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Differential expression of cytokine genes during natural course of lumpy skin disease virus infection in cattle with varied clinical presentation of the disease

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Abstract
Lumpy skin disease (LSD) is a re-emerging transboundary viral disease of cattle caused by the lumpy skin disease virus (LSDV), leading to significant economic losses in endemic regions. Clinical manifestation of LSDV infection in cattle varies amongst the hosts and results in high morbidity, while mortality is usually low. This study investigates the host cytokine response to LSDV during natural infections in cattle with varying disease severity. Blood samples were collected from mild and severely affected animals across different day post-symptoms (DPS). Cytokine profiling (IL-2, IL-4, IL-6, IL-10, TNF-α, IFN-γ) by a quantitative PCR (qPCR) revealed stronger Th1 and Th2 responses (notably IL-2, IL-4, IL-10, and IFN- Lumpy Skin Disease Virus; Cytokine expression, Natural infection; Cattle γ) in mildly affected animals, indicating effective immune activation. In contrast, severely affected animals exhibited elevated IL-6 and TNF-α with delayed IL-2 response suggestive of immune dysregulation. The results indicate a key role of cell-mediated immunity in disease severity of LSDV infection in cattle. The findings provide valuable insight to understand LSDV pathogenesis and will help to device suitable strategies for clinical management and immunotherapeutic interventions.

Keywords: Lumpy skin disease virus, cytokine expression, natural infection, cattle

Introduction

Lumpy skin disease (LSD) has emerged as a significant transboundary viral threat to cattle and water buffaloes hindering productivity and profitability. Known by various names such as 'Neethling virus disease' and 'pseudo-urticaria', LSD is categorized as a 'notifiable' disease by the World Organization for Animal Health (WOAH) due to its severe economic impact (Tuppurainen and Oura, 2012) [33]. Economic losses include reduced milk and meat production, stunted growth, reproductive issues and restrictions on animal movement and trade (Alemayehu *et al*., 2013 [2]; Babiuk *et al*., 2008 [3]; Sevik and Dogan, 2017) [28]. LSD was first reported in Zambia in 1929 (Morris, 1930) [23] and subsequently spread across Africa, the Middle East, Europe, and Asia (Tuppurainen *et al*., 2015; Eom *et al*., 2023) [9]. In India, the first outbreak occurred in Odisha in 2019 and rapidly expanded to other states, affecting both northern and southern regions by 2022 (Kumar *et al*., 2021 [16]; Topan, 2023) [32]. The factors enabling its introduction remain unclear, but its rapid spread has been associated with the movement of infected animals and arthropod vectors including stable flies, mosquitoes, and ticks (Tuppurainen *et al*., 2013a [34]; Lubinga *et al*., 2014b) [19].

LSD is caused by the Lumpy Skin Disease Virus (LSDV), a member of the *Capripoxvirus* genus in the *Poxviridae* family. Sharing 97% genetic homology with sheep pox and goat pox viruses, LSDV primarily infects cattle and Asian water buffalo, although sporadic cases have been reported in other species, such as giraffes, gazelles, camels, Yak (Sudhakar *et al*., 2020 [29]; Kumar *et al*., 2021) [16]. The disease typically manifests with fever, skin nodules, lymphadenopathy and necrotic lesions. Morbidity ranges around 10%, while mortality is generally low (1-3%), though higher rates of morbidity (5 to 45 %) and mortality (1 and 5 %) were observed during recent outbreaks in India in the year 2021-2022 (Mathivanan *et al*.,

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2023) [20]. Primary diagnosis of the disease based on characteristic clinical symptoms requires laboratory confirmation via molecular techniques like PCR and virus isolation, alongside serological assays (Tuppurainen *et al*.,2011 [36]; Kresic *et al*., 2020) [15]. Control measures include early detection, quarantine, vaccination, and vector management.

During recent years, several outbreaks of LSD were reported across India with variable morbidity, mortality and clinical outcomes (Mathivanan *et al*., 2023) [20]. It has been noted that the immune response to LSDV infection varies from individual animal to animal and strain of the virus involved (Tuppurainen *et al*., 2017) [37]. Studies on host responses to LSDV infection are therefore essential for comprehensive understanding of the disease pathogenesis for effective prevention and clinical management of susceptible hosts. Role of both humoral and cell mediated immunity (CMI) has been noted for limiting the LSDV infection (Wallace *et al*., 2020) [39]. CMI response involving interferon-gamma release assays (IGRA), enzyme-linked immunospot (ELISpot), and intracellular cytokine staining indicates the involvement of CD4+ and CD8+ T cells in response to LSDV infections (Kara *et al*., 2018) [12]. However, most of such investigations were done in the experimental animals and there is limited such information available for natural infection of the virus in cattle (Kara *et al*., 2018 [12]; Wallace *et al*., 2020) [39]. This study was undertaken to investigate the host immune response to LSDV infection through evaluation of the cytokine profile of cattle showing varied clinical severity of the disease during a natural course of LSDV infections.

