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Differential expression of *TLR7* and *iNOS* in chicken experimentally infected with a nephropathogenic infectious bronchitis virus isolate from Kerala

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Abstract

Infectious bronchitis virus (IBV) is a major pathogen of poultry, with nephropathogenic variants causing persistent infection and economic losses. This study investigated the expression of Toll-like receptor 7 (TLR7) and inducible nitric oxide synthase (iNOS) in broiler chicken experimentally infected with a nephropathogenic IBV isolate from Kerala. One-week-old broilers were inoculated oculonasally with 1×10^4 EID_{50} of the isolate, and trachea, lung and kidney tissues were collected at 3-, 7-, and 14- days post-infection (dpi). The relative gene expression was determined by quantitative real-time PCR (qPCR) using the $2^{-\Delta\Delta Ct}$ method. The TLR7 expression was significantly upregulated in the trachea at 3 dpi (~7-fold) and in the kidneys at both 3 and 7 dpi (~3-4 fold), before declining at 14 dpi. Pulmonary TLR7 expression was delayed, showing a two-fold rise at 7 dpi. The iNOS expression was markedly elevated in renal tissue (~6-fold at 3 and 7 dpi) and in lungs (~4-fold at 7 dpi), whereas tracheal expression was initially suppressed and recovered later. The findings of the study suggested that nephropathogenic IBV activates TLR7-mediated viral recognition and iNOS-driven inflammatory responses in a tissue and time-dependent manner. The results also pointed out that the virus's ability to evade or modulate the initial immune alarm system is a critical determinant of disease outcome.

Keywords: *Infectious bronchitis virus, TLR7, iNOS, nephropathogenic isolate*

TLR7 and *iNOS* expression in Nephropathogenic IBV

Infectious bronchitis virus (IBV), a gammacoronavirus of poultry, is responsible for respiratory, renal and reproductive disease worldwide (Cavanagh, 2007). Among its pathotypes, nephropathogenic variants are of particular concern in India, where they contribute to economic losses by reducing the growth performance, impairing renal function and predisposing birds to secondary infections (Bayry *et al,* 2005).

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The host innate immune system plays a central role in determining the disease outcome. The Toll-like receptor 7 (*TLR7*), recognises single-stranded viral RNA, activating downstream antiviral responses, while inducible nitric oxide synthase (*iNOS)* produces nitric oxide, which can exert both antiviral and immunopathological effects (Chhabra *et al,* 2016; Okino *et al*, 2017). Although the pathological features of nephropathogenic IBV are well characterised, little information is available on the dynamics of innate immune gene expression in chicken infected with the field isolates.

The present study aimed to characterise the expression of *TLR7* and *iNOS* in trachea, lung and kidney of broiler chicken experimentally infected with a nephropathogenic IBV isolate from Kerala, thereby providing insight into the molecular basis of host–virus interactions.

Materials and methods

A nephropathogenic IBV isolate was obtained from field outbreaks in Kerala and propagated in 9–10-day-old embryonated chicken eggs *via* the allantoic route. The infectivity titre was determined by Reed and Muench method and found to be 1×10^5 EID_{50}/mL. One-week-old broiler chicken was divided into two groups: infected and control. The infected group received 0.1 mL of the viral inoculum oculonasally, while controls received sterile phosphate-buffered saline. The bird experiment was carried out as per the approval of Institutional Animal Ethics Committee (CVAS/MTY/IAEC/25/102 dated 14/02/2025). Three birds from each group were sacrificed at 3, 7 and 14 dpi for tissue collection.

The reverse transcriptase-polymerase chain reaction (RT-PCR) was done targeting the 5' UTR of IBV genome (Callison *et al.,* 2006). The RT-PCR was performed in a Bio-Rad T100 thermal cycler with the following conditions: initial denaturation at 94 °C for 10 min; 35 cycles of denaturation at 95 °C for 45 s, annealing at 56 °C for 1 min, and extension at 72 °C for 1 min; followed by a final extension at 72 °C for 10 min. Amplicons were resolved by electrophoresis on a 2 % agarose gel in 1X TBE buffer containing ethidium bromide (5 µg/mL), at 80 V for 45 min. Bands were visualised under UV illumination using a gel documentation system.

