RESEARCH ARTICLE Indian Journal of Animal Research, Volume 58 Issue 5: 834-840 (May 2024)

Inflammasome Activation and Pro-inflammatory Cytokine Genes Expression Following Exposure of PPRV in Vero Cells

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10.18805/IJAR.B-5102

ABSTRACT

Background: *Peste des petits ruminants* (PPR), a contagious and fatal infection of small ruminants, caused by PPR virus (PPRV). Detection of microbes is mediated by pattern recognition receptors (PRRs). These PRRs trigger the assembly of a multi-protein signaling platform known as inflammasome. It detects the pathogens and induces the synthesis of pro-inflammatory cytokines interleukins (IL) *i.e.* IL-1β and IL-18. Aim of present study was to assess the expression of inflammasome related genes, following exposure to PPRV in Vero Cells and was to assess the effect of activation of inflammasome on downstream pro-inflammatory cytokines IL-1β and IL-18 gene expression.

Methods: Vero cells were infected with PPRV. The cells were incubated for different periods. Total RNA was reverse transcribed and used for amplification by quantitative real time PCR (qRT-PCR) to detect the relative change in expression of NLRP3, ASC, Caspase-1 and cytokines IL-1β and IL-18 genes.

Result: It was revealed that 1 MOI of PPRV was appropriate for NLRP3 inflammasome activation. NLRP3 and ASC showed an increased level of expression at 2-4 hrs post inoculation (hpi). While, the Caspase-1 showed biphasic expression on 2-4 and 24 hpi, further, in the synchrony to it, IL-1β and IL-18 gene expression were also found increased at 2-4 and 24 hpi.

Key words: ASC, Caspase-1, IL-18 expression, IL-1β, Inflammasome, NLRP3, PPRV.

INTRODUCTION

PPR is a highly contagious disease that causes high mortality in sheep and goats resulting in heavy economic losses to the small ruminant industry (Kumar *et al*., 2017). PPR is currently endemic across Asia and Africa (de Vries *et al*., 2015; Baron *et al*., 2016). The causative agent, PPRV is classified as a member of Family: *Paramyxoviridae* Genus: *Morbillivirus* and Species: *Small ruminant morbillivirus* (Kumar *et al*., 2017). There are excessive inflammatory responses in PPRV infection that results in heavy mortality and these inflammatory responses against viral infection must be optimized to clear the pathogen without tissue damage (Vijay *et al*., 2017).

Upon PPRV infection, the Pathogen associated molecules (PAMs) are activated and induce the extensive inflammatory reactions and damage in PPRV-infected tissues such as lungs and Gastro-intestinal tract (GIT) mucous membrane (Truong *et al.,* 2014). These are believed to be the most important pathogenic events leading to increase insusceptibility to secondary infection and even to death.

Nevertheless, the mechanism by which PPRV initiates PAM-activated inflammation is unknown. However, recent studies in other viral infections have revealed that macrophage-mediated tissue inflammation is related to a multi-protein complex called the inflammasome, which initiates innate immune responses by activation of caspase-1. This caspase-1 is a protease, which processes pro-interleukin-1β (pro-IL-1β) and pro-interleukin-18 (pro-IL-18), by removing the amino-terminal amino acids to release mature and active forms of the cytokines, the most significant inflammatory cytokines in triggering tissue inflammation (Gram *et al*., 2012).

The Inflammasome is a multi-protein oligomer, which consists mainly of centrally located NLRs such as NLRP1,

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How to cite this article: Sharma, D.K., Joseph, B., Singathia, R., Gaurav, A. and Kumar, V. (2023). Inflammasome Activation and Pro-Inflammatory Cytokine Genes expression following Exposure of PPRV in Vero Cells. Indian Journal of Animal Research. 58(5): 834-840. doi: 10.18805/IJAR.B-5102.