2. Materials and Methods

2.1. Collection of samples

Blood samples from 31 cattle were collected from Bareilly District of Uttar Pradesh during the investigation of LSD outbreaks in 2022. Clinical history of the disease with a special note on the onset of the first clinical symptoms and the case severity were noted along with basic epidemiological information. The clinical course of the disease was noted as days post symptoms (dps) based on the clinical history and observations during the investigations. Animals were also grouped as mild and severely affected based on clinical parameters including food intake, nasal discharge, and nodule dissemination as mentioned elsewhere (Aerts *et al*., 2021) [1]. The animals were considered as mildly affected, having slightly reduced food intake, mild nasal discharge, and localized nodules (<10) whereas, severely affected animals were grouped based on noticeable reduction of food intake, purulent nasal discharge, and generalized nodules (>20).

Blood samples (5 ml each) were collected aseptically with anticoagulant (0.1% EDTA) from the cattle grouped as mildly affected (n= 23) and severely affected (n= 8) based on clinical presentation of the disease. Blood samples were collected for the animals at 1-3dps (early phase), 14-16 dps (mid phase), 21-23 dps (late phase). Whole blood samples were screened for confirmation of viraemia and amplification of targeted cytokine genes by qPCR. Blood samples collected from clinically healthy and non-viraemic cattle (n=6) were used as control for evaluation of the cytokine profile.

2.2. Screening of suspected blood samples for viraemia

Genomic DNA was extracted from blood samples using the DNeasy Blood and Tissue Kit (Qiagen) following the manufacturer's protocol. PCR amplification of extracted DNA was performed using WOAH recommended primers (Ireland and Binepal, 1998) [11] targeting the viral attachment protein gene (VAP). The reaction mixtures were prepared in a 25 µl volume consisting of 12.5 µl 2X SapphireAmp Fast PCR Master Mix (Takara), 1 µl each of forward and reverse primers (10 pM), 2 µl of DNA template, and 8.5 µlof nuclease-free water. The thermal conditions included an initial denaturation at 95°C for 3 minutes, followed by 35 cycles of denaturation at 95°C for 30 seconds, annealing at 56°C for 30 seconds, extension at 72°C for 30 seconds and final extension at 72°C for 10 minutes were followed for amplification of target sequence as recommended by WOAH. PCR products were analysed via 1.5% agarose gel electrophoresis.

2.3. Cytokine Profiling using qPCR

2.3.1. Extraction of RNA and cDNA synthesis

Total RNA was extracted from whole blood using TRI Reagent (Sigma-Aldrich, USA) following standard protocols. RNA yield and purity were assessed using a Nano-Drop spectrophotometer (Thermo Fisher Scientific, USA) before storage at -80°C.

Extracted RNA was treated with DNase I (Thermo Scientific, Lithuania) to remove genomic DNA contamination. For cDNA synthesis, 1 µg of DNase-treated RNA was reverse transcribed using the RevertAid first-Strand cDNA synthesis kit (Thermo Scientific, USA). The cDNA was diluted (1:10) with nuclease-free water for subsequent qPCR analysis.

2.3.2. Real-Time PCR (RT-PCR)

Relative expression levels of six bovine cytokines namely, IFN-γ, TNF-α, IL-2, IL-6, IL-4, and IL-10 along with the housekeeping gene (β-actin) were quantified by qPCR. Primers optimized for annealing temperature and specificity mentioned in (Table 1).

Table 1: List of cytokine primers and their annealing temperature

Target Genes	Primer sets (5' to 3')	Amplicon size	Annealing Temp (°C)	Reference
β-Actin:	CGCACCACTGGCATTGTCAT TCCAAGGCGACGTAGCAGAG	227bp	60	Konnai *et al*., 2003 [14]
IL-4:	GTGCTGGTCTGCTTACTGGT CGTTGTGAGGATGTTCAGCG	102bp	60	Rashid *et al*., 2019 [26]
IL-10:	AAGGTGAAGAGAGTCTTCAGTGAGC TGCATCTTCGTTGTCATGTAGG	110bp	62	O'Gorman *et al*., 2006 [24]
IFN-γ:	TCAAATTCCGGTGGATGATCT CTTCTCTTCCGCTTTCTGAGG	108bp	60	Ohtsuka *et al*.,2019 [25]
TNF-α:	TCTTCTCAAGCCTCAAGTAACAAGC CCATGAGGGCATTGGCATAC	418bp	60	Lee *et al*., 2006
IL-2:	CAAACGGTGCACCTACTTCA CCTTGGGCACGTAAAAGTCA	153bp	62	Kaur *et al*., 2023 [13]
IL-6:	CCTTCACTCCATTCGCTGTC CGACCATGCGCTTAATGAGA	162bp	62	Kaur *et al*., 2023 [13]

Real-time PCR was performed using Power Up SYBR Green Master Mix (Thermo Scientific, USA) on a Qiaquant 96 5-plex real-time thermal cycler (Germany). Each reaction was run in duplicate, and data acquisition was carried out using Qiaquant 96 Software (v1.0.3). The reaction mixture (20 µl) consisted of 10 µl of 2X Master Mix, 1 µl each of forward and reverse primers (10 pM), 1.5 µl of cDNA template and 6.5 µl of nuclease-free water. Thermal cycling conditions included an initial denaturation step at 50°C for 2 minutes and 95°C for 2 minutes, followed by 35 cycles of denaturation (95°C for 15 seconds), annealing (60°C for IL-4, IFN-γ, β-actin, and TNF-α; 62°C for IL-2, IL-6, and IL-10 for 30 seconds), and extension 72°C for 30 seconds. A final hold step was set at 4°C.

