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Determination of the Pathogenicity of the Variant UPM 1432/2019 IBDV in SPF Chicken Eggs and Chicken Fibroblast Cell Line

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ABSTRACT

The infectious bursal disease virus (IBDV) is a nuisance to chicken productivity in the global poultry industry due to its high pathogenicity. In this study, the pathogenicity of the UPM 1432/2019 variant was evaluated using specific pathogen-free (SPF) chicken eggs and fibroblast cell lines. This viral strain was isolated from chickens displaying symptoms of watery diarrhoea and ruffled feathers. Following inoculation, the SPF chicken eggs, and cell lines were meticulously monitored for indicators of viral replication and the onset of cytopathic effects (CPE). The results showed that the virus could replicate in the SPF eggs and cell lines due to the presence of CPE in some cells. In addition, virus-induced weight loss in infected embryonated chicken eggs was evaluated. The UPM 1432/2019 and UPM 1219/2019 strains demonstrated similar mortality rates and CPE features. These findings indicate that the UPM 1432/2019 variant is pathogenic in SPF chicken eggs and fibroblast cell lines. Furthermore, the new UPM 1432/2019 variant caused IBD with pathogenic characteristics similar to the traditional UPM 1219/2019 variant. The emergence of the UPM 1432/2019 variant poses a risk to the economy and poultry production and is potentially resistant to the existing bursal disease vaccines. Consequently, the UPM 1432/2019 variant may significantly contribute to economic losses for chicken farm operators. Moreover, this study provides essential information on the potential virulence of the UPM 1432/2019 variant of IBDV and its potential impact on the poultry industry.

Keywords: CEF cells, cytopathic, embryonated, IBDV, inoculated, pathogenicity

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INTRODUCTION

Infectious bursal disease (IBD) is highly contagious and potentially fatal for

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chickens. This viral disease is caused by the infectious bursal disease virus (IBDV) from the Avibirnavirus genus and Birnaviridae family, which targets the bursa of Fabricius imperative for immune cell development (Huang et al., 2021). The IBDV is a double-stranded RNA virus that codes for five viral proteins: VP1, VP2, VP3, VP4, and VP5 (Thai et al., 2021). The VP2 protein is the primary factor determining the virus's pathogenicity and ability to elicit a protective immune response. Therefore, sequencing the hVP2 gene and pathogenicity testing are the most reliable methods for identifying IBDV strains (Deorao et al., 2021). The first IBD outbreak in Malaysia was reported in 1991, where the very virulent IBDV (vvIBDV) strain resulted in catastrophic mortality in poultry farms (Aliyu et al., 2021). Local chicken farm operators experienced devastating economic losses, estimated at more than RM72 million annually. The symptoms of IBD include depression, watery diarrhoea, ruffled feathers, dehydration, and immunosuppression in chickens aged between three and six weeks (Eterradossi et al., 1999).

A critical pathological consequence of IBD is the bursa of Fabricius atrophy, characterised by diminished organ size and function. Consequently, the host's immune competence is compromised, making the chicken susceptible to secondary infections. Therefore, management and prevention strategies, particularly vaccination regimens, have been established to combat the threats posed by the disease on the poultry industry (Taghavian et *al*., 2013). Attenuated live, inactivated immune complex and recombinant vaccines have been developed to specifically target virulent infectious bursal disease Virus (vIBDV), classical virulent IBDV (clIBDV), and antigenic variant IBDV (avIBDV) strains, respectively (Jin et al., 2005).

