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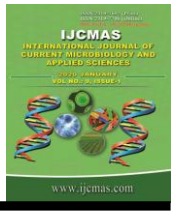

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# Expression of *RelA*, *IKBα* and *IKKβ* Genes in *peste des petits* Ruminants Virus Infected Cells

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**A B S T R A C T**

Peste des petits ruminants (PPRV) is a highly contagious transboundary viral disease of small ruminants with widespread distribution and poorly characterized molecular pathogenesis. The present study aimed at delineating the role of NF-kB signalling pathway during PPRV infection in the *in-vitro* system. For this the gene expression of three major regulatory proteins of NF-kB pathway *viz*. IKBα, IKKβ and RelA were analysed in the PPRV infected and mock infected Vero cells as a function of time using SYBR-green based one-step RT-qPCR. The results genes revealed downregulation of IKBα gene and upregulation of RelA and IKKβ genes in the PPRV infected group as against mock infected during initial hours of infection.

## Introduction

NF-κB is considered as a hallmark of most viral infections and confers resistance to viral infections due to its ability to promote the expression of numerous proteins involved in innate and adaptive immunity. Therefore, NF-κB activation during viral infection has been interpreted as a protective response of the host to the viral pathogen. Furthermore, this hypothesis is supported by the fact that mice deficient in different members of the NF-κB family are more susceptible to infection (Tato and Hunter, 2002). However, it is now evident that viruses can directly activate NF-κB and can utilize it in different ways. Studies have indicated that NF-κB activation could be a strategy evolved by different viruses to block apoptosis and prolong survival of the host cell in order to gain time for replication

and increase viral progeny production. This has been shown for several viruses, including HIV, herpesviruses, HCV and encephalo myocarditis virus (EMCV) (Roulston *et al.*, 1999).

Members of morbillivirus genus have been reported to up-regulate or down-regulate the expression of NF-κB family of proteins. The P, V, and C proteins of MV can bind and inhibit NF-κB-dependent gene expression, particularly the binding of the V protein with NF-κB subunits p65 (Schuhmann *et al.*, 2011). Furthermore, the rinderpest virus has been incriminated in interfering innate immune response by blocking the activity of IRF3 and NF-kB (Boxer *et al.*, 2009). The information on the role of NF-kB in the pathogenesis of peste des petits ruminants (PPRV) is meagre and only modulation of NF-kB regulating miRNAs has been reported in a transcriptomic study (Pandey *et al.*, 2017). More importantly, no information is available on the expression profile of the major regulatory gene of this pathway in the PPRV infection. The present study aimed at determining the gene expression pattern of three major regulatory proteins *viz*. IκBα, IKKβ and RelA in PPRV infected cells in the *in-vitro* system.

## Materials and Methods

### Virus and cells

A highly virulent form of PPRV (Palampur/16) available at the National Morbillivirus Referral Laboratory, IVRI, Mukteshwar campus was used in the present study. Vero cells were propagated using EMEM supplemented with 10% FBS and L-Glutamine. Antibiotic-antimycotic solution containing penicillin (10,000 IU/mL), streptomycin (10 mg/mL), and amphotericin B (25 µg/mL) were included in the media. The cells were maintained at 37 °C in the presence of 5% $CO_2$.

### Infection of Vero cell with PPRV

The PPRV was subjected to virus titration in 96 well microtiter plates using Vero cells through $TCID_{50}$ method (Sannat *et al.*, 2014) by cell-ELISA (Sarkar *et al.*, 2012). The cells were infected with PPRV at a concentration of 0.01 m.o.i. Cells mock-infected with EMEM served as control. The cells were incubated at 37 °C with gentle shaking and the cells were collected on an hourly basis till 5 hours post-infection (hpi). The cells were centrifuged and the pelleted cells were used for RNA extraction.