The cycle threshold (Ct) values for target and housekeeping genes were recorded, and relative expression was calculated using the ΔΔCt method (Livak and Schmittgen, 2001) [18], with results expressed as fold changes compared to the control group. Statistical analyses were performed using GraphPad PRISM (v5.0; GraphPad Software, Inc., San Diego, CA).

3. Results

3.1. Confirmation of LSDV-associated viraemia

All 31 animals categorised under the naturally infected group were found positive for LSDV genomic DNA in conventional PCR indicating viremia. The positive samples revealed a distinct amplicon of 192 bp in an agarose gel electrophoresis (Fig 1). Blood samples collected from clinically healthy (non-viraemic) animals were confirmed as negative for the presence of LSDV genomic DNA in PCR.

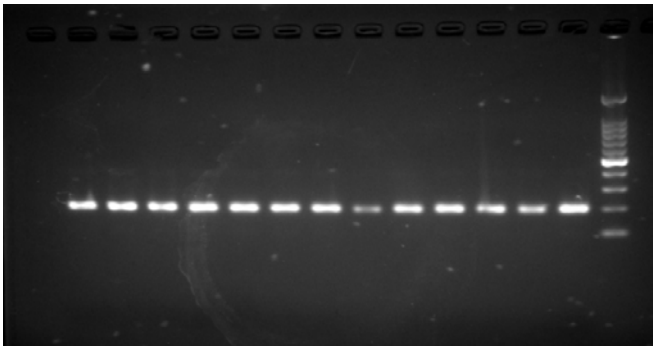


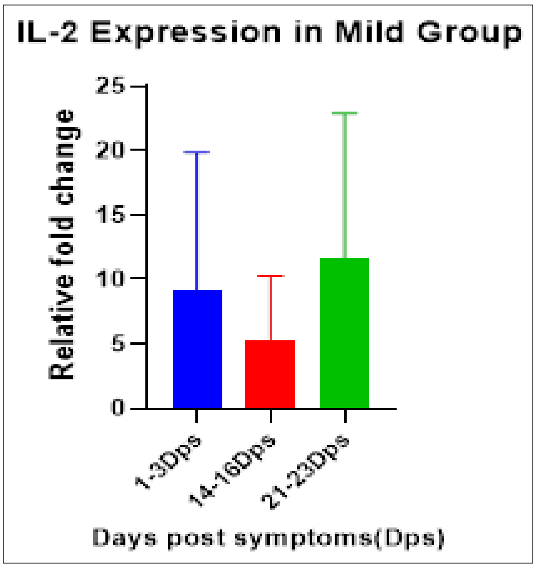
Fig 1: Agarose gel showing 192bp amplified product for LSDV.L1: 100bp ladder; L2: positive control; L3-L14: samples from viraemic animals; L15: negative control.

3.2. Cytokine profiling through qPCR

Overall, the cytokine profiles revealed significant differences between mild and severely affected animals, underscoring the variability in immune responses to LSDV infection. A significant difference in the cytokine expression level was also evident at different stages of the clinical disease.

3.2.1. Expression of cytokine genes in mild and severely affected animals

Mildly affected animals showed higher overall expression of IL-2, IL-4, IL-6, IL-10, and TNF-α, IFN -γ compared to severely affected and control animals, indicating a more balanced and regulated immune response**.** IL-2 expression was higher during the early phase, followed by a decline in the mid-phase and resurgence in the late phase. Notably, the mild group exhibited higher IL-2 expression in the early phase compared to the severe group (Fig.2). IL-4 levels remained consistently high in mildly affected group during the course of clinical disease. In contrary to the severely affected animals IL4 levels peaked during the early and mid-phases (Fig.3) whereas; IL-6 levels found to be declined during the late phase (Fig.4). IL-10 levels were found be declined with progression of the disease in mildly affected animals. However, the peak level of IL10 expression was evident during the mid-phase of the disease in severely affected animals (Fig.5). Expression of TNF-α was gradually higher from the early through late phase of the disease in mildly affected animals whereas; the expression level peaked during the mid-phase of the disease in severely affected animals (Fig.6). The level of IFN-γ was found to be significantly up-regulated during the mid-phase of the disease in both mild and severely affected animals (Fig. 7).



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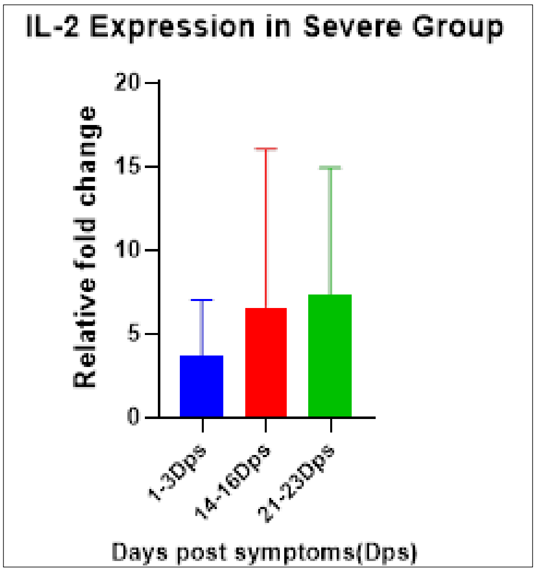

