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**Short Communication**

# Mycotoxin deoxynivalenol (DON) increases replication of the lumpy skin disease virus in MDBK cells

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## Abstract

**Recent outbreaks of lumpy skin disease (LSD), involving cattle devastated livestock sector with high economic losses. LSD virus (LSDV) is a capripoxvirus that was earlier endemic in Africa but has since 2019 caused major outbreaks across India. Simultaneously, livestock are continuously exposed to mycotoxin through contaminated feed, increasing the burden on animal health. Among mycotoxins, deoxynivalenol (DON) is frequently reported in wide-variety of feeds produced by *Fusarium* species globally including India. Microbial degradation of DON in rumen makes ruminants comparatively less sensitive to DON. However, before ingestion, direct exposure to skin could have a toxic effect on dermal tissue, making skin more sensitive than other organs. Indeed, mycotoxin contamination in feed is shown to increase viral replication, infection and virus-related damage like that of influenza and porcine circovirus. However, to date, the interrelation between DON and skin viruses, particularly LSDV, is relatively lacking. Thus, using *in-vitro* MDBK cells, we tested DON from 0.4 to 0.00156 µg//mL and observed for cytopathic effect (CPE) for assaying the viral replication rate and infectivity. Interestingly, at 0.0125 µg/mL of DON, we found CPE by the end of 72 hrs post-infection, whereas at the same time period, viral control showed minor changes in morphology comparatively, indicating that the presence of DON enhanced the viral replication rate in MDBK cells. Testing LSDV infectivity in different doses and combinations of mycotoxins using different models (*ex vivo* per-fused tissue, *in vivo*, etc) is a sequential step forward. Further, conducting prospective-epidemiological studies relating to mycotoxin prevalence and LSD occurrence in a particular geographical area would strengthen the understanding of the role of mycotoxins in host-pathogen interaction.**

**Keywords:** Cytopathic effect (CPE), Infectivity, Lumpy skin disease virus, MDBK cells, Viral replication

## Highlights

- Lumpy skin disease (LSD) caused major outbreaks recently.
- Mycotoxins, particularly deoxynivalenol, are frequently found in animal feed.
- Inter-relation between simultaneous DON and LSD exposure was not known.
- We found DON increases LSD replication *in vitro*.

India is the largest milk producer in the world, attributing to nearly 23% of global milk production. But, recent outbreaks of lumpy skin disease (LSD) involving cattle and buffalo devastated livestock industries with high economic losses (Akther *et al.*, 2023). These economic losses were attributed to reduced milk production, abortion, damage to skin/ hide, weight loss, temporary sterility in bulls and cows, draft power loss, mortality and treatment/ prevention costs (Molla *et al.*, 2017). LSD virus (LSDV) is a capripoxvirus (Pox-viridae family) and was considered endemic in Africa. However, after first appearing in South Asia in 2019, since then, it caused major outbreaks across India also. It is a highly contagious disease, with characteristic multiple raised nodular lesions on the skin, with high morbidity and sometimes lethal infection (Mathivanan *et al.*, 2023). Currently, there are no specific treatments for LSD, but only include preventive measures like skin wound care, vaccination, implementing bio-security and quarantining.

Deoxynivalenol (DON), a mycotoxin, belongs to the trichothecenes group found in a wide variety of foods and feeds produced by *Fusarium* species and is one of the most frequently found mycotoxins globally (Streit *et al.*, 2013). As a result, livestock are continuously exposed to DON through contaminated feed. Previous studies indicated that DON exposure produces potent gastrointestinal toxicity (Sobrova *et al.*, 2010). However, microbial degradation of DON in rumen makes ruminants comparatively less sensitive

