Transcriptional response of chicken embryo cells to Newcastle disease virus (D58 strain) infection

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Abstract Newcastle disease virus (NDV), the causative agent of Newcastle disease (ND) in chicken causes significant economic loss for the poultry industry worldwide. The mechanism involved in host response to NDV infection is not well understood. For better understanding of the virus–host interaction; transcriptional profile of some genes of chicken embryo (CE) cells infected with NDV vaccine strain D58 was established using quantitative RT-PCR SYBR Green method. The relative standard curve method was used to measure the level of transcripts of the cellular genes against an endogenous control (β actin) gene. Among the genes studied, IFN α , IFN γ , MHC I and DDX 1 were up-regulated while IL 6 was down regulated. The expression of viral genes (M and F) in the infected CE cells was also confirmed by relative quantification. The host cellular genes involved in pro-inflammatory response, interferon-regulated proteins and the cellular immune response were affected by NDV infection, indicating involvement of complex signaling pathways of host cell responses to the infection. Thus, this study contributes to the understanding of the pathogenesis of ND and provides an insight into the virus–host interaction.

Keywords Newcastle disease virus · CE cell culture · Quantitative PCR · Gene expression · Transcriptional profile

Introduction

Newcastle disease (ND) [9] is considered as one of the most important avian diseases in Asian countries. The disease has been reported to be present in endemic form in many Asian countries with frequent outbreaks in chickens and other birds. Newcastle disease virus (NDV) isolates have been classified into five pathotypes namely viscerotropic velogenic, neurotropic velogenic, mesogenic, lentogenic and apathogenic. The intra cerebral pathogenicity index (ICPI), intra venous pathogenicity index (IVPI), mean death time (MDT) and deduced amino acid sequences at the F protein cleavage site (FPCS) form the basis for grouping of NDV isolates into different pathotypes. NDV with an ICPI value of more than 0.7 has been reported to cause ND with increased morbidity and mortality [2]. As per the Terrestrial Manual of OIE-2012, lentogenic and apathogenic strains alone should be used as seed virus in live and inactivated vaccines. The genome of NDV is single stranded negative sense RNA and has been reported to be made up of approximately 15,186 nucleotides [7, 14] comprising of genes coding for nucleoprotein (N), phosphoprotein (P), matrix protein (M), haemagglutinin-neuraminidase (HN), fusion (F) and large protein (L). Of these proteins, fusion (F) protein has been reported to mediate the fusion of viral envelope with the host cell membrane and considered as the major determinant of virulence [25]. A pair of basic amino acids lysine (K) and/or arginine (R) at positions 116 and 115 with a phenylalanine (F) at position 117 and arginine at position 113 has been reported to determine the virulence of the virus for chicken [1]. The M protein has been reported to be responsible for the budding of virus [6, 12, 24] and viral assembly making interaction with HN and F proteins, envelope and the nucleocapsid [28, 32].