Submitted: 24-02-2023 **Accepted:** 07-06-2023 **Online:** 14-07-2023

NLRP3, NLRC4, NLRP6 and NLRP12, N terminal Pyrin and/or Caspase-1, apoptosis-associated speck-like protein containing CARD (ASC) and at C terminal leucine rich region (LRRs) (Broz and Dixit, 2016; Anand *et al*., 2011; Kanneganti *et al*., 2007). Among various types of inflammasomes the NLRP3 inflammasome, is the most studied and comprises NLRP3, the adaptor protein ASC and procaspase-1. On activation, NLRP3 forms a homo-oligomer *via* its NACHT domain and directly interacts with ASC through its Pyrin domain (PYD). ASC directly interacts with procaspase-1 through the CARD domain. Formation of the NLRP3 inflammasome induces activation of Caspase-1 that eventually results in the production of mature IL-1β and IL-18 respectively from pro IL-1β and pro IL-18 (Chen and Ichinohe, 2015). IL-1β and IL-18 mount an immune response against invading pathogen (Sharma and Kanneganti, 2016). Dynamics of inflammasome and the mechanism by which the inflammasome gets activated have been characterized in many RNA as well as DNA viruses (Shil *et al*., 2018 and Zannetti *et al*., 2016). However dynamics of inflammasome has not been reported following PPRV infection in Vero cells. The present study has given glimpse of understanding the kinetics of inflammasome following PPRV infection in Vero cells (Fig 1).

MATERIALS AND METHODS

The present study was conducted in Department of Veterinary Microbiology, CVAS, Navania, Udaipur, Rajasthan, from 2017 to 2019 as part of PhD thesis of first author.

Vero cells and viruses

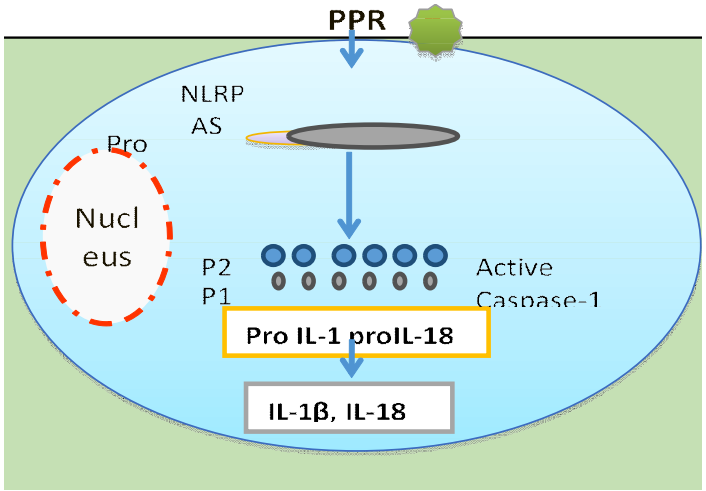
Vero cells were obtained from the National Center of Veterinary Type Cultures (NCVTC), Hisar, India. These cells were cultured and maintained in Dulbecco's modified Eagle medium (DMEM, Himedia, Mumbai, India) with 10% fetal bovine serum (FBS), (Himedia, Mumbai, India) as previously described (Ammerman *et al*., 2008). PPRV/Shahjadpur/2015 was obtained from NCVTC, Hisar, India and was cultivate in Vero cells, as described [Kumar *et al*., 2016]. Standard plaque titrations were performed on a confluent monolayer of Vero cells and the virus titers were obtained in the range of ~10^7 pfu/ml.

Dose response assay

Further for determination of the MOI of PPRV required for secretion of pro-inflammatory cytokines, a dose response assay has been performed by infecting Vero cells with PPRV at three different MOIs of 0.1, 1 and 10, followed by quantitative estimation of respective mRNA of IL-1β and IL-18 by qRT-PCR. Each test was performed in triplicates.

Infections and inflammasome activation

All infections were carried out at a MOI of 1 for PPRV infection. Confluent monolayer of Vero cells was infected with PPRV of one MOI (as we receive optimum quantity required for activation if 1 MOI in dose response assay). The cells were washed after 1hr incubation with Phosphate Buffer saline (PBS) and addition of fresh media. The cells were incubated for different time periods *i.e*. 2, 4, 8, 12, 24 and 48 hrs. The cells were scrapped and cell lysates were



Inflammasome may be activated by wide variety of ligands including viral components. On activation, NLRP3 forms a homo-oligomer domain and directly interacts with ASC through its Pyrin domain. ASC directly interacts with procaspase-1 through its CARD domain. Formation of the NLRP3 inflammasome results in activation of procaspase-1 in to caspase-1 which in turn leads to conversion of pro IL-1β and pro IL-18 in to active IL-1β and IL-18.