The variant UPM1432/2019 IBDV was isolated from vaccinated poultry with watery diarrhoea and ruffled feathers. This Malaysian variant is more persistent in the bursa of chickens than the vvIBDV and UPM1056/2018 strains (Aliyu et al., 2022). The bursa atrophy caused by IBDV is a significant complication with possible long-term effects on chicken health and immunity (Yang et al., 2021). Thus, this research aims to determine the effects of the propagated UPM1432/2019 variant on specific pathogen-free (SPF) chicken eggs and fibroblast cell lines. The study findings could shed light on the pathogenic severity of the UPM1432/2019 variant compared to other IBDV strains. In addition, this study ascertained the 50% tissue culture infectious dose ($TCID_{50}$) of the UPM1432/2019 variant in fibroblast cells of SPF chicken embryonated eggs. It determined the virus's mortality, persistence, and cytopathic effect on SPF chicken eggs and chicken embryonated fibroblast cells.

MATERIALS AND METHODS

Samples

A total of 70 SPF eggs and three viral isolates were used in this study. Two strains, UPM1219/2019 (accession no. MT431215) and UPM1432/2019 (accession no. MT505343), were obtained with other isolates from different commercial broiler farms across five states

in Malaysia between 2017 and 2019. The bursa samples exhibited haemorrhages, oedema, and lesions (Aliyu et al., 2021). Meanwhile, UPM081 (accession no. AY520910), a very virulent strain isolated from an outbreak in Malaysia back in 2000 (Lawal et al., 2018), was obtained as CAM homogenates and used as a comparison with the other isolates.

Titration ($TCID_{50}$) of UPM1432/2019 Variant Isolates

The $TCID_{50}$ was used to determine the number of virus particles required to infect 50% of the host cell. This assay measures a virus's virulence and compares the infectivity of different strains. First, the UPM1432/2019 variant was serially diluted, and each dilution was added to a separate well containing the host cell, the chicken embryo fibroblast (CEF) cells. The plate was incubated for a specified period, followed by a cell count of infected cells to determine the dilution at which 50% of the wells were infected (Jin et al., 2005).

Pathogenicity in Embryonated SPF Chicken Eggs

Three cycles of freeze-thaw and homogenisation of the bursa tissues were performed in a sterile mortar and pestle containing 20% (w/v) suspension of sterile, pH 7.4 phosphate-buffered saline (PBS). Subsequently, the homogenised tissue suspension was centrifuged at 4,000 x *g* for 20 min at 4°C (Ebrahimi et al., 2013). The supernatant was filtered using 0.22 µm sterile syringe filters (Micro, Lab Scientific, China) and stored for 60 to 90 min at 40°C. Finally, the virus was inoculated in the 9–11-day-old SPF embryonated chicken eggs using the chorioallantois membrane (CAM) method and incubated at 37°C for eight days while continuously checking for mortality.

Primary Chicken Embryonated Fibroblast (CEF) Cell Culture

Fibroblast cells obtained from 9–11-day-old SPF embryonated chicken eggs were used for cell culture preparation (Rekha et al., 2014). First, the CEF tissue was extracted and washed using 1 ml of PBS. The fibroblast tissue was cut into smaller pieces, transferred into a 15 ml tube containing 0.25% trypsin, mixed, and shaken for 20 min. Subsequently, the DMEM growth medium (high glucose, L-glutamine, and sodium pyruvate, Biosera, France) was supplemented with 10% fetal bovine serum (FBS) (Hyclone, USA) to stop the trypsinisation process. The cells were centrifuged at 350 x *g* for 5 min at 4°C, and the supernatant was discarded. The growth medium was then added to the cell pellets. The cell suspension (5 ml) was adjusted to a concentration of 5.0 x 10^6 cells/ml (SPL Lifesciences, South Korea) and seeded into a 25 ml flask. Complete monolayers in the flask were infected with 0.1 ml of the UPM1432/2019 variant and incubated at 37°C in 5% carbon dioxide (CO_2). The cytopathic effect (CPE) was monitored daily, and the flasks were freeze-thawed thrice and centrifuged at 350 x *g* for 5 min upon CPE detection. The supernatants were collected and stored at -20°C.

