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Large-Scale Field Evaluation of a Chicken Embryo Fibroblast Adapted Live Attenuated Vaccine Against Duck Viral Enteritis

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Abstract

Duck viral enteritis (DVE) causes significant economic losses to duck industries in endemic regions, and existing control measures, including embryonated-egg-based vaccines, are limited by batch variability and restricted scalability. In this context, the present study reports the development of a cell culture-based live attenuated duck enteritis virus (DEV) vaccine and its evaluation under both experimental and field conditions. The Holland strain of DEV was adapted to chicken embryo fibroblast (CEF) cells and cultured through 60 serial passages, achieving a titer of $10^{6.88}$ 50% tissue culture-infective dose ($TCID_{50}$)/mL. Evaluation in seronegative ducklings demonstrated the safety and immunogenicity of the vaccine, with no adverse clinical signs. Field evaluation in 9,000 ducklings across diverse farming systems showed consistent humoral immune responses under varying conditions. The vaccine's efficacy was further assessed under controlled-challenge conditions, where it conferred complete protection against a virulent field isolate of DEV (DP/As/Km/19). Overall, the findings indicate that the CEF-adapted live attenuated vaccine is safe, highly immunogenic, and suitable for large-scale production, highlighting its potential as a practical and scalable strategy for controlling DVE in endemic regions.

Keywords: Duck viral enteritis; Holland strain; chicken embryo fibroblast; live attenuated vaccine; field evaluation; immunogenicity

1. Introduction

Duck rearing plays a vital role in the rural economy of India, particularly among small and marginal farmers. According to the 19th Livestock Census, India has approximately 23.54 million ducks, with Assam contributing the largest share (7.31 million), highlighting its regional importance. Despite increasing demand for duck meat and eggs, the sector's growth is constrained by recurring outbreaks of infectious diseases, which cause significant economic losses. Among these, Duck Plague (DP), also known as Duck Viral Enteritis (DVE), remains one of the most devastating viral diseases affecting domestic and wild waterfowl. Since its first report in the Netherlands [1], DVE has been widely reported across India, including Assam and other duck-rearing regions [2,3], underscoring its endemic nature. DVE is caused by *Anatid alphaherpesvirus 1*, a member of the genus *Mardivirus* within the subfamily *Alphaherpesvirinae* [4]. The virus possesses a linear double-stranded DNA genome of approximately 160 kbp [5]

Vaccination remains the most effective strategy for controlling DVE. Currently, control programs rely largely on live attenuated vaccines produced in embryonated chicken eggs. However, these vaccines are associated with several limitations, including batch-to-batch variability due to

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difficulty in measuring viral concentration, high production costs, biosafety concerns related to potential contamination with adventitious agents, and suboptimal immunogenicity [6]. Moreover, reliance on embryonated eggs limits rapid scalability during outbreaks. Recent research has focused on developing cell culture-based vaccines as safer and more scalable alternatives. In particular, adaptation of duck enteritis virus (DEV) to chicken embryo fibroblasts (CEF) and other cell systems has shown promising results for vaccine production [3]. A recent study by [7] reported the development of a CEF-adapted live attenuated DEV vaccine with high viral titers and complete protection under experimental conditions. However, such studies are largely confined to controlled laboratory settings, with limited evidence on field-level performance, long-term immune response, and applicability under diverse farming conditions. Therefore, a critical gap remains in translating laboratory-scale vaccine development into real-world application, particularly in endemic regions with heterogeneous farming practices.

With this background, this study aimed to develop a CEF-adapted live attenuated DVE vaccine to evaluate its safety, immunogenicity, and efficacy. More importantly, this study takes a practical step and demonstrates the effectiveness of the developed vaccine by conducting extensive field trials involving more than 9,000 ducklings from Northeast India.

2. Materials and Methods

2.1. Virus Source and Propagation in Embryonated Eggs

The freeze-dried egg-adapted Holland strain of duck enteritis virus (DEV) was obtained from the Department of Veterinary Microbiology, Assam Agricultural University, Khanapara. The vaccine vial was reconstituted in 1 mL sterile distilled water, filtered through a 0.45 µm membrane filter, and supplemented with antibiotics (penicillin 100 IU/mL, streptomycin 100 µg/mL, and amphotericin B 25 µg/mL; Sigma-Aldrich, USA).

The virus was propagated in 9-11-day-old embryonated chicken eggs via the chorioallantoic membrane (CAM) route using 0.2 mL inoculum per egg. Inoculated eggs were incubated at 37°C and monitored daily. At 4-5 days post-inoculation, CAM tissues were harvested and homogenized in phosphate-buffered saline (PBS; pH 7.2) to obtain a 20% (w/v) suspension. The homogenate was clarified by centrifugation at 840 × *g* for 15 min and filtered before further use.