Fig 1: Inflammasome: Inflammasome is a multi-protein complex consisting of NLRP3, the adaptor protein apoptosis-associated speck-like protein containing a CARD (ASC) and procaspase-1.

prepared to isolate the total RNA and used for downstream assays. Each assay was performed in triplicates.

qRT-PCR

Total RNA was extracted from cells using the TRIzol (Sigma-Aldrich, St. Louis, U.S.A.) as described (Rio *et al*., 2010) and RNA was quantified by bio spectrometer (Eppendrof, Humburg, Germany). The cDNA was synthesized using reagents (Invitrogen, Life Technologies, CA, U.S.A.) in a total reaction volume of 20 µl as follows: RNA: 5 µl, Oligo dT : 1 µl, RNAse/ DNAse free water: 6 µl. Mixed and incubated at 65°C for 5 minutes, then chilled on ice. The reaction mixture was spun briefly and added- 5X RT buffer: 4 µl, 10 mM dNTPs: 2 µl, 40U/µl RNAse inhibitors: 1 µl, 200U/µl Revert Aid H Minus Reverse Transcriptase: 1µl. Mixed and incubated at 25°C for 10 minutes followed by 42°C for 1 hour (hr) and 70°C for 10 minutes in thermal cycler machine (BioRed Pvt. Ltd California U.S.A). The c DNA prepared was stored at -20°C till further use. qPCR was performed on Aria-Mx real time PCR instrument (Agilent Technology, Santa Clara, CA, U.S.A.) with SYBR Green ER qPCR Super Mix Universal (Invitrogen, Life Technologies, CA, U.S.A.). The primers used in the study have been described in Table 1. β actin (ACTB) was used to normalize the data. Quantitation was performed by normalizing target gene mRNA levels to ACTB levels and infected sample values were expressed relative to the mean of mock values. Statistical significance between-groups were calculated with $^{\Delta}C_T$ values that provides the estimates of $^{\Delta\Delta}C_T$ values (Log 2 fold change) (Karlen *et al*., 2007; Pabinger *et al*., 2014).

Statistical analysis

Student's *t* test was performed for statistical comparison of gene expression among different time points. Statistical analysis was performed on the results of each individual experiment unless otherwise noted in the figure legend.

RESULTS AND DISCUSSION

In the present study, we evaluated whether PPRV activates the expression of NLRP3 inflammasome and its effect on downstream proinflammatory cytokines in PPRV -Vero Cells infection model.

Dose response assay

The Vero cells were infected with different MOIs of PPRV and it was observed that PPRV induced IL-1β (Fig 2a) and IL-18 production (Fig 2b) in a MOI-dependent manner. It was concluded that in order to induce inflammasome activation, Vero cells must be infected with PPRV at MOI of equivalent or more than one. These findings are in agreement with the previous workers who have observed the highest induction of IL-1β and IL18 secretion at MOI of >1 (Sagoo *et al*., 2016; Wang *et al*., 2016).

Inflammasome activation

The activation of NLRP3, ASC and Caspase-1 in PPRV infected Vero Cells was observed in a synchronous manner (Fig 3 a, b and c). As compared to the mock-infected cells, a major increase (~30-fold, $p<0.01$) in NLRP3 transcript level was observed at 2 hours post pinfection (hpi) (Fig 3a). This might be due to the synthesis of viral proteins which might have hampered NLRP3 expression. A study in HPV type-3 also indicated early activation of NLRP3 inflammasome followed by its quick abrogation. The authors reported that the inactivation in NLRP3 activity is mediated via the C protein of this HPV (Shil *et al*., 2018).

ASC plays an essential role in completing assembly of inflammasome as an adapter molecule between NLRP3 and procaspase-1, activation of procaspase-1 into active caspase-1 (Guo *et al*., 2015; Rathinam and Fitzgerald, 2016). In our study, ASC transcript level was also increased ~11-fold ($p<0.01$) at 2 hpi (Fig 3b). Activation of ASC was shown to be synchronized with NLRP3. In a recent study

Table 1: Primers sequences of Inflammasome, IL-1β and IL-18 gene-.