Virus Inoculation in Chicken Embryonated Fibroblast (CEF) Cell Culture

A full monolayer was formed in the flasks after 24 h. The flasks were washed thrice with 1 ml of pH 7.4 PBS and inoculated with 0.1 ml of the UPM1432/2019 variant. The flasks were incubated at 37 °C for 1 h to allow virus attachment. Subsequently, maintenance media containing DMEM, 2% FBS, and antibiotics were added to the flask. The cells were observed at 24, 48, and 72-h intervals and checked daily for cytopathic effects. Once CPE was visible, the cells were centrifuged, and the supernatant was stored at -20 °C for viral RNA extraction.

Immunofluorescence Assay (IFA)

As mentioned, the CEF cells injected with wild-type UPM1432/2019 were prepared to identify their morphological characteristics. Immunofluorescent staining was performed on the cells at 24 and 48 hr. First, the plates were fixed for 10 min with 4% paraformaldehyde (Biotium, USA), followed by washing with 1 ml of PBS thrice every 5 min and carefully aspirated. Subsequently, 0.5 ml of 0.3% triton X-100 was added to each well for 10 min to catalyse permeabilisation and washed gently with PBS thrice at 5 min intervals. After adding 500 µl of 1% bovine serum albumin (Sigma-Aldrich, Germany) to each well for 30 mins, the plate was washed thrice at 5 min intervals using PBST (Thermo Scientific, USA). Each well received 40 µl of mouse primary IBDV antibody (Fitzgerald, USA) and incubated at 4°C overnight in the dark. All plates were covered with aluminium foil to maintain the dark condition.

The plates were thoroughly rinsed thrice with 1 ml of PBS after 18 hr, followed by the addition of 40 µl secondary rabbit antibody against mouse IgG1 conjugated with fluorescein isothiocyanate (FITC) (Abcam, UK) to each well. The plates were incubated for 2 hr at 4°C in the dark. After three PBS washes, the plates were left to dry. Once dried, the coverslip was carefully removed from the plates, mounted with 20 µl of Fluoroshield mounting media with Dapi (Abcam, UK), and rested for 30 min. Finally, the slides were examined under a fluorescence microscope. Fluorescent signals of varying magnitude are indicative of a successful IFA. The viral overlap in the nucleus was identified and captured using a camera (Axiocam MRm, Germany) to assess the localisation features of the UPM1432/2019 variant within the CEF cells.

RESULTS

Figure 1 compares the pathogenicity of SPF chicken eggs inoculated with three strains (UPM1219, UPM081, and UPM1432/2019). The mortality rate was observed from day 1 to 8 post-infection, where UPM1432/2019 IBDV showed an increased mortality rate of about 30% from day seven post-infection.

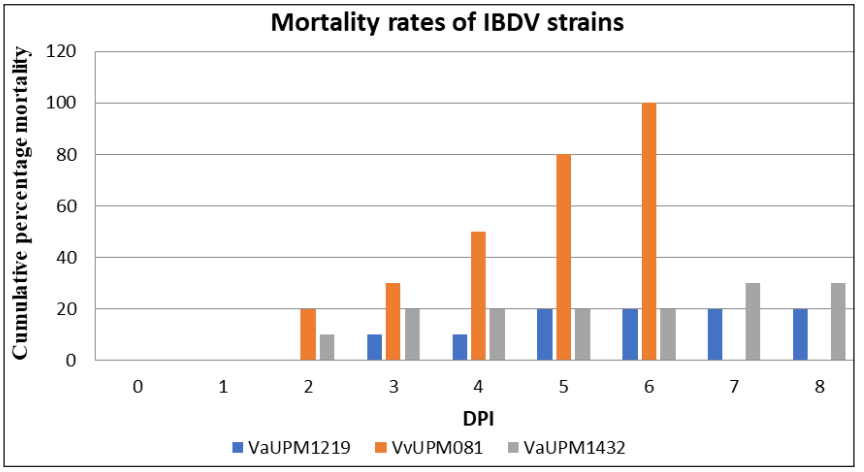

Figure 1. Cumulative mortality rates of IBDV variants inoculated in specific pathogen free (SPF) chicken eggs

Figure 2 shows gross lesions of the UPM 1432/2019, UPM 1219/2019, and UPM 081 in nine-day-old embryonated chicken eggs.