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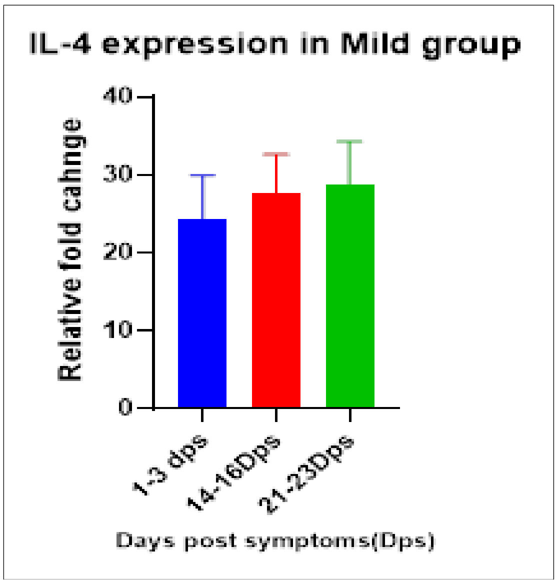

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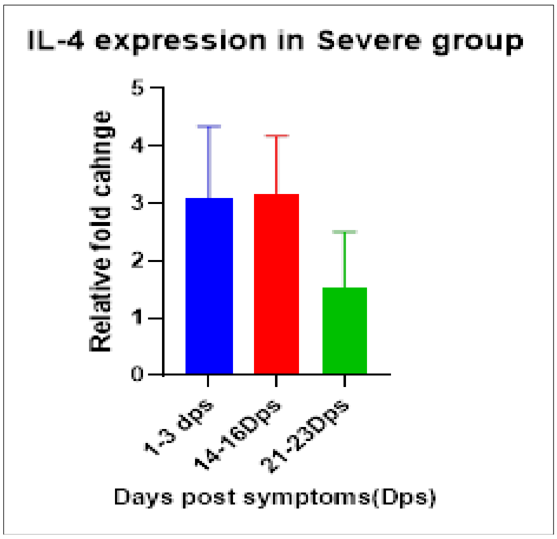
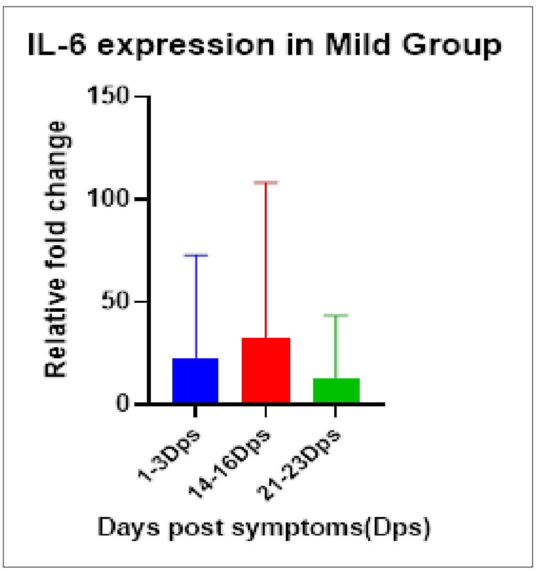


d

Fig 3: Expression of IL-4 in mildly affected (a) and severely affected (b) cattle across different phases of disease. Bar graph showing the increase in relative fold change of IL-4 mRNA expression at early (1-3 dps), mid (14-16 dps) and late (21-23 dps) phase of infection. Data is represented as mean ± standard deviation.



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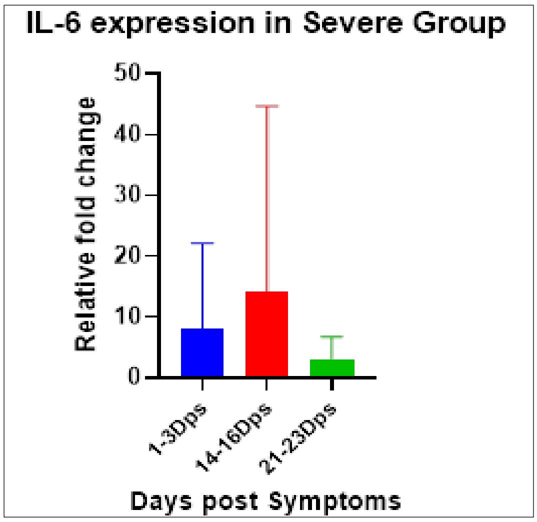

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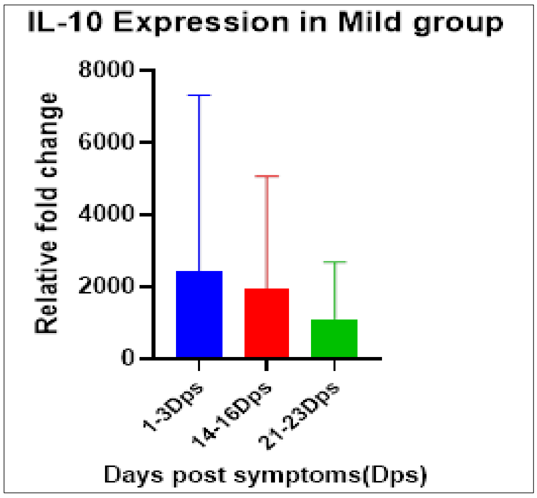
Fig 4: Expression of IL-6 in mildly affected (a) and severely affected (b) cattle across different phases of disease. Bar graph showing the increase in relative fold change of IL-6 mRNA expression at early (1-3 dps), mid (14-16 dps) and late (21-23 dps) phase of infection. Data is represented as mean ± standard deviation.