Gene	Product size (bp)	Name designated	Sequence	Melting temperature (°C)	Annealing (°C)	Reference
Humb-Actin	409	NRG25 (Fp)	5′-CCCCAGCCATGTACGTTGCTATCC-3′	66	54	Khandelwal *et al.,* 2017
		NRG25 (Rp)	5′-GCCTCAGGGCAGCGGAACCGCTCA-3′	65.6		
NLRP3	300	NRG173 (Fp)	5′-CCAGAAGCTGGTGGAACTGGACC-3′	60.9	58	Sharma *et al.,* 2019
		NRG174 (Rp)	5′-GTAAGGCCAGAATTCACCAACCCC-3′	60.9		
ASC	306	NRG177 (Fp)	5′-CGCGACATGGGCATGCAGGAG-3′	63.6	58	
		NRG178 (Rp)	5′-GCTCCAGGTCGTCCACCAGGTAG-3′	63.1		
Caspase-1	209	NRG179 (Fp)	5′-GAGAGGGATTTTATTGCTTTCTGCTCTTC-3′	56.3	55	
		NRG180 (Rp)	5′-GTCCTGATGTACCAGTTAGGG-3′	57.7		
IL-1β	210	NRG181 (Rp)	5′-CAAGATTCCTGTGGCCTTGG-3′	56.2	55	Sharma *et al.,* 2020
		NRG182 (Rp)	5′-GTGCTGATGTACCAGTTAGGG-3′	55.1		
IL-18	333	NRG183 (fp)	5′-CCTGGAATCAGATCACTTTGGC-3′	55.9	54	
		NRG184 (Rp)	5′-CCTGGAACACTTCTCTGAAAGAATATG-3′	55.4		

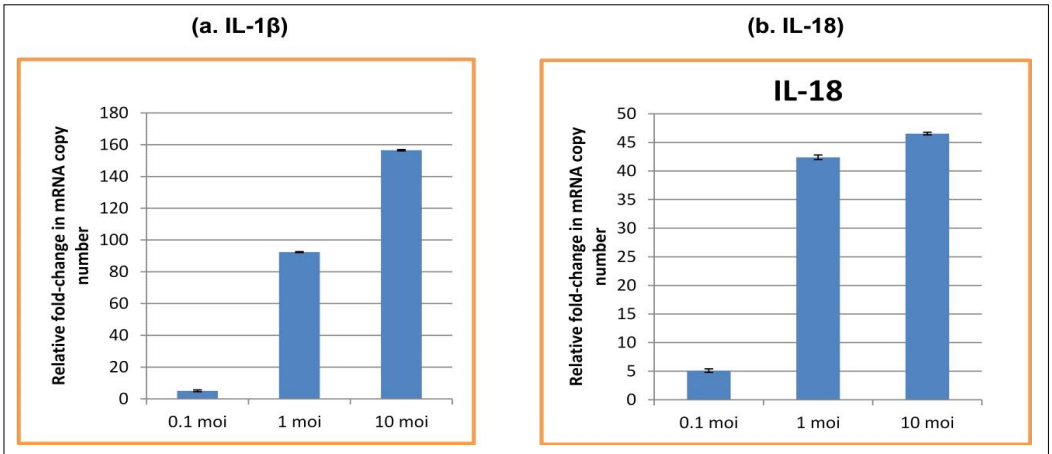
with Modified vaccinia virus in mice, ASC was shown to form specks in lymphocytes, thereby attracting innate and adaptive immune cells towards the infection site (Sagoo *et al*., 2016). These inflows of immune cells increase the interaction of adaptive immune cells (Naive T cells) for priming with antigen, which boosts the antigen specific T-cell mediated adaptive immune response (Ippagunta *et al*., 2011 and Sagoo *et al*., 2016). These findings showed the importance of ASC as an inflammasome component and as a link between innate and adaptive immune systems.