Gross lesions

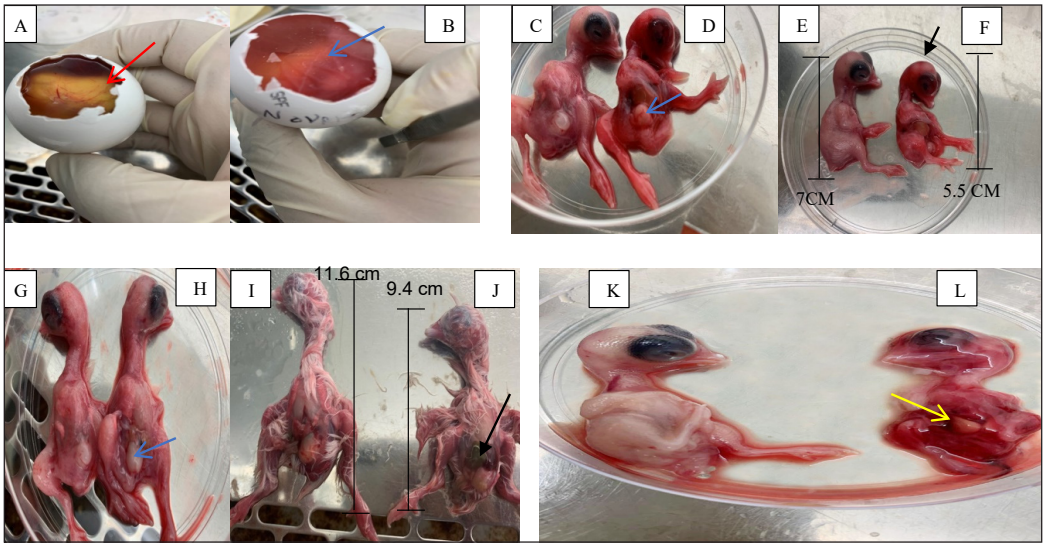

Figure 2. Gross lesions of UPM 1432/2019, UPM 1219/2019, and UPM 081 in nine-day-old embryonated chicken eggs. (A, C, E, G, I, and K): the control uninoculated embryo presents normal conditions. (A) shows normal transparent CAM (red arrow), (B, D, and F) infected group with UPM 1432/2019 displayed typical lesions on the bursa. Also, it showed cloudy CAM in the embryo (B) (blue arrow), head haemorrhagic (F) (black arrow), and swollen bursa (D) (blue arrow). (H and J) show chicken embryonated eggs inoculated with UPM 1219/2019, which represent swollen bursa (blue arrow) (H), dwarfing growth, and liver abnormality (black arrow) (J). (L) represent gross lesions in the infected nine-day-old embryonated chicken eggs and display congestion in the liver (yellow arrow) due to infection with the UPM 081 strain

The 50% tissue culture infectious dose ($TCID_{50}$) value of the UPM 1432/2019 variant strain was also used to compare its infectivity to other IBDV strains and to monitor changes in virulence over time. The $TCID_{50}$ value was calculated using experimental data shown in Table 1.