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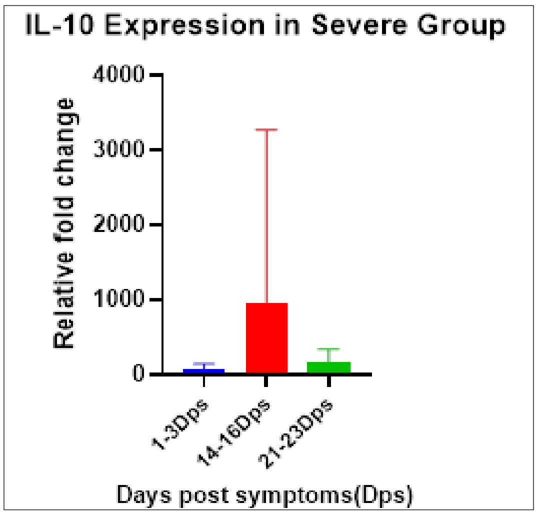
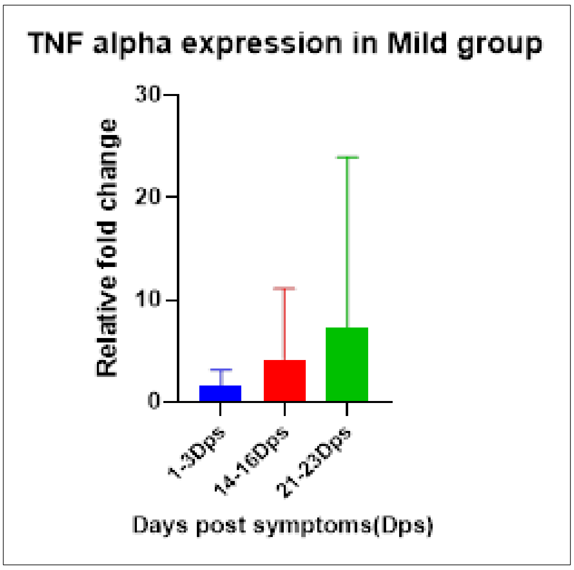


B

Fig 5: Expression of IL-10 in mildly affected (a) and severely affected (b) cattle across different phases of disease. Bar graph showing the increase in relative fold change of IL-10 mRNA expression at early (1-3 dps), mid (14-16 dps) and late (21-23 dps) phase of infection. Data is represented as mean ± standard deviation.



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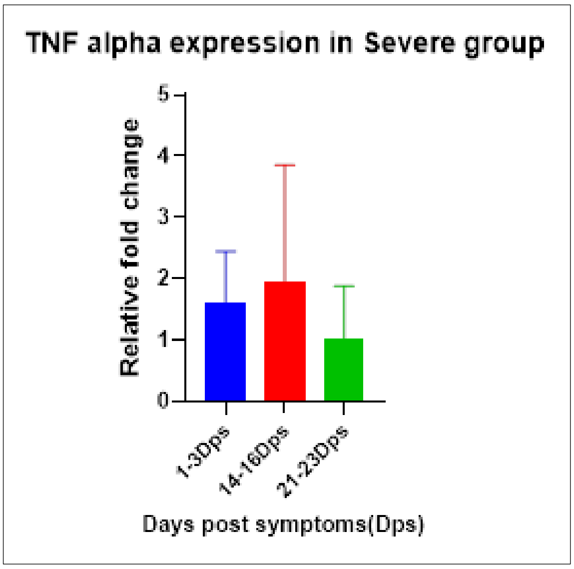
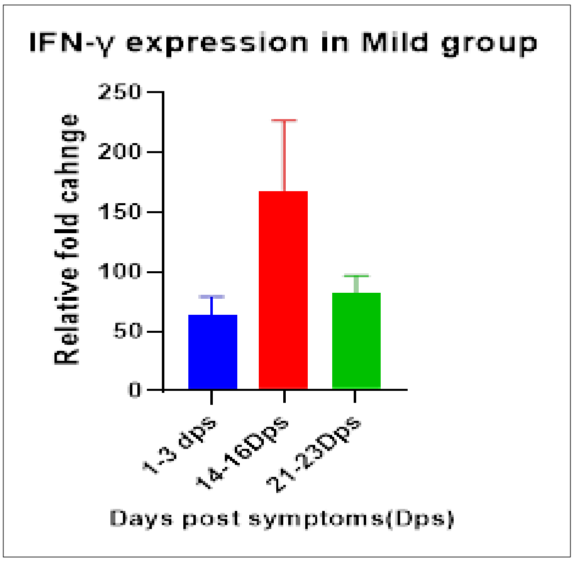


b

Fig 6: Expression of TNF-*α* in mildly affected (a) and severely affected (b) cattle across different phases of disease. Bar graph showing the increase in relative fold change of TNF-*α* mRNA expression at early (1-3 dps), mid (14-16 dps) and late (21-23 dps) phase of infection. Data is represented as mean ± standard deviation.



