpath.2013.05.009.
26. Yang, X.; Arslan, M.; Liu, X.; Song, H.; Du, M.; Li, Y.; Zhang, Z. IFN-γ Establishes Interferon-Stimulated Gene-Mediated Antiviral State against Newcastle Disease Virus in Chicken Fibroblasts. *Acta Biochim Biophys Sin Shanghai* **2020**, *52*, 268–280, doi:10.1093/abbs/gmz158.
27. Bashir, K.; Kappala, D.; Singh, Y. Combination of TLR2 and TLR3 Agonists Derepress Infectious Bursal Disease Virus Vaccine-Induced Immunosuppression in the Chicken. *Sci Rep* **2019**, *9*, 8197, doi:10.1038/s41598-019-44578-5.
28. Li, X.Y.; Qu, L.J.; Hou, Z.C.; Yao, J.F.; Xu, G.Y.; Yang, N. Genomic Structure and Diversity of the Chicken Mx Gene. *Poult. Sci.* **2007**, *86*, 786–789, doi:10.1093/ps/86.4.786.
29. Zhao, X.; Li, J.; Winkler, C.A.; P, A.; Guo, J.-T. IFITM Genes, Variants, and Their Roles in the Control and Pathogenesis of Viral Infections. *Front Microbiol* **2019**, *9*, 3228, doi:10.3389/fmicb.2018.03228.
30. Q., W.; X., Y.; C., L.; H., D.; C, W. Distribution of IFITM3 in Yellow-Feathered Broilers and Inhibition of Avian Reovirus Multiplication by IFITM3. *Braz. J. Poult. Sci.* **2018**, *20*, 377–386, doi:10.1590/1806-9061-2017-0662.
31. Friedlová, N.; Zavadil Kokáš, F.; Hupp, T.R.; Vojtěšek, B.; Nekulová, M. IFITM Protein Regulation and Functions: Far beyond the Fight against Viruses. *Front. Immunol.* **2022**, *13*.
32. Whitehead, T.J. Characterisation of Chicken Interferon-Inducible Transmembrane Proteins: Locus Architecture, Gene Expression and Viral Restriction. Doctoral thesis (Ph. D), 2018.
33. Haller, O.; Kochs, G. Mx Genes: Host Determinants Controlling Influenza Virus Infection and Trans-Species Transmission. *Hum. Genet.* **2020**, *139*, 695–705, doi:10.1007/s00439-019-02092-8.
34. Blyth, G.A.D.; Chan, W.F.; Webster, R.G.; Magor, K.E. Duck Interferon-Inducible Transmembrane Protein 3 Mediates Restriction of Influenza Viruses. *J. Virol.* **2016**, *90*, 103–116, doi:10.1128/JVI.01593-15.
35. Perreira, J.M.; Chin, C.R.; Feeley, E.M.; Brass, A.L. IFITMs Restrict the Replication of Multiple Pathogenic Viruses. *J. Mol. Biol.* **2013**, *425*, 4937–4955, doi:10.1016/j.jmb.2013.09.024.
36. Desai, T.M.; Marin, M.; Chin, C.R.; Savidis, G.; Brass, A.L.; Melikyan, G.B. IFITM3 Restricts Influenza A Virus Entry by Blocking the Formation of Fusion Pores Following Virus-Endosome Hemifusion. *PLoS Pathog* **2014**, *10*, 1004048.
37. Amini, B.O.S.; Choi, Y.J.; Lee, J.H.; Shi, M.; Huang, I.C.; Farzan, M.; Jung, J.U. The Antiviral Effector IFITM3 Disrupts Intracellular Cholesterol Homeostasis to Block Viral Entry. **2013**, 452–464.
38. Ravindra, P.V.; Tiwari, A.K.; Ratta, B.; Chaturvedi, U.; Palia, S.K.; Chauhan, R.S. Newcastle Disease Virus-Induced Cytopathic Effect in Infected Cells Is Caused by Apoptosis. *Virus Res* **2009**, *141*, 13–20, doi:10.1016/j.virusres.2008.12.008.
39. Li, X.; Su, S.; Cui, N.; Zhou, H.; Liu, X.; Cui, Z. Transcriptome Analysis of Chicken Embryo Fibroblast Cell Infected with Marek's Disease Virus of GX0101ΔLTR. *Braz. J. Poult. Sci.* **2017**, *19*, 179–184, doi:10.1590/1806-9061-2016-0329.

40. Siegrist, F.; Ebeling, M.; Certa, U. The Small Interferon-Induced Transmembrane Genes and Proteins. *J Interferon Cytokine Res* **2011**, *31*, 183–197, doi:10.1089/jir.2010.0112.
41. Anjum, F.R.; Rahman, S.U.; Aslam, M.A.; S, Q.A. Antiviral Potential and Stability Analysis of Chicken Interferon-α Produced by Newcastle Disease Virus in Chicken Embryo Fibroblast Cells. *Vet Med-Czech* **2021**, *66*, 197–207.