The relative quantification of messenger RNA (mRNA) expression of target genes in the tissues was determined by qPCR using SYBR Green I chemistry on a CFX Opus 96 PCR machine (Bio-Rad, USA). Each reaction was performed in a 20 µL mixture containing 1.0 µL of cDNA, 5.0 µL of 2× SYBR Green I master mix (BioRad, USA), and 10 pmol of gene-specific primers (Table 1). The thermal cycling protocol consisted of an initial denaturation at 95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 15 s, primer annealing at 55–60°C for 60 s, and extension at 72°C for 30 s. After amplification, melting curve analysis was performed by gradually increasing the temperature from 65°C to 95°C in 0.2°C increments with a 1 s hold at each step. Primers were designed using Primer3 software based on the sequences available in GenBank. The relative gene expression was calculated as the fold change compared with the negative control group at the corresponding time point. For normalisation, the expression levels of each sample were standardised using the Ct value of *β-actin* as the internal reference.

Data were expressed as mean ± SE and analysed using repeated measures of ANOVA. Differences were considered significant at $p < 0.05$.

Results and discussion

The RT-PCR amplification of the 5′ UTR confirmed

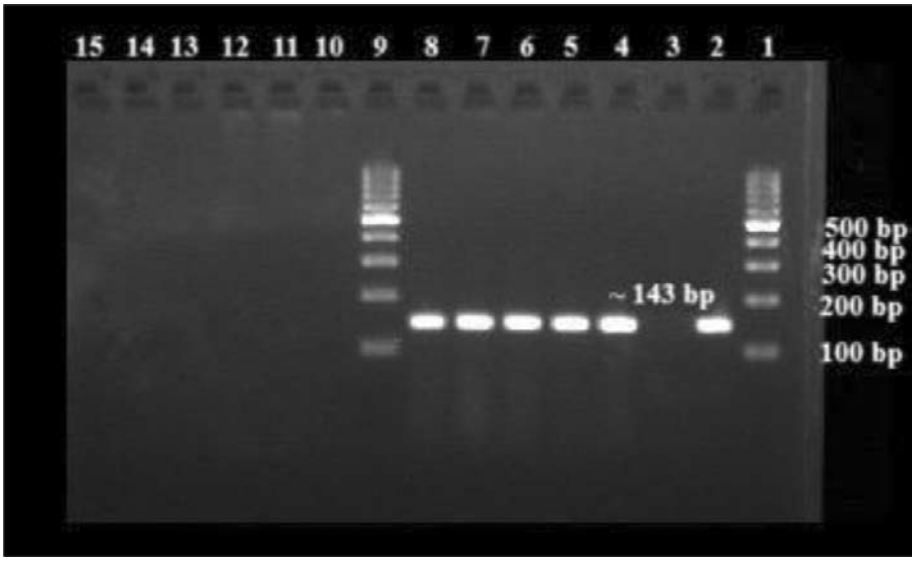

Fig. 1. Agarose gel electrophoresed image of the amplicons targeting 5' UTR (~143bp)