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Generally, host–virus interactions include a complex interplay of molecular pathways directed by the host to prevent viral replication and counter measures by the virus to favor its propagation. Elucidation of these responses with regard to both virus and host could provide new insights into the molecular mechanisms governing host antiviral strategies, the pathogenesis of NDV infection and the gene expression markers in chicken embryo (CE) cell cultures after NDV infection. Further, information on actual mechanism of viral pathogenesis as a result of such a study will be helpful in identification of suitable strategies to control ND. To characterize the host response to an infection, a profile of genes whose expression is affected by the infection is therefore critical. In the present study, the CE cell cultures were infected with NDV D58 strain and the expression of genes of interest was then quantitated in a 5-day time course using the qPCR applying SYBR green chemistry. This D58 strain belongs to lentogenic pathotype with an ICPI value of 0.14 and has amino acids -112 G-R-Q-G-R-L 117- at the FPCS region and is being used as vaccine strain in priming the chickens [3]. To study the expression profile certain cytokine and cellular genes have been identified. The identification of genes namely interferon-IFN α and IFN γ ; interleukin—IL6, Major histocompatibility complex-MHC1, RNA metabolism-related proteins-DDX1, viral genes-F and M is based on their property and role played by them in establishing infection in the host. The IFN α gene has been selected since this has been reported to have antiviral function and toll like receptor (TLR) mediated activation of macrophages and dendritic cells in responses to viruses [17], IFN- γ has been identified for its role in the regulation of macrophage functions and in the expression of major histocompatibility class I and II antigens (MHC-I and MHC-II) and Fc receptor inducing an antiviral state [21]. The cytokine IL-6 has been selected because it has been reported to be one of the major factors necessary for the terminal differentiation of activated B cells into immunoglobulin secreting cells [23] and MHC I gene has been selected since MHC-I antigen presentation pathway provides the immune system with a way to detect intracellular pathogens by displaying intracellular protein fragments on the cell surface [29]. Further, dead box protein gene (DDX1) has been selected for its role in RNA metabolism [30]. The viral genes F and M have been selected for their role in virulence and virus assembly respectively. The expression levels of β actin gene has been used as internal control since its suitability has already been demonstrated in CE cell cultures after experimental infection with infectious bursal disease virus (IBDV) by qPCR [18]. The results of this study demonstrated that NDV infection alters the expression of most of the genes tested. The function and relationship between these genes and their potential in the antiviral response are discussed.

Materials and Methods

Virus

A thermo-stabilized low intracerebral pathogenicity index (ICPI 0.14) isolate of NDV (D58) obtained from apparently normal village chicken was selected [3]. The virus was adapted to CE cell cultures by serial passaging and the seventh passaged virus was used in this study.

CE cell culture and adaption of NDV (D58)

CE cell cultures were prepared from 10 day-old CEs of specific-pathogen-free (SPF) eggs (Venkys Hatchery, India) as per the procedure prescribed by Freshney [11]. The cell culture medium, MEM (Invitrogen, USA) containing 10 and 1 % foetal bovine serum (HiMedia, India) was used as growth and maintenance media respectively. The virus was adapted to CE cell by serial passaging. The CE cells were prepared in 25 cm² tissue culture flasks (Jet-Biofilm) and confluent cell monolayer was inoculated with NDV (D58) infected amnio-allantoic fluid (AAF). The cells were incubated at 37 °C for 1 h for virus adsorption. The unadsorbed virus was removed after incubation and fresh maintenance medium was added, and the cells were incubated for 4–5 days at 37 °C with 5 % CO₂ in CO₂ Incubator (Thermo Scientific, USA). Regular microscopic examination was carried out to assess the development of cytopathic effects (CPE) and the virus was harvested after 5 days post infection. After every passage, the tissue culture fluid was tested for the presence of NDV by haemagglutination (HA) test and polymerase chain reaction (PCR) for the FPCS region of the F gene [31]. The harvested virus was stored at –80 °C and used for subsequent passaging.

Sample preparation for gene expression study

The CE cells were cultured in six well tissue culture plates (Nunc, Denmark) into two groups for preparation of samples. After confluent monolayer formation, the CE cells of one well from each group were harvested as the sample of day 0 and the CE cells of treatment group were infected with 7th passaged NDV (D58) virus while of control group with MEM. The incubation of the CE cells and adsorption of the virus was performed as described above. The CE cells of one well from each group were harvested at 24-hour intervals during a 5-day time course. To harvest the cells, the cultures were quickly rinsed twice in pre-warmed PBS and trypsinized with 250 μ l of 0.1 % trypsin (1:250) (Invitrogen, Canada) and 0.5 mM EDTA (Life-Technologies, USA) solution (prepared in PBS) for 2–3 min. To stop the trypsinization, 750 μ l growth medium

was added. The cells were collected by centrifugation at 500 g (~2,400 rpm) for 10 min at room temperature (25 °C) and were preserved at -40 °C for RNA isolation.