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to DON. However, before ingestion, direct exposure of skin could have a toxic effect on dermal tissue. This makes dermal toxicity particularly more deleterious and dermal tissue more sensitive among other organs. Indeed, dermal exposure to mycotoxins could lead to immunosuppression, allergic dermatitis (Aihara *et al.*, 2020) and even carcinogenic effects (Mishra *et al.*, 2016). Further, mycotoxin (like aflatoxin B1, ochratoxin A, DON) contamination in feed is shown to increase viral replication, infection and virus-related damage like that of influenza, porcine circovirus (Gan *et al.*, 2015; Sun *et al.*, 2018). But, to date, little is known regarding the interaction between DON and cattle LSD virus. Further, the relation between mycotoxin dermal exposure and viruses causing skin infection, particularly LSDV, remains un-investigated. Thus, the current research was conducted to understand the interrelationship between DON and LSDV, employing cytopathic effect (CPE) through *in vitro* studies.

Lumpy skin disease (LSD) virus and MDBK (Madin-Darby bovine kidney) were maintained at ICAR-National Institute of Veterinary Epidemiology and Disease Informatics (NIVEDI). The cells were cultured in minimum essential medium (MEM; Invitrogen, USA) in 10% fetal bovine serum (FBS) with 1% antibiotic (penicillin 100 U/mL) and streptomycin 100 µg/mL)) and incubated at 37°C with 5% $CO_2$ in a humidified atmosphere. Initially, cells were seeded in cell culture flasks (approx 2.2 million cells). After 48 h of incubation, monolayers were formed. Then, the MDBK cells in the flask were trypsinised and seeded to the 96-well cell-culture plate ($2 \times 10^4$ cells per well) in 100 µL/well and allowed for 1 day (24 h) for the monolayers to form. After 24 h the media was decanted. The mycotoxin (DON, Sigma-Aldrich) in required concentrations ranging from 0.4 µg/mL to 0.025 µg/mL (in 50 µL) and LSD virus (50 µL of 100 TCID50 virus) together forming 100 µL/well were added to the wells and incubated at 37°C with 5% $CO_2$. In all cases, the cells were monitored at least once daily. Simultaneously, DON control-DCG (MDBK cells + DON) and viral control-VCG (MDBK cells + LSDV) and cell control-CCG (MDBK monolayer) groups were maintained. Considering the doses of DON tested in the literature review, we used a two-fold serial dilution of DON from 0.4 µg/mL to 0.025 µg//mL in the initial stage. Cells were monitored every day for cytopathic effect (CPE) visualization using an inverted microscope. Since we noticed cell deaths at all the above doses tested within 48-72 h (data not shown), we decided to further dilute the DON. Thus, in the second stage, DON was used from 0.025 µg/mL, 0.0125 µg/mL, 0.00625 µg/mL, 0.00312 µg/mL and 0.00156 µg/mL. Interestingly, at 0.0125 µg/mL of DON, CPE was observed by the end of 72 h of post-infection (pi), whereas at the same time period, VCG showed minor changes in morphology comparatively. This indicates that the presence of DON enhanced the viral replication rate in MDBK cells compared to viral control. There were no previous studies to compare simultaneous effect of DON and LSDV in MDBK cells. However, these results are consistent with the increased replication of porcine epidemic diarrhoea virus (PEDV) by DON, wherein DON at low concentrations augmented PEDV infection and replication (Liu *et al.*, 2020). Subsequently, prominent CPE was observed in

**Table 1. Results of cell growth (death) and cytopathic effect (CPE) in MDBK cells following incubation with DON (0.025 µg/mL to 0.00156 µg/mL) and LSD virus**

| Groups | DON Concentration | | | | |
|---|---|---|---|---|---|
| | **0.025 µg/mL** | **0.0125 µg/mL** | **0.00625 µg/mL** | **0.00312µg/mL** | **0.00156µg//mL** |
| | 1 | 2 | 3 | 4 | 5 |
| A | CD | + | + | + | + |
| B | CD | + | + | + | + |
| C | CD | + | + | + | + |
| D | CD | + | + | + | + |
| | | | DON control | | |
| E | CD | - | - | - | - |
| | | | Virus control | | |
| F | + | + | + | + | + |
| G | + | + | + | + | + |
| | | | Cell control | | |
| H | - | - | - | - | - |