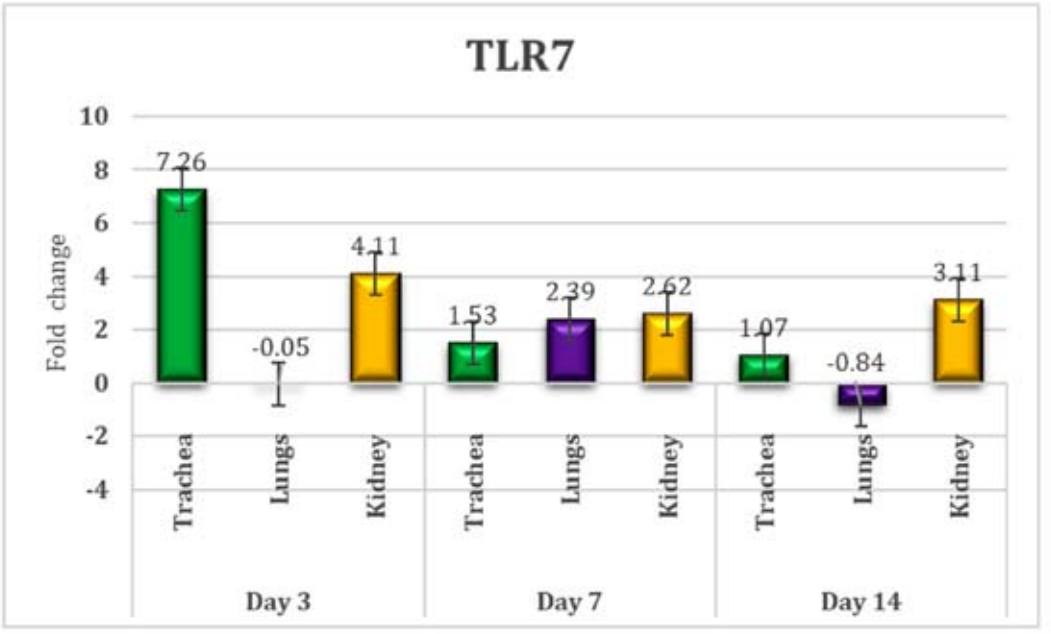

Fig. 2. Fold change of *TLR7* for trachea, lung and kidney at 3, 7 and 14 dpi

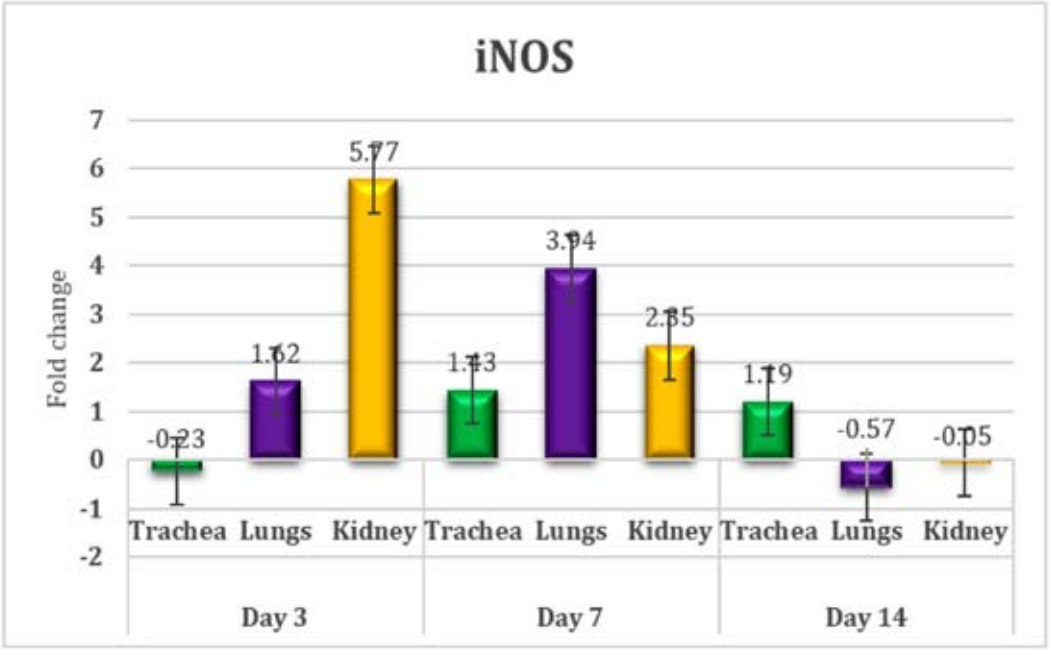

Fig. 3. Fold change of *iNOS* for trachea, lung and kidney at 3, 7 and 14 dpi

the presence of IBV in the experimentally infected birds. The extracted RNA showed good yield (6000–7000 ng/µL) and purity (average A260/280 ratio of 2.01). A 143 bp amplicon was obtained in infected samples, which was visualised by agarose gel electrophoresis (Fig. 1). The samples were subsequently subjected to qRT-PCR analysis using β-actin as the housekeeping gene.