Viral RNA extraction and reverse transcription

The total RNA including cell and virus was extracted from preserved CE cells using TRIZOL® LS reagent (Invitrogen, USA) as per manufacturer's instructions. Reverse transcription was carried out with random hexamer using iScript™ cDNA Synthesis kit (Bio-Rad, USA) following manufacturer's instructions. After cDNA synthesis, 10 µl of the cDNA from each sample was pooled, serially diluted, quantified and used for a standard curve in relative quantitative PCR assay and another 10 µl of the cDNA was diluted 10 times and stored at -40 °C for further use for gene expression study.

Quantitative PCR (qPCR) and gene expression data analysis

Quantitative PCR (qPCR) was carried out using SYBR® Green JumpStart™ Taq Ready Mix™ (Sigma, USA) following manufacturer's instructions. For the quantification of the genes, real time PCR was carried out in Real time Thermocycler (Realplex, Eppendorf, Germany) [19]. The primers used are provided as Table 1. The relative standard curve method was used for expression of cellular genes relative to reference (or endogenous control) gene in response to NDV (D58) infection and the presence of viral genes were detected by relative quantification.

Results

During passaging of the NDV into the CE cells, no characteristic changes could be observed up to 120 h post infection (hpi) for five passages. From sixth passage virus specific changes like rounding, syncytia formation and peeling of cells from the surface could be observed after 72 hpi. After every passage, the tissue culture fluid was found positive for virus both in HA test and in PCR for the FPCS region of the F gene. The qPCR assay were carried out for the expression of β actin gene and IFN α , IFN γ , IL 6, MHC I and DDX 1 genes at 0, 24, 48, 72, 96 and 120 hpi in NDV infected CE cells and in uninfected control CE at same time points. The Ct value obtained during the expression of these genes at time points mentioned above for both infected and uninfected control were normalized to "Sample negative" values and the expression ratio and percentage along with standard deviation (SD) and coefficient of variation (COV) were calculated. The standard curve was drawn with Ct value and log10 concentration of

cDNA (ng/reaction) with slope, intercept and r^2 value. The specificity of these genes of interest was confirmed by the melting curve. A threefold increase from the expression ratio of day 0 was considered as significant increase in expression of the gene and it was said to be up-regulated. Thus, significant increase in expression was noticed for IFN α and γ genes from 72 hpi, whereas the expression of IL 6 gene was slightly decreased throughout the study period. Among cellular genes, MHC I was strongly expressed on 72 hpi with 85 % increase in the expression ratio. The DDX1 gene expression was increased from 96 hpi. The expression ratio of IFN α , IFN γ , IL 6, MHC I and DDX 1 genes from 24 to 120 hpi at 24 h interval are depicted in Fig. 1. The fold increase in expression by keeping the expression level of 0 hpi as 1 $\times$ is provided in Table 2. The transcripts of M and F gene were identified from 24 and 48 hpi. The results of the qPCR assay for the expression of viral genes M and F are provided in Table 3.

Discussion

The main purpose of this study was to find out the transcriptional profile of D58 strain of NDV in chicken cells with an aim to know the alterations in host cell gene expressions and to understand the molecular mechanisms of viral pathogenesis. Previously such a type of work has been carried out in CE cells for IBDV [19]. In the present study the isolate of NDV used was obtained from a normal village chicken and used as priming vaccine in chicks. It has been reported to produce significant humoral, cell mediated and mucosal immune responses in chickens [33].

In the present study, representative genes for interferon—IFN α , and IFN γ , for interleukins—IL6, MHC I, and for RNA metabolism—DDX 1 were identified. Their transcripts were measured to find out their up/down regulation or the time of their expression in terms of hours post infection. Interferons have been reported to be pleiotropic cytokines with immune modulatory, antiviral, antitumor and growth inhibitory properties. At 72 hpi, the expression ratio of IFN α gene was at least sixfolds more than that at 0 day level and at 96 and 120 hpi, there were no significant increase in expression ratio. More or less constant expression of the gene up to 72 h post infection and again after 96 hpi could be due to the concentration of V protein which is an interferon antagonist and a product of the P gene [13]. However, in a study with virulent Texas GB strain of NDV, the virus was reported to up-regulate the IFN α gene from 36 hpi and no change was observed up to 24 hpi [22]. A constant expression of the IFN α gene was observed up to 7 days post infection with IBDV [19]. IFN γ has been reported to be involved in macrophage activation and inhibition of virus replication [8]. A protective