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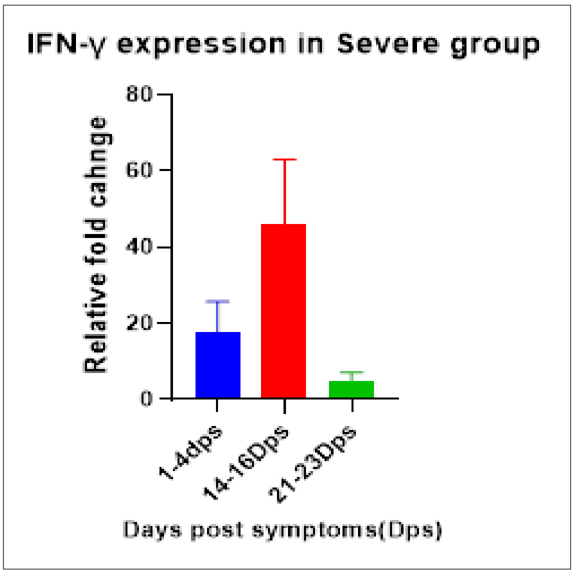


b

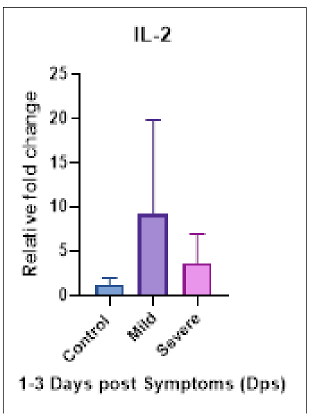
Fig 7: Expression of IFN-γ in mildly affected (a) and severely affected (b) cattle across different phases of disease. Bar graph showing the increase in relative fold change of IFN-γ mRNA expression at early (1-3 dps), mid (14-16 dps) and late (21-23 dps) phase of infection. Data is represented as mean ± standard deviation.

3.2.3. Expression of cytokines at different stages of clinical disease

During the early phase (1-3 dps), significantly higher levels of IL-2, IL-4, IL-6, IL-10, and IFN-γ were observed in mildly affected animals compared to the severely affected group. In contrast, TNF-α levels were elevated in the severe group relative to the mild group. The levels of cytokines remained consistently low in the control group in comparison to both the groups (Fig.8).

In the mid phase (14-16 dps) of infection, the expression levels of IL-4, TNF-α, IFN-γ, and IL-10 were notably higher in mildly affected animals compared to the severe group. Conversely, IL-2 and IL-6 levels were elevated in the severe group during this phase. The levels of cytokines remained consistently low in the control group in comparison to both the groups (Fig. 9)

By the late phase (21-23 dps) of infection, the levels of most of the cytokines analyzed (IL-2, IL-4, IL-6, IL-10, TNF-α, IFN-γ) were higher in the mildly affected group than in the severe group. The levels of cytokines remained consistently low in the control group in comparison to both the groups (Fig.10)



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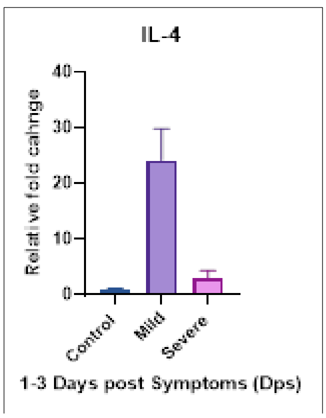

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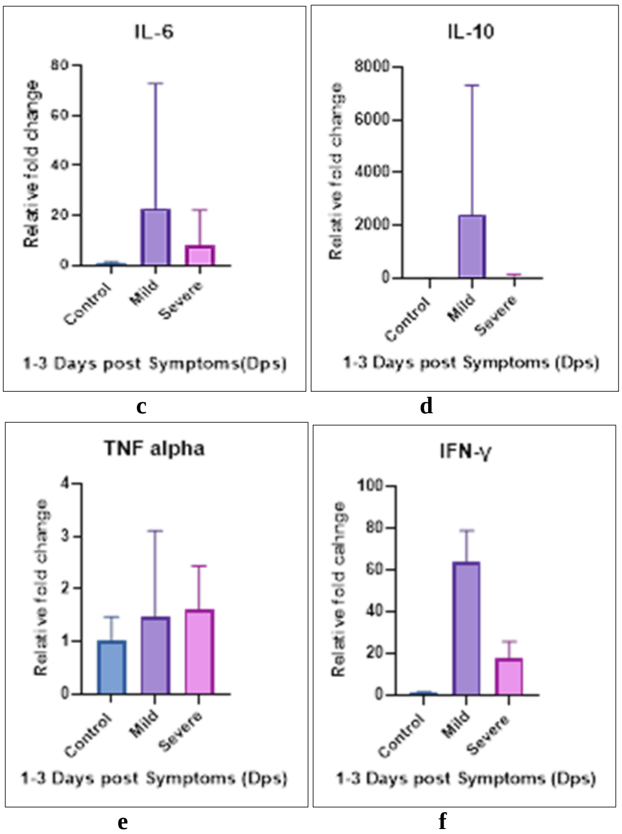
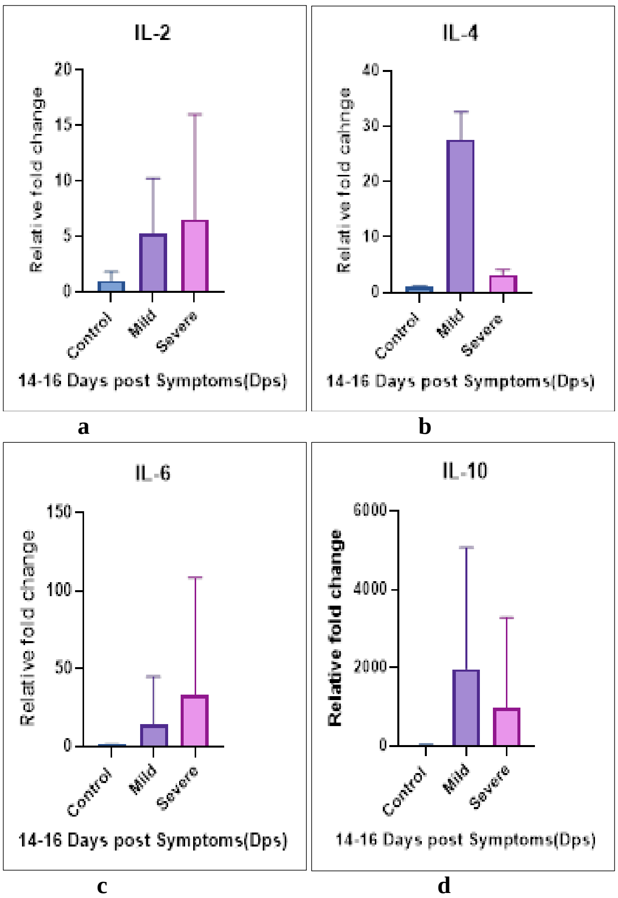