Note: CD = Cell Destruction, '+' = CPE observed, '-' = No CPE
CPE in 0.0125 µg/mL was seen in 72 h and remaining wells observed in 96 h

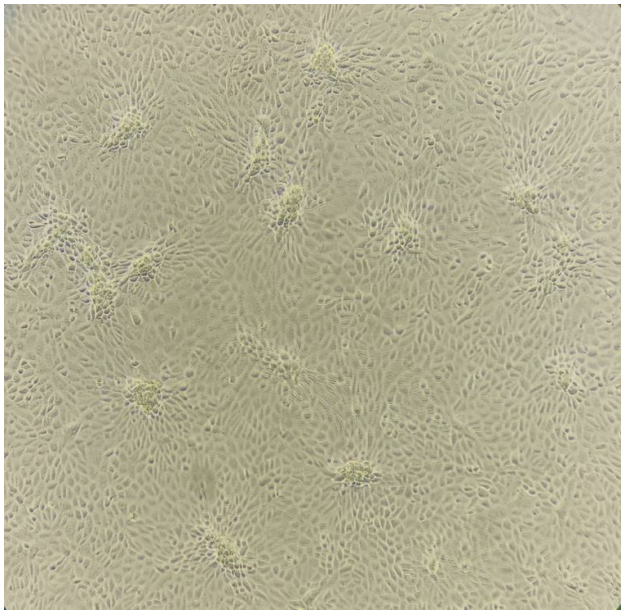

**Fig. 1. MDBK cells of DON group 0.0125 μg/mL showing many cytopathic effects at 96 h post infection**

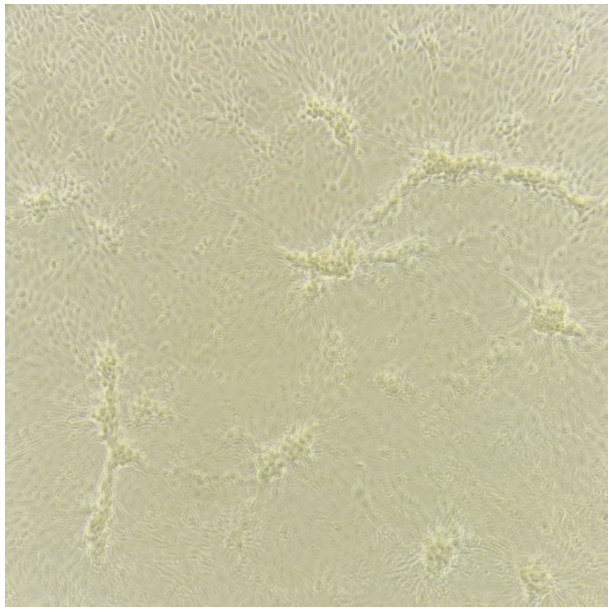

**Fig. 2. MDBK cells of VCG showing few cytopathic effects at 96 h post infection**

DON concentration of 0.00625 μg/mL to 0.00156 μg/mL and even in VCG (Table 1) only at 96 h.

The CPE of MDBK cells at 0.0125 μg/mL of DON and VCG at 96 h is shown in Fig. 1 and Fig. 2, respectively. Despite the occurrence of CPE in VCG at 96 h, in the visual (qualitative) scale, the number of CPE observed in DON group 0.0125 μg/mL was higher at 96 h (approximately 15 per field, Fig. 2) as compared to VCG (7 per field, Fig. 1), per visualized field of inverted microscope. Our results are also similar to the influence of OTA (ochratoxin A) on porcine circovirus-2 (PCV2), in which OTA at 0.01, 0.05, 0.1 and 0.5 μg/mL increased PCV2 replication (Gan *et al.*, 2015). Similar to our experiment, even in that experiment, higher doses (1.0 μg/mL) of OTA had no significant increase PCV2 infection. The maximum increase in PCV2 replication was observed in OTA at 0.05 μg/mL, similar to 0.0125 μg/mL of DON. However, in our experiment, no increase was observed below 0.0125 μg/mL of DON. This could be because, in that experiment, they used real-time PCR and immuno-fluorescent technique (IFA) to quantify the viral load, whereas, in our experiment, we employed CPE.