Table 1 Sequences of primers used in the study

Target gene	Primer sequence	Annealing temp (0 °C)	Product size (bp)	References
β-actin	β-actin F-GAG AAA TTG TGC GTG ACA TCA	60	152	[19]
	β-actin R-CCT GAACCT CTC ATT GCC A			
IFN-α	IFNAF-CCA GCA CCT CGA GCA AT	60	133	
	IFNAR-GGC GCT GTA ATC GTT GTC T			
IFN-γ	IFNYF-CAA GTC AAA GCC GCA CAT C	59	135	
	IFNYR-CGC TGGATT CTC AAG TCG TT			
MHC-I	MHCIF-CTT CAT TGC CTT CGA CAA A	64	137	
	MHCIR-GCC ACT CCA CGC AGG T			
DDX1	DDX1F-CGG CGT TCT CGG AAA TG	62	187	
	DDX1R-ACA ATT TGG ATA ACC GGA ATG C			
IL-6	IL-6 F-GTT CGC CTT TCA GAC CTA C	60	138	
	IL-6 R-ACC ACT TCA TCG GGA TTT A			
M gene	MF-GAA GCC ACT GTC GGC ATG ATC	59	204	This study
	MR-GCT TTG CAG CAC TTG GGG TG			
F gene	Sense Primer-CCT TGG TGA'ITC TAT CCG IAG	52	254	[32]
	CTG CCA CTG CTA GTT GIG ATA ATC C			

Fig. 1 Transcription profile of certain cytokine (IFN-α, IFN-γ, IL-6) and Cellular (MHC-I & DDXI) genes in chicken embryo cells infected with Newcastle disease virus (D58 strain)

role of IFN γ against NDV infection has also been demonstrated [26]. In this study the expression ratio of IFN γ gene was constant up to 48 h, but was observed at least 16-folds more at 72 hpi than at 24 hpi. At 96 and 120 hpi, the increase was up to four and sixfolds more, respectively. IFN γ was reported to be up-regulated from 24 hpi showing induction of immune response [27].

IL 6 has been reported to be responsible for regulation of inflammation with pronounced effect on host defense mechanisms. An elevation of IL 6 has been reported in peripheral mononuclear cells (PBMC) in chickens infected with *Salmonella enteritidis* [34]. However, elevation has

not been reported in an in vitro study with IBVDV on CE cells [19]. Data on elevation of IL 6 upon infection with NDV is inconsistent. In a study elevation of IL 6 was noticed with virulent velogenic viscerotropic strains of NDV namely California and rZJ1. However, in a study involving an atypical velogenic viscerotropic virus (Australia strain), two velogenic neurotropic viruses (Turkey ND and Texas GB) and a mesogenic virus (Anhinga strain), the IL 6 response to infection was reported to be reduced [10]. In the present study, no elevation in the expression level of IL 6 was reported from that of 0 hpi. This could be due to the fact that D58 strain is a lentogenic strain. This finding correlated

Table 2 Transcription profiles of certain cytokine and cellular genes in chicken embryo cells infected with Newcastle disease virus (D58 strain)

Hours post infection	Cytokine genes			Cellular genes	
	IFN α	IFN γ	IL 6	MHC I	DDX 1
0	0.99	0.10	0.93	0.87	1.00
24	1.13	0.11	0.85	1.49	0.54
48	2.62	1.63	0.50	9.65	0.61
72	6.72	16.38	0.28	87.18	0.86
96	1.90	3.54	0.13	4.07	5.32
120	2.46	6.05	0.34	12.72	4.84

with an earlier work, wherein up-regulation of IL 6 genes in splenocytes in chickens infected with virulent ND from 6 hpi in comparison to lentogenic strain LaSota was reported [27]. The cytokine IL 6 being pro-inflammatory and D58 being a lentogenic strain, the constant expression of IL 6 at the levels of 0 h post infection is justified.