### Quantification of genes

Three major regulatory genes, namely, IKKβ, IKBα and RelA were amplified in both infected and control samples at different time points through SYBR green chemistry-based one-step RT-qPCR. Beta-actin was included as an endogenous control. The primers were designed against the above-said genes using Primer Select software (DNAstar) with following sequences:

RNA from the cell pellet was isolated using SV Total RNA isolation kit (Promega). The one-step RT-qPCR was performed using One Step TB Green PCR kit (Takara) through StepOnePlus Real-Time PCR system (Applied Biosystem).

A 10 µl total reaction included 0.4 µM of forward and reverse primers and 2.5 µl of RNA template. The cycling condition was as follows: RT step - 42 °C for 5 min; initial heat activation - 95 °C for 10 sec; 40 cycles of denaturation at 95 °C for 5 sec and combined annealing/extension at 60 °C for 30 sec. The melt curve profiling was left as default for the apparatus. The specificity of the product was ascertained by the overlapping of the melt curves at single melting temperature.

## Statistical analysis

The data pertaining to the relative quantification of NF-kB pathway genes among infected and mock-infected cells was analyzed using two-way ANOVA using SPSS software. A p-value of 5% or lower was considered statistically significant.

## Results and Discussion

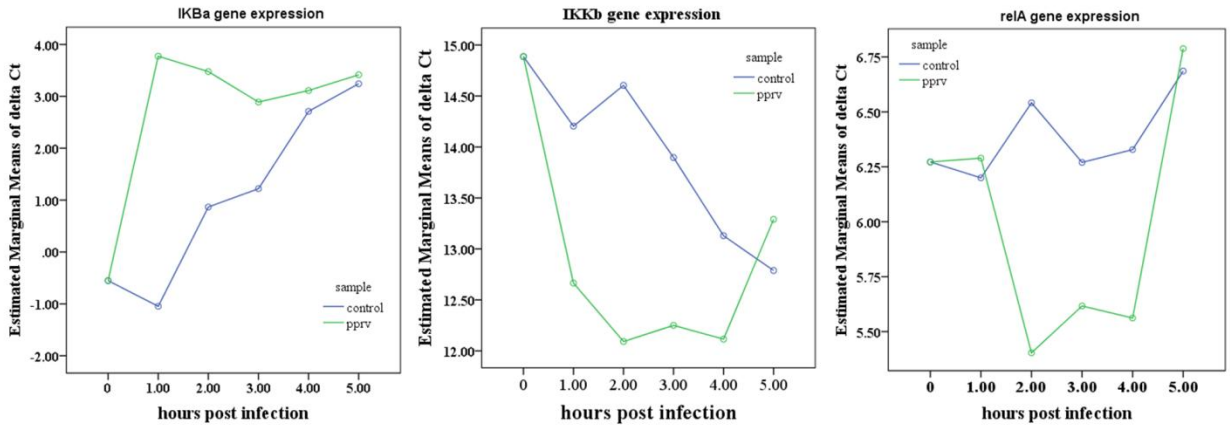
PPR is a highly contagious, acute and febrile disease of small ruminants with severe mortality and morbidity. PPR is endemic in several developing countries of Africa (Padhi and Ma, 2014) and Asia, including India (Muthuchelvan *et al.,* 2014), Bangladesh (Rahman *et al.,* 2018), Pakistan (Khan *et al.,* 2018) and Afghanistan (Banyard *et al.,* 2010). PPR has a huge impact on the socio-economic status of poor marginalised livestock owners. It is therefore important to understand the pathogenesis of PPRV and the mechanisms through which could evade the host immune response. For this reason, the present study was conducted to evaluate the gene expression of three major regulatory proteins of the NF-kB pathway.