Table 1
Estimation of fifty per cent tissue culture infective dose ($TCID_{50}$) recorded UPM 1432/2019 variant virus data

10-fold dilution	No. of positive CPE well	No. of negative CPE well	Accumulative	Number	Total number	Percentage
			Positive (A)	Negative (B)	A+B	A/(A+B)X 100%
10^{-1}	10	0	61	0	61	100.0
10^{-2}	10	0	52	1	53	98.0
10^{-3}	10	0	45	3	48	93.7
10^{-4}	10	0	37	5	42	88.0
10^{-5}	10	0	29	6	35	82.0
10^{-6}	9.0	0	21	9	30	70.0
10^{-7}	7.0	4	18	12	30	60.0
10^{-8}	5.0	6	12	19	31	38.0
10^{-9}	4.0	8	7.0	22	29	24.0
10^{-10}	2.0	8	2.0	29	31	6.0

Note. It is important to note that the $TCID_{50}$ assay is a simplified measure of virulence and does not consider other factors that may affect the ability of a virus to infect a host cell culture (Wang et al., 2021)

At dilution 10^7, the dilution percentage immediately above 50% = 60%. At dilution 10^8, the percentage of dilution immediately below 50% = 38%.

$$Index\ calculation = \frac{\text{Percentage on } \textit{infectivity at dilution above } 50\% - 50}{\text{Percentage on } \textit{infectivity at dilution above } 50\% - \text{Percentage on infectivity below } 50\%}$$

$$= \frac{60 - 5}{60 - 38} = 0.45$$

$$10^{7.45}/0.1\ ml = 10^{8.45}\ TCID_{50}/ML$$

It is important to note that the $TCID_{50}$ assay is a simplified measure of virulence and does not consider other factors that may affect the ability of a virus to infect a host cell culture (Wang et al., 2021).

Figure 3 shows specific features of each strain's CPE, such as cytoplasmic vacuolation, apoptosis, and necrosis. These features were used to differentiate between IBDV strains.

Figure 4 shows that a microscopic examination of a CEF monolayer demonstrates alterations in the nucleolus morphology of chicken embryo fibroblast cells post-infection with the UPM 1432/2019 variant virus at 24- and 48-hours post-infection (hpi).

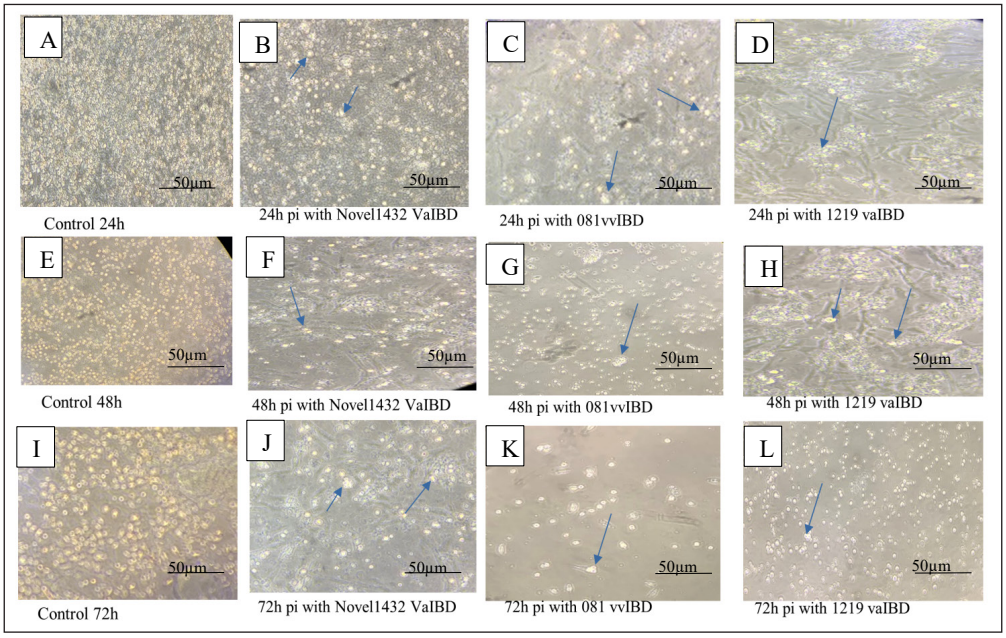

Figure 3. Microscopic view of chicken embryo fibroblast (CEF) cells monolayer after 24, 48, and 72-hours post-infection (hpi) by three different infectious bursal disease virus (IBDV) strains (variant UPM 1432/2019, very virulent UPM 081, and variant UPM 1219/2019). (A) indicates the control group