The genetic control of disease resistance and immune response in chicken has been attributed to MHC, which was first discovered as blood group locus [5]. It has also been attributed as a marker for disease resistance [16]. The MHCs in chicken have been reported to perform the crucial functions of cellular communications in the immune response [15]. It has been reported that virulent NDV increases the expression of MHC I gene in splenocytes by threefolds 48 hpi [27]. In the present study a ninefold increase in expression of MHC I was reported at 48 hpi and 87-fold increase at 72 hpi. In another study using vaccine virus of IBDV, MHC I expression was more than

threefolds from 24 hpi and a gradual increase was observed up to 7 days [19]. The strong expression of MHC I with many folds compared to a virulent virus [27] could be due to the fact that D58 strain is a lentogenic strain and do not cause extensive damage to cells as done by virulent NDV.

DEAD box proteins have been reported to be a family of putative RNA helicases containing the core amino acid sequence DEAD (Asp-Glu-Ala-Asp) [20] and associated with all aspects of RNA metabolism, including translation, ribosome biogenesis, and pre-mRNA splicing. To the best of our knowledge we could not come across any paper dealing with expression of DDX 1 gene in in vivo or in vitro systems infected with NDV. In the present study, the expression of DDX1 gene was found to increase more than threefolds only on 96 hpi. In a study involving vaccine virus of IBD, the expression of this gene was reported to be at constant level [19]. We attribute that the slight up-regulation of this gene at later stage of infection; 96 hpi could be due to accumulation of double stranded viral RNA replicative intermediates as this protein is reported to be involved in RNA metabolisms and possess RNA helicase activity [4].

Based on our results, we conclude that the D58 strain used in this study belongs to lentogenic pathotype as per the expression levels of IFN α , IL 6 and MHC I developed in this study. This also concurs with the previous work carried out for these genes using velogenic, mesogenic and lentogenic strains. Further, expression profile of more genes and correlation of these with biological tests will throw more light into the exact mechanism of pathogenesis of NDV.

Table 3 Expression of viral M and F gene in chicken embryo cells infected by Newcastle disease virus (D58 strain)

Gene	Hours post infection	cDNA ng/rxn	Ct value					Melting temperature					Positive/Negative
			Ct 1	Ct 2	Mean	SD	COV	1	2	Mean	SD	COV	
M Gene	0	10	UD	UD	UD	UD	UD	UD	UD	UD	UD	UD	Negative
	24	10	22.58	22.53	22.56	0.04	0.16	85.1	85.1	85.1	0.0	0.0	Positive
	48	10	19.36	18.98	19.17	0.27	1.40	85.1	85	85.1	0.1	0.1	Positive
	72	10	19.01	19.25	19.13	0.17	0.89	85	85.1	85.1	0.1	0.1	Positive
	96	10	16.82	16.61	16.72	0.15	0.89	84.7	84.7	84.7	0.0	0.0	Positive
	120	10	18.98	18.01	18.50	0.69	3.71	84.6	84.6	84.6	0.0	0.0	Positive
F Gene	0	10	UD	UD	UD	UD	UD	UD	UD	UD	UD	UD	Negative
	24	10	25.54	25.33	25.44	0.15	0.58	UD	UD	UD	UD	UD	Negative
	48	10	22.72	22.96	22.84	0.17	0.74	86.5	86.3	86.4	0.1	0.2	Positive
	72	10	22.09	21.91	22.00	0.13	0.58	86.3	86.5	86.4	0.1	0.2	Positive
	96	10	19.82	19.78	19.80	0.03	0.14	86.3	86.3	86.3	0.0	0.0	Positive
	120	10	21.91	22.01	21.96	0.07	0.32	86.6	86.6	86.6	0.0	0.0	Positive

UD undetermined

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