The *TLR7*, a key endosomal sensor of single-stranded RNA viruses, was significantly upregulated in the trachea at day three ($p<0.01$), showing nearly a ten-fold increase, indicating a strong early response. By day seven ($p<0.01$), expression was downregulated. At day 14 ($p<0.01$), transcript levels dropped below baseline, reflecting a decline in immune stimulation over time. This sharp decline in later stages suggested rapid activation and subsequent resolution of the local antiviral response. Similar early tracheal activation of *TLR7* had been reported by Kameka *et al.* (2014) in chicken infected with the Connecticut strain, although the persistence of activation observed here suggested possible strain-specific or host-dependent differences. In the lung, *TLR7* expression was markedly downregulated at day three ($p<0.01$). By day seven ($p<0.01$), expression rose modestly with

a two-fold increase, indicating delayed activation of pattern recognition receptors (PRRs) in response to viral replication and PAMP accumulation (Guo *et al.*, 2008). At 14 dpi, expression remained suppressed without statistical significance (p=0.1), reflecting a return towards baseline consistent with the resolution phase observed in other viral infections (Wang *et al.,* 2006; Guo *et al.,* 2008). In the kidney, *TLR7* expression was strongly upregulated at day three (p<0.01), with more than a four-fold increase. At day seven (p<0.01), expression remained elevated at over a two-fold rise. By day 14 (p<0.01), expression continued to be upregulated, showing more than a three-fold increase, indicating the persistence of the renal immune response. This prolonged renal activation aligned with the findings of Chhabra *et al.* (2016), who reported similar patterns in nephropathogenic strains, and highlighted the strong renal tropism and pathology associated with these viruses.

The expression of *iNOS*, an effector enzyme that mediates nitric oxide production with both antiviral and tissue-damaging effects, also revealed distinct patterns. In the trachea, *iNOS* expression was significantly downregulated at day three (p<0.01), indicating an early-phase suppression. By day seven (p<0.01), expression was slightly upregulated, suggesting a delayed activation. At day 14 (p<0.01), expression showed a mild increase of about two-fold, reflecting activation of *iNOS* responses in the upper respiratory tract. This staggered induction, contrasting with the early-phase *iNOS* activation reported for classical IBV strains (Okino *et al.,* 2014), suggests modulation of the respiratory immune environment by nephropathogenic variants. In the lung, *iNOS* expression was significantly upregulated at day three (two-fold; p<0.01), indicating an early pulmonary immune activation. By day seven (p<0.01), expression further increased to nearly four-fold, reflecting a prolonged inflammatory response. At 14 dpi, expression remained suppressed, although without statistical significance (p=0.4). This pattern of rapid induction and peak at seven dpi aligns with previous studies linking pulmonary *iNOS* expression to the severity of inflammatory pathology during IBV infection (Chhabra *et al.,* 2016). In the kidney, *iNOS* expression was markedly upregulated at day three (six-fold; p<0.01), representing a strong early renal response. At day seven (p<0.01), expression remained upregulated at more than two-fold. By day 14 (p<0.01), expression decreased markedly, indicating the termination of active immune response. The pronounced induction and early persistence of renal *iNOS* align with previous reports linking sustained *iNOS* activity to tissue damage and lesion severity in nephropathogenic IBV (Asif *et al*., 2007; Chhabra *et al.,* 2018), supporting its role in mediating kidney pathology during infection.

Early innate immune pathways shape the host response to IBV, influencing viral clearance and tissue injury. The intensity and timing of these responses vary with viral strain and host–virus interactions, with severe infections marked by rapid replication, excessive inflammation, and delayed antiviral activation (Zhong *et al*., 2016; Kameka *et al.,* 2014; Okino *et al*., 2014)

In summary, nephropathogenic IBV induced a prolonged innate immune activation, notably in the kidney, with sustained *TLR7* and *iNOS* expression corresponding to viral tropism and lesion development. In contrast, respiratory tissues exhibited transient or delayed activation, indicating the differential regulation of innate pathways across infection sites. The findings of the current study underscored the importance of tissue-specific immune responses in determining disease outcome during nephropathogenic IBV infection.

Conclusion

Nephropathogenic IBV infection triggers robust and tissue-specific innate immune responses. The expression of *TLR7* and *iNOS* were strong and persistently upregulated in the kidney, reflecting the virus's renal tropism and persistence. In contrast, respiratory tissues exhibited transient or delayed activation, highlighting the differential regulation of antiviral pathways across the infection sites. The findings of the study demonstrated that the timing, magnitude, and tissue distribution of early immune responses play a critical role in viral clearance and disease outcome during nephropathogenic IBV infection.

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Conflict of interest

The authors declare no conflict of interest.

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