2.2. Preparation of Chicken Embryo Fibroblast (CEF) Cells and Virus Adaptation

CEF cells were prepared from 9-11-day-old embryonated chicken eggs as described previously [8] with minor modifications. Cells were isolated by trypsinization (0.25%), filtered, and centrifuged at 100 × *g* for 10 min. The pellet was resuspended in Dulbecco's Modified Eagle Medium (DMEM) supplemented with antibiotics, and cell viability was assessed using trypan blue exclusion.

Cells were seeded into culture flasks and incubated at 37°C in a humidified atmosphere containing 5% CO_2 until a confluent monolayer was formed. Monolayers (~70% confluence) were infected with chick embryo (CE)-passaged DEV and incubated for 1 h. After adsorption, maintenance medium (2% serum) was added, and cultures were incubated under standard conditions. Cells were observed daily for cytopathic effects (CPE).

The virus was harvested by three freeze-thaw cycles, clarified by centrifugation at 2630 × *g* for 30 min, and used for subsequent passages. Serial passaging was continued until stable and consistent CPE was observed.

2.3. Virus Titration

Virus titers were determined using the median tissue culture infective dose ($TCID_{50}$) method [9]. Tenfold serial dilutions (10^{-1} to 10^{-7}) were inoculated into CEF monolayers in 96-well plates (100 µL/well, 10 replicates per dilution). Plates were incubated at 37°C and observed for CPE for 4–5 days. $TCID_{50}$ values were calculated using the Karber method.

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2.4. Virus Purification

Virus purification was performed by sucrose cushion ultracentrifugation as described by [10] with minor modifications. Virus suspensions were layered over 30% (w/v) sucrose and centrifuged at 100,000 × *g* for 2 h at 4°C. The resulting pellet was resuspended in GTNE buffer (0.2 M glycine, 50 mM Tris-HCl, 100 mM NaCl, and 1 mM EDTA; pH 7.4) and stored at -80°C.

2.5. Vaccine Preparation, Storage, Purity, and Sterility Testing

The purified DEV suspension was formulated as a live attenuated vaccine preparation under aseptic conditions. The prepared vaccine was stored at 4°C until use. Vaccine vials were protected from repeated freeze–thaw cycles and direct light exposure throughout storage.

Purity testing of the vaccine preparation was performed to assess the presence of extraneous cellular debris and visible contaminants after ultracentrifugation. Filtration through a 0.45 μm membrane filter was performed to remove residual debris prior to vaccine formulation.

Sterility testing was performed by inoculating aliquots of the final vaccine preparation onto nutrient agar, blood agar, and Sabouraud dextrose agar, then incubating under standard microbiological conditions.

2.6. DNA Extraction and Polymerase Chain Reaction (PCR)

Viral DNA was extracted using TRIzol LS reagent (Invitrogen) according to the manufacturer's instructions. PCR targeting the UL30 gene was performed using previously described primers [11]. Each reaction included appropriate negative and positive controls.

Cycling conditions consisted of initial denaturation at 95°C for 3 min, followed by 30 cycles of denaturation (94°C for 30 s), annealing (57°C for 60 s), and extension (72°C for 60 s), with a final extension at 72°C for 8 min. Amplified products were visualized by agarose gel electrophoresis.

2.7. Serological Assays

Indirect enzyme-linked immunosorbent assay (ELISA) was performed as described previously, with modifications [12]. Microtiter plates were coated with purified antigen (10 μg/well), blocked, then incubated with serially diluted serum samples. Bound antibodies were detected using anti-duck IgG-horseradish peroxidase (HRPO) conjugate (1:300; KPL, USA), and absorbance was measured at 492 nm. Antibody titers were expressed as the reciprocal of the highest serum dilution above the cut-off value. The ELISA cut-off value was determined as the mean optical density (OD) value of negative control sera plus three standard deviations (Mean negative OD + 3SD). The calculated cut-off value for seropositivity was 0.285, and samples with OD values above this threshold were considered positive.

Serum neutralization tests (SNT) were performed by incubating serially diluted, heat-inactivated sera with 100 $TCID_{50}$ of DEV as described [9]. The mixture was then inoculated onto CEF monolayers and incubated at 37°C. Neutralizing antibody titers were defined as the highest serum dilution that completely inhibited CPE.