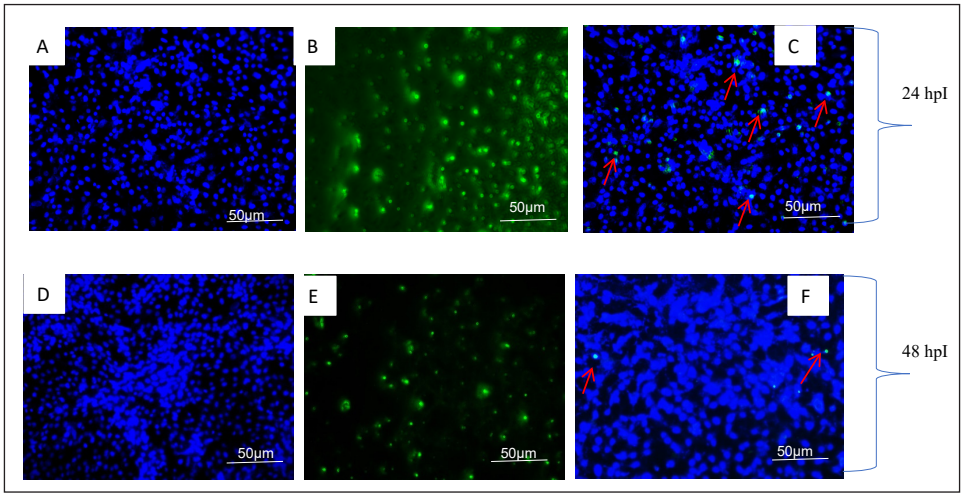

Figure 4. A microscopic examination of a chicken embryo fibroblast (CEF) monolayer demonstrates alterations in the nucleolus morphology of chicken embryo fibroblast cells post-infection with the UPM 1432 variant infectious bursal disease virus (IBDV) at 24 and 48-hours post-infection (hpi). The control group (A and D) displays a nucleus labelled with Dapi (blue) at 24 and 48 hpi, respectively, while infected CEFs (B and E) reveal the virus labelled with FITC in green at 24 and 48 hpi. Additionally, mixed staining of infected CEFs (C and F) exhibits a nucleus stained blue and the variant UPM1432 IBD virus stained with fluorescein isothiocyanate (FITC) green at 24 hpi and 48 hpi

DISCUSSION

This study successfully propagated the UPM 1432/2019 and UPM 1219/2019 strains and UPM 081 virulent strains in SPF chicken embryonated eggs. The mortality rates of the IBDV strains varied depending on factors such as the chickens' age and immune states (Dey et al., 2019). The disease caused by vvIBDV is often more severe than those caused by avIBDV or clIBDV, where the former is characterised by severe depression, diarrhoea, and high mortality (Fan et al., 2020). The mortality rate for the clIBDV-infected chicken is typically low in SPF eggs. Conversely, a significant death rate may be evident among chickens infected with vvIBDV strain (Dey et al., 2019). Approximately 20% and 30% of the SPF eggs infected with UPM 1432/2019 and UPM 1219/2019 died on day 8 post-infection (Figure 1). Meanwhile, 100% mortality was observed in the SPF eggs at day 6 post-infection with the UPM 081 strain.