The absence of effect on DON on LSD viral replication below 0.0125 μg/mL could also be because low concentrations of DON could be inhibiting the viral replication, as found on HSV-1 (herpes simplex virus type 1) replication (Tani *et al.*, 1995). Moreover, not all doses of mycotoxins increase replication; rather, in some cases, inhibition of viral replication, such as rabies viral inhibition by DON, is also noticed (Liu *et al.*, 2023). These results indicate that the effect of mycotoxin on viral replication is, on one hand, dose-dependent, and also the effect varies based on the virus and the host system (cell-line used).

However, cell death at DON doses (from 0.4 - 0.025 μg/mL) within 48-72 h is in contrast to earlier studies wherein cytotoxicity in MDBK cells was noted only from 2.25 μg/mL (Bailey *et al.*, 2019). Contrary to the use of CPE in our study, the cell death in that study was measured by the release of lactate dehydrogenase (LDH). Recently, it was shown that exposure of human epidermal cells to DON altered lipid biosynthesis machinery and impaired the structural integrity needed for skin barrier function (Cho *et al.*, 2017; Del Favero *et al.*, 2021). This could partly explain the mechanism for the cell death noticed in our study. Further, DON exposure to the skin increased levels of inflammatory cytokines (IFN-γ, TNF-α, IL-6, IL-17, and IL-1). Further, other inflammatory markers (PGE2, COX2, iNOS) were also augmented and resulted in the up-regulation of inflammatory reactions and acute inflammation. Additionally, an increase in these inflammatory markers by DON was correlated to the phosphorylation of ERKs, p38 MAPK, and JNKs (Mishra *et al.*, 2020) and apoptosis. Thus, combined exposure of DON with LSDV might have increased the cell death rate in MDBK cells at high doses.

LSDV exhibits a cytoplasmic replication cycle, and its replication is known to occur in keratinocytes,

hair follicle epithelium, pericytes in the skin and also in secondary organs, like lungs, kidneys, etc. *In vitro*, LSDV has been classically propagated in MDBK cells and shows a cytopathic effect (CPE) with foci-type plaques that were used to assay the viral replication rate and infectivity. Since we have provided the proof-of-concept on the positive correlation between LSDV replication (infectivity) in culture (MDBK) cell lines that are currently used in LSDV vaccine production, further progress in this area would be focussed on testing the infectivity of LSDV in epithelial cells derived from dermal tissues or *ex vivo* per-fused tissue. Additionally, testing different combinations of mycotoxins and their impact on viral infection is a sequential step forward. After these are established, testing it in animal models and conducting prospective epidemiological studies relating to mycotoxin prevalence and the LSD occurrence in a particular geographical area would strengthen the understanding of the role of mycotoxins in host-pathogen interaction. Moreover, considering the endemic nature of other skin pathogen infections such as foot-and-mouth disease and the simultaneous burden of mycotoxins in global feed, particularly in India, investigations on the interaction between FMD and other skin pathogens with mycotoxins (in different doses and combinations) are also needed.

**Conflict of interest:** Authors have no conflict of interest in this study.

**Author's contributions:** PKK: Conceptualized the idea and hypothesis, conceived the experimental endpoints and analysis, designed the experimental protocol and wrote the paper; SN: Conducted the experiments and collected the data; SMP: Collected the data; MNBN, PN: Reviewed the experimental design and the paper; GBMR: Coordinated the experiment, fine-tuned the experimental protocol, interpreted the results and reviewed the paper.

**Data availability statement:** Data from research are given in the paper, and if any data is needed, it shall be available upon request.

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