In our study, Caspase-1 showed a biphasic pattern of activation it's, transcript level was increased 1.2-fold (P>0.05) at 2 hpi, 1.8-fold (P<0.05) at 4 hpi and major increase, ~4-fold (P<0.01) was observed at 24 hpi as compared to mock-infected cells (Fig 3c). Concomitant with the enhanced expression of other inflammasome components, the initial activation (2-4 hpi) of Caspase-1 appears to be due to early events of viral infection. The second peak of activation might be due to the synthesis/accumulation of viral protein in virus infected cells (Sagoo *et al*., 2016 and Strittmatter *et al*., 2016). Caspase-1 exerts enzymatic activity and cleaves the pro-IL-1β and pro-IL18 in active IL-1β and IL-18 (Lupfer *et al*., 2015; Malik and Kanneganti, 2017). In our study, we observed that caspase-1 expression activated at 4 hpi, almost at the same time when the highest activity of IL-1β and IL-18 was observed. Our above finding suggests that oligomerization of inflammasome assembly occurs somewhere around 4 hpi to produce mature IL-1β and IL-18 at the same time (Shi *et al*., 2015) (Fig 3e).

IL-1β and IL18 expression

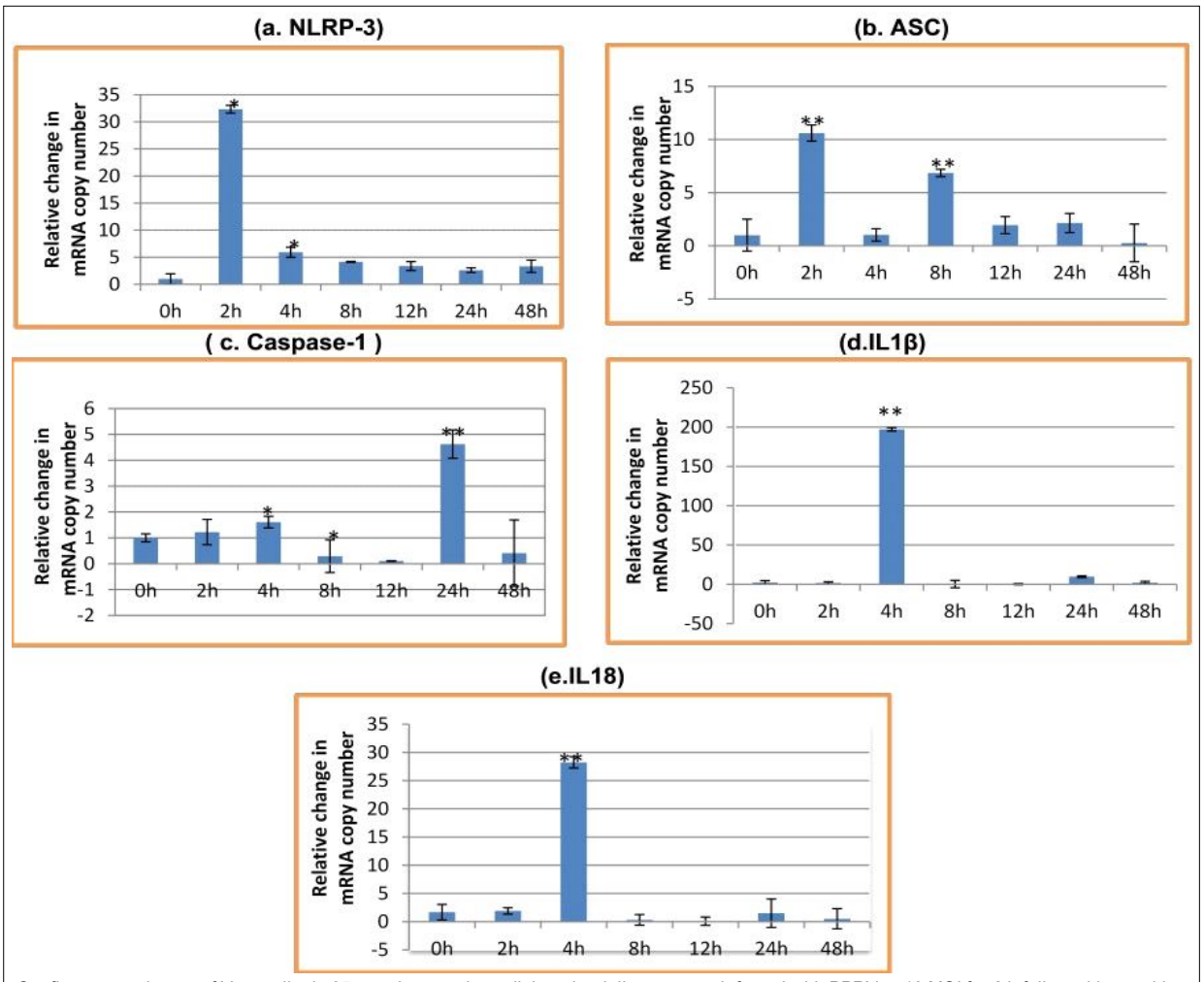
Following PPRV infection major increase of IL-1β transcript levels as compared to mock-infected cells, was at 2 and 4 hpi (~9-fold (P>0.05), 142-fold (p<0.01) it remained elevated at rest time points *i.e.* 8, 12 and 48 hpi (Fig 3d). Likewise, as compared to mock-infected cells, IL-18 transcript level was at peak level at 2 and 4 hpi *i.e.* 1.90 (P>0.05) and 28 (p<0.01) fold respectively (Fig 3e).