2.8. Animal Experiments and Study Design

All animal experiments were conducted in accordance with institutional ethical guidelines. Seronegative ducklings (~2 months old) were randomly allocated to experimental groups, and clinical assessments were performed in a blinded manner wherever feasible. Three vaccinated groups (A, B, and C), each containing 50 ducklings, received different vaccine doses, while one control group (D; n = 50) received sterile PBS. Random allocation was performed to minimize selection bias.

Safety and potency studies were conducted in accordance with previously described guidelines [13]. For safety evaluation, ducklings (n = 10 per group) were inoculated subcutaneously with DEV at doses of 10^3, 10^4, and 10^5 $TCID_{50}$ and monitored for 14 days for adverse effects.

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For immunogenicity and potency evaluation, ducklings (n = 50 per group) were vaccinated with 10^3 $TCID_{50}$ and boosted at 30 days post-primary immunization. Blood samples were collected at defined intervals up to 150 days post-vaccination, and antibody titers were evaluated in the sera. Viral shedding was assessed in tracheal and cloacal swabs during the early post-vaccination period by PCR.

2.9. Challenge Study

Challenge studies were conducted using a virulent DEV field isolate (DP/As/Km/19). At 60 days post-immunization, ducklings were challenged intramuscularly with 100 DID_{50} and monitored for clinical signs and mortality.

A subset of animals (n = 10) was randomly selected for post-mortem examination and molecular detection of viral genome in tissues, while the remaining animals were monitored for protection and survival.

2.10. Clinical Scoring

Clinical evaluation was performed using a standardized scoring system [14,15] based on parameters including body condition, activity, feed intake, ocular/nasal discharge, and fecal consistency. Scores ranged from 0 (normal) to 3 (severe clinical manifestation).

2.11. Field Evaluation

Field evaluation was conducted across 30 farms located in Assam, Manipur, and Meghalaya under both organized and backyard farming systems.

Farms were selected based on geographic representation, accessibility, flock size, willingness of farmers to participate, and absence of prior vaccination against duck viral enteritis. Both organized and backyard farming systems were included to evaluate vaccine performance under diverse management and environmental conditions. Farms with active severe concurrent infectious disease outbreaks at the time of vaccination were excluded from the study.

A total of over 9,000 ducklings were vaccinated subcutaneously with 10^3 $TCID_{50}$, followed by a booster dose at 30 days post-vaccination. Blood samples were collected at regular intervals up to 180 days post-vaccination, and antibody responses were evaluated using ELISA.

2.12. Statistical Analysis

Data were analyzed using GraphPad Prism (version 9.0) and expressed as mean ± standard deviation. Differences between groups were analyzed using one-way or two-way ANOVA followed by Tukey's multiple comparison test. Correlation between ELISA and SNT titers was evaluated using Pearson's correlation coefficient. A p-value < 0.05 was considered statistically significant. The individual animal was considered the experimental unit.

3. Results

3.1. Propagation of DEV in Embryonated Chicken Eggs

The DEV vaccine strain was successfully revived in 9-11-day-old embryonated chicken eggs. No visible changes were observed in the chorioallantoic membrane (CAM) or embryos during the first two passages. From the third passage onwards, distinct pathological changes were observed, including thickening and hemorrhages of the CAM, along with generalized congestion in infected embryos (Figure 1A). Embryo mortality was consistently recorded at 4 days post-inoculation. The severity of lesions increased progressively with subsequent passages, indicating efficient viral adaptation. Viral nucleic acid was detected up to the 7th passage by PCR (Figure 1B).

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3.2. Adaptation of DEV in CEF Cells

The DEV strain was successfully adapted to chicken embryo fibroblast (CEF) cells through serial passaging up to passage 60. Cytopathic effects (CPE) were first observed at the third passage and included cell rounding, syncytia formation, cytoplasmic granulation, and intracellular vacuolation (Figure 1C). Occasional inclusion bodies were also noted.

The onset of CPE occurred at approximately 48 h post-infection (hpi) during passages 3-5 and was reduced to 24 hpi from passage 6 onwards, suggesting enhanced viral adaptation and replication efficiency. The presence of DEV was consistently confirmed by PCR amplification of the UL30 gene across passages (Figure 1B). The viral titer at passage 60 reached $10^{6.88}$ $TCID_{50}$/mL.