Fig 8: Expression of different cytokines at early phase (1-3dps) of the clinical disease. Bar graphs showing the higher expression levels of IL-2 (a), IL-4 (b), IL-6 (c), IL-10 (d),TNF-α (e) and IFN-γ (f), were notable in mildly affected animals TNF-α (e) levels were elevated in the severe group.



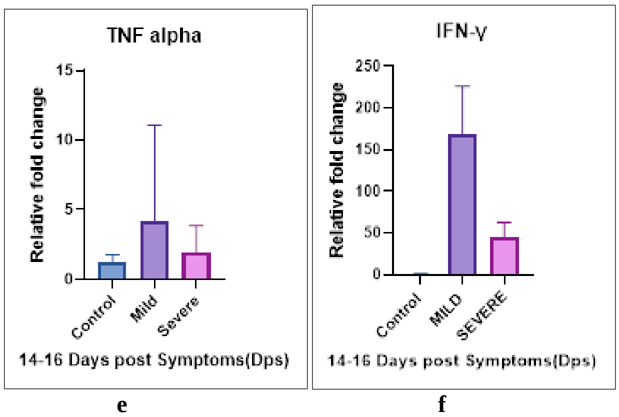


Fig 9: Expression of cytokines at mid phase (14-16dps) of the clinical disease. Expression levels of IL-4 (b), TNF-α (e), IFN-γ (f), and IL-10 (d) were notably higher in mildly affected animals compared to the severe group. IL-2 (a) and IL-6 (c) levels were elevated in the severe group.

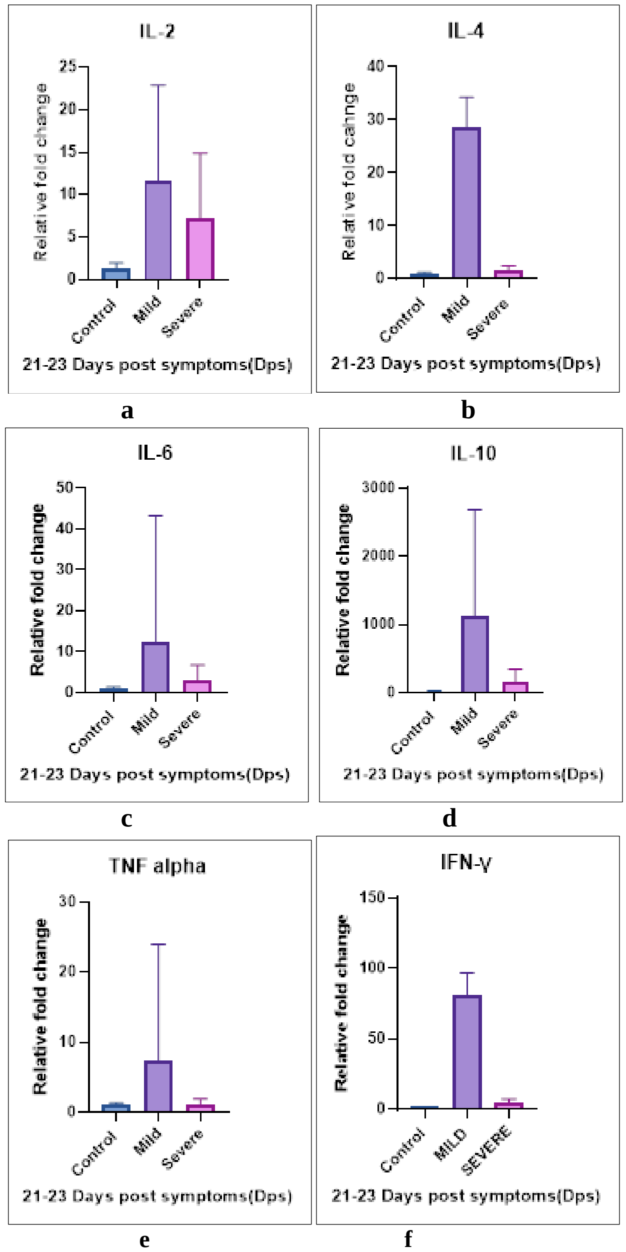


Fig 10: Expression of cytokines at late phase (21-23 dps) of the clinical disease. IL-2 (a), IL-4 (b), IL-6 (c), IL-10 (d), TNF-α (e), and IFN-γ (f) were consistently higher in the mildly affected group than in the severe group.