At nine days old, the SPF chicken embryonated eggs inoculated with the UPM1432/2019 variant demonstrated cloudy CAM in the embryo (Figure 2B), swelling, and a haemorrhagic in the bursa (Figure 2D). In contrast, those infected with UPM 081 had significant lesions in their bursa and liver (Figure 2L). Figure 1 also indicated that the UPM 1432/2019 infection did not result in high mortality, and the embryonated chicken could continue to propagate eight days post-infection. However, lesions were observed at their bursa (Figure 2D). This finding confirmed that the impacts of UPM 1432/2019 are more persistent and limited to the bursa, while the UPM 081 strain spreads its pathogenicity to other organs, such as the liver. Likewise, the SPF eggs infected with UPM 1432/2019 and UPM 1219/2019 also exhibited similar outcomes (Figure 2 D & H). Earlier studies reported that IBDV mainly targets the bursa of Fabricius in chickens, a reservoir for B lymphocytes (Reddy et al., 2022).

The CPE refers to changes in host cell post-viral infection, particularly in the cellular morphology and function. These alterations vary depending on the viral strain and host cell. The present study described the CPE of three viral strains on CEF cell culture (Figure 3). At 24 h post-infection (hpi) with the UPM 1432/2019 variant, the microscopic view of the CEF cell monolayer appeared relatively normal (Figure 3B). The cells are well-spread and adhered to the culture dish, with a normal cytoplasmic appearance. Meanwhile, cells inoculated with the UPM 081 strain had a slightly rounded appearance (Figure 3C). At 48 hpi (Figure 3G), the UPM 081-infected CEF cell monolayer was significantly altered and indicative of severe CPE, characterised by a rounded appearance and detachment from the culture dish. No morphological changes were observed in CEF infected with UPM 1432/2019 and UPM 1219/2019 strains after 48 h (Figure 3F & H). At 72 hpi, the monolayer of the UPM 081-infected CEF cells exhibited prominent CPE, evident from the rounded cells detached from the culture plate (Figure 3K). The cytoplasm of the infected cells was vacuolated and swollen, with a substantial decrease in cell proliferation compared to UPM 1432/2019 and UPM 1219/2019 CEFs, which demonstrated mild CPE and a slight reduction in cells (Figure 3J & L) (Deorao et al., 2021).

Similar CPE patterns were observed in the IFA (Figure 4). At 24 hpi, the CEF infected with the UPM1432/2019 variant overlapped with the nucleus (Figure 4C), acquired CPE, fully integrated into the CEF nucleus, and began to transform morphologically by 48 hpi (Figure 4F). Overall, the microscopic view of the CEF cell monolayer post-infection with the three IBDV strains exhibited clear CPE progression over time. The UPM1432/2019 and UPM1219/2019 IBDV strains caused mild CPE, whereas the UPM081 very virulent strain caused severe CPE (Aliyu et al., 2021). These changes in the CEF cell monolayer are a valuable tool for differentiating the IBDV strains. The IBDV disease severity is associated with strain virulence, and novel IBDV variants can possess stronger virulence than their predecessors (Aliyu et al., 2021). Moreover, vvIBDV can cause severe immunosuppression and high mortality rates, particularly in young birds. These strains also cause more severe bursal damage, loss, and systemic disease (Agnihotri et al., 2022). Besides the bursal lesions, other organs, such as the thymus, spleen, and liver, could be affected by vvIBDV, which is indicated by organ enlargement and congestion. The kidneys may also be impacted, indicated by tubular necrosis.

CONCLUSION

The emergence of new IBDV variants continues to concern various stakeholders in the poultry industry. Controlling the spread of these variants is crucial to prevent severe disease spread and economic losses. In the current study, the UPM 1432/2019 variant was successfully propagated in SPF chicken embryonated eggs. The results indicate that this strain induces IBD with pathological features mimicking the conventional UPM 1219/2019 strain and UPM081 virulent strain. This finding highlighted the potential threat of the UPM 1432/2019 to the economy and poultry farming, as the existing IBD vaccines may not be effective against this variant. Therefore, it is recommended for future studies to develop a new and safe IBD vaccine that targets UPM 1432/2019, UPM 1219/2019, and UPM 081 IBDV to protect chickens against the disease.

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