This synchronized increase in the transcripts of various inflammasome components concomitant with enhanced synthesis of the effecter molecules- IL-1β and IL-18 strongly suggest that PPRV infection indeed activates the inflammasome. The inflammasome complex activation results in the secretion of IL-1β and IL-18 (Kanneganti *et al*., 2006; Lamkanfi and Dixit 2012; Sutterwala *et al*., 2014). These cytokines generate signaling cascades that lead to activation of tumor necrosis factor alpha (TNF-α), *Interferon* gamma (IFN-γ) and other cytokines. This activation recruits lymphocytes and act as a vital bridge between the innate and adaptive immune responses to control the invading pathogen (Kanneganti 2010; Davis *et al*., 2011; Dhanasekaran *et al*., 2014; Rathinam and Fitzgerald, 2016). Nevertheless, role of inflammasome has been recognized in Influenza virus (Ichinohe *et al*., 2010), hepatitis C virus (Zannetti *et al*., 2016), Rift valley virus (Ermler *et al*., 2014), Paramyxoviruses like oncolytic Newcastle disease virus (NDV) (Wang *et al*., 2016), Sendai virus (SV) (Kanneganti *et al*., 2006) and in human parainfluenza virus (HPV) (Shil *et al*., 2018). Though TLR3/7 is activated by PPRV infection (Dhanshekharan *et al*., 2014), we provide the glimpse intothe dynamics of inflammasome activation in Vero cell lines following PPRV infection. This study adds PPRV as an example to the list of viruses that interact with the inflammasome.



Confluent monolayers of Vero cells, in 35 mm tissue culture dishes, in triplicates were infected with indicated concentrations of PPRV for 2 h followed by washing with PBS and addition of fresh DMEM (without serum). Cells were scraped at 4 hpi by cell scrapper and washed with PBS. Total RNA was extracted by Triazol. cDNA was synthesized using oligo-dT primers and relative fold-change in mRNA copy number was determined by qRT-PCR. Relative fold-change (compared to mock-infected) in mRNA copy number in Il-1β (a) and IL-18 (b) is shown. Error bars indicate SD.

Fig 2: Dose dependent effect of PPRV on Gene expression of IL-1β and IL-18 in Vero cells.



Confluent monolayers of Vero cells, in 35 mm tissue culture dishes, in triplicates were infected with PPRV at 10 MOI for 2 h followed by washing with PBS and addition of fresh DMEM (without serum). At indicated time points, cells were scraped by cell scrapper and washed with PBS. The cell pellet was stored at -80°C till use. Total RNA was extracted by Triazol. cDNA was synthesized using oligo-dT primers and relative fold-change in mRNA copy number was determined by qRT-PCR and Relative fold-change in mRNA copy number in inflammasome component-NLRP3, (Fig a), ASC (Fig b), Caspase-1 (Fig c.) and IL1β (Fig d.) and IL18 expression (Fig e.) is shown. Error bars indicate SD. Pair-wise statistical comparisons were performed using Student's t-test (* P<0.05% and ** P<0.01).

Fig 3: Evaluation of inflammasome components NLRP3, ASC, Caspase-1 activation and IL1b and IL18 cytokine expression following PPRV infection to Vero cells following PPRV infection to Vero cells.

CONCLUSION

It was clearly observed in our study that PPRV activates the inflammasome components at an early stage of infection (2-4 hpi) in a synchronous manner pro-inflammatory cytokines also increased substantially.

Conflict of interest

All authors declare that they have no conflict of interest.

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