IKBα is an inhibitory protein that prevents the activation and translocation of p65 protein to the nucleus. The normalized gene expression of IKBα among the PPRV infected and control samples at different time intervals revealed a highly significant difference from 1 to 3 hpi. Although differences also existed at 4 hpi, the difference was less pronounced (Figure 1). Furthermore, the fold change analysis revealed a significant decrease in the expression of IKBα in PPRV infected cells from 1 to 3 hpi as compared to control samples. Degradation of IKBα protein is essential for the activation of the NF-kB pathway. However, it has been observed that several viruses prevent the degradation of this protein (Mansur *et al.,* 2013; Morelli *et al.,* 2015). Our finding of reduced gene expression of IKBα is in accordance with the general notion that the level of IKBα is reduced during viral infections, particularly during initial phases of infection. IKKβ is the kinase responsible for the activation of the NF-kB pathway. Comparison of normalized IKKβ gene expression (delta $C_T$ values) at different time intervals revealed a significant difference between the control and PPRV infected samples. Likewise IKBα, the differences were marked from 1 to 3 hpi and less pronounced at 4 hpi (Figure 1). No difference was apparent at 5 hpi. The differences between the two groups existed due to the upregulation of IKKβ expression in PPRV infected samples as compared to mock-infected cells. The protein product of IKKβ gene is responsible for the phosphorylation mediated degradation of the IKBα. IKKβ itself gets activated by several stimuli, of which viral infections are the major ones (Amaya *et al.,* 2014). Virus mediated activation of NK-kB pathway for enhancing its replication by targeting IKKβ protein has been reported previously (Wu *et al.,* 2014). In the present study, it was not examined whether the upregulation of IKKβ gene is mediated by PPRV for its own benefit or is due to the innate immune response of the host cells. However, our results validated that following PPRV infection in the *in-vitro* system, the expression of IKKβ gene is upregulated. A similar observation was recorded for the RelA (p65) gene which encodes for the transcriptional factor protein that actually translocates to the nucleus and induces transcription of pro-inflammatory and anti-viral genes. Gene expression profile of RelA among PPRV infected and control group revealed significant differences at 2 to 4 hpi (Figure 1). The expression of RelA was markedly elevated in PPRV infected cells as compared to the control group which is further highlighted by the fold change analysis.

**Table.1** Details of primers used for SYBR green-based one-step RT-qPCR

| S.No. | Target gene | Primer | Primer sequence (5'- 3') | Amplicon size (bp) |
|---|---|---|---|---|
| **1** | IKKβ | Forward | 5' - GCTGTGGGCGGGAGAATGAAGT- 3' | 77 |
| | | Reverse | 5'- CTGCAAGTCCACAATGTCGGTCTG - 3' | |
| **2** | IKBα | Forward | 5'- CCATCCTGAAAGCTACCAACTACA-3' | 103 |
| | | Reverse | 5'- TCAGCACCCAAGGATACCAAAAG -3' | |
| **3** | RelA | Forward | 5'- GGCGAGAGGAGCACAGATAC- 3' | 151 |
| | | Reverse | 5'- CATCCCGGCAGTCTTTTCCT- 3' | |
| **4** | β-actin | Forward | 5'- GAGAAGCTGTGCTACGTCGC -3' | 263 |
| | | Reverse | 5'- CCAGACAGCACTGTGTTGGC -3' | |

**Figure.1** Comparison of mean delta $C_T$ values of IKBα, IKKβ and RelA gene expression between control and PPRV infected samples




Results of RT-qPCR assay suggested a significant decrease in the transcription of IkBα and a significant increase in the transcription of IKKβ and RelA in PPRV infected cells as compared to the control group, particularly during initial hours of infection. However, there is no literature available to support or to oppose our RT-qPCR results. Despite extensive studies and reviewing of the NF-kB pathway, literature pertaining to the mRNA transcription of the key regulatory gene of this pathway is primarily lacking. The majority of studies are directed against evaluating the activation of the NF-kB pathway rather than the estimation of mRNA transcript. The most obvious justification for such discrepancy could be the immediate early response effect of this pathway. The transcription of target pro-inflammatory and anti-viral genes relies on the activation of *de novo* proteins present in the cytoplasm rather than the transcription and translation of newly synthesized proteins. Nonetheless, the information generated in the

present study regarding the mRNA profiling and detection of key regulatory proteins of NF-kB pathways appears to be primitive in the case of PPRV infection.

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