4. Discussion

Natural outbreaks of LSD in bovines present a unique opportunity to study immune responses under near-uniform environmental and physiological conditions. Such studies provide valuable insights into the genetic and immunological mechanisms driving disease resistance or susceptibility. Evaluating immune responses in natural hosts under field conditions provides a more accurate understanding of host-pathogen interactions than in *vitro* or experimental models; as such conditions reflect the complexity of immune dynamics (Urbina *et al*., 2003 [38]; Boraschi *et al*., 2021) [4]. However, such investigations on hosts' response during a natural infection are difficult and limited to the occurrence of natural outbreaks and logistic constraints of periodic sampling; unlike an experimental model. Our study focuses the expression profiles of cytokines IL-2, IL-4, IL-6, IL-10, TNF-α, and IFN-γ in LSDV-infected cattle to elucidate the role of immune responses in disease progression and control.

The results revealed an overall higher cytokine expression levels in mildly affected animals compared to the severely affected and control groups. IL-2, a pivotal cytokine for T-cell activation, showed higher expression in the early phase of infection in the mildly affected group, reflecting a robust initial immune response. Conversely, in the severely affected group, IL-2 expression increased gradually over time, suggesting a delayed and prolonged immune activation, potentially due to viral immuno-modulation (Hilton *et al*., 2002) [10]. IL-4 levels peaked in the mid and late phases of the disease in the mildly affected group, aligning with its role in promoting humoral immunity (Chen *et al*., 2017) [8]. In severe cases, although IL-4 levels increased during the early and mid-phase the response appeared insufficient for effective viral clearance, contributing to disease severity (Moran *et al*., 1996 [22]; Chen *et al*., 2011) [7].

Pro-inflammatory cytokines IL-6 and TNF-α were elevated in the early and mid-phases of infection, particularly in severely affected animals, highlighting their role in acute inflammation and immune cell recruitment (Miller and Madsen, 2021 [21]; Seo and Webster, 2002) [27]. However, the unregulated inflammatory response in severe cases may exacerbate tissue damage resulting into severe form of the disease. In the mildly affected group, a more controlled cytokine response was observed, contributing to better disease outcomes. It is noteworthy that, IL-10, an anti-inflammatory cytokine, was elevated in the mildly affected group during the early and mid-phases, indicating an active mechanism to balance inflammation and prevent tissue damage (Suwankitwat *et al*., 2023) [30]. This regulatory response was less effective in severe cases, where excessive pro-inflammatory cytokines likely overshadowed IL-10's protective effects.

IFN-γ, a key Th1 cytokine, peaked during the mid-phase of infection in both mild and severely affected groups, consistent with its role in controlling intracellular pathogens like LSDV (Wallace *et al*., 2020 [39]; Boshra *et al*., 2015) [5]. However, the higher levels of TNF-α and IL-6 in severe cases may have led to immune dysregulation, limiting IFN-γ's efficacy. Findings of the present study support the notion indicating mild infections are associated with a balanced immune response, while severe cases exhibit dysregulated cytokine dynamics (Bradley, 2008 [6]; Tanaka *et al*., 2014 [31]; Suwankitwat *et al*., 2023) [30]. These results contribute to a better understanding of the immune mechanisms driving LSDV infection, particularly the differential cytokine responses in mild and severe cases. They emphasize the importance of balanced immune regulation in preventing disease severity and highlight potential targets for therapeutic interventions aimed at modulating cytokine responses.

Investigations on natural infections provide valuable insights into the hosts' response to the virus as it represents the original epidemiological scenario, unlike an experimental infection model. However, there are certain limitations in studying natural infections due to probable inherent variability amongst the target population. Tracing and periodic sampling from the targeted animals was the major challenge during the course of the study. Further, the estimation of dps was based on the history and clinical observations rather than precise infection timing which can only be recorded in an experimental infection.

In conclusion, the present study emphasizes differential expression of cytokines in cattle showing varied clinical severity of LSD during a natural infection. Mildly affected animals showed an early and balanced immune response, particularly with timely expression of IL-2, IL-10, and IFN-γ, while severe cases demonstrated a delayed but stronger inflammatory response that probably contributed to the disease severity. The observations support the notions of cell-mediated immune components as a key factor in controlling LSDV infection and helps for better understanding of the hosts' response to LSDV in a natural infection. The findings of the present study will also be helpful to device suitable strategies for immunotherapeutic interventions and clinical management of the disease.

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Ethical approval

The authors confirm that the ethical policies of the journal, as noted on the journal's author guidelines page, have been adhered to.

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

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