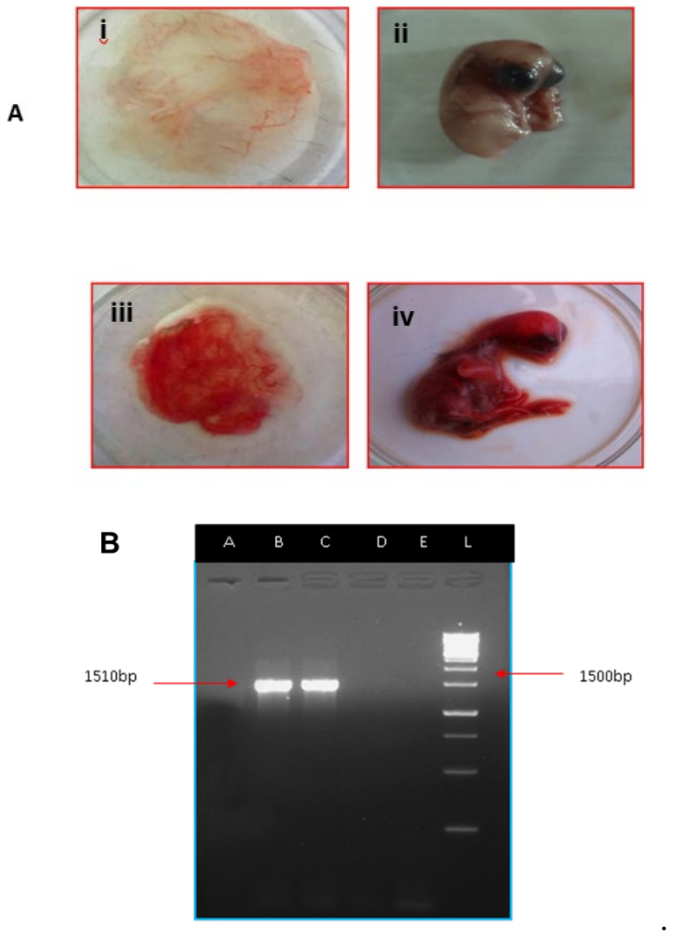

Figure 1. Propagation, adaptation, and molecular confirmation of duck enteritis virus (DEV). (A) Propagation of DEV in 9-11-day-old embryonated chicken eggs following chorioallantoic membrane (CAM) inoculation. Representative images of uninfected control CAM and embryos (i, ii) and DEV-infected CAM and embryos (iii, iv) at 4 days post-inoculation (dpi). Infected embryos exhibited hemorrhage, congestion, and thickening of the CAM. (B) PCR-based confirmation of DEV targeting the UL30 gene. Lane A: empty lane; Lane B: positive control (original virus stock); Lane C: CEF-adapted DEV (60th passage); Lane D: negative control (no template); L: 1 kb DNA ladder. (C) Cytopathic effects in chicken embryo fibroblast (CEF) cells. Uninfected control monolayer (left) and DEV-infected monolayer (right) at 24 h post-infection (hpi), stained with hematoxylin and eosin, showing cell rounding, syncytia formation, cytoplasmic vacuolation, and inclusion bodies.

3.3. Vaccine Stability, Purity, and Sterility

The prepared vaccine remained stable, maintaining acceptable potency and immunogenicity for up to 6 months when stored at 4 °C.

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Purity assessment revealed no visible extraneous contaminants following purification procedures.

Sterility testing showed no detectable bacterial or fungal growth on nutrient agar, blood agar, or Sabouraud dextrose agar media following incubation.

3.3. Safety and Immunogenicity

No clinical signs or adverse effects were observed in ducklings inoculated with CEF-adapted DEV at doses of 10^3, 10^4, and 10^5 $TCID_{50}$ up to 10 days post-inoculation. Minimal to undetectable levels of viral DNA were observed in tracheal and cloacal swabs of vaccinated birds during the observation period.

Serological analysis revealed detectable antibody responses by day 30 post-vaccination, which increased further following booster immunization. iELISA titers were comparable between the 10^3 and 10^4 dose groups, with no statistically significant difference ($p > 0.05$), whereas significantly higher titers were observed in the 10^5 group at both day 30 and day 60 ($p < 0.05$). In contrast, neutralization antibody titers remained comparable across all dose groups, with no significant differences (Table 1).

Longitudinal analysis demonstrated that antibody titers peaked at day 90 post-vaccination and declined gradually thereafter, while remaining detectable up to day 150 (Table 2).

Table 1. Antibody titer (mean ± SD) in ducklings vaccinated with CEF-adapted DEV with different doses and at different post-vaccination days, as determined by indirect ELISA (iELISA) and serum neutralization test (SNT).

	Day 0		Day 30		Day 60	
Dose	**iELISAtitre**	**SNT titre**	**iELISAtitre**	**SNT titre**	**iELISAtitre**	**SNT titre**
10^3	<20	<10	158.4 ± 19.73	32.8 ±4.33	320 ±43.82	121.6 ±7.6
10^4	<20	<10	166.4 ±10.45	33.4 ±3.15	331.2 ±54.38	121.6 ±7.6
10^5	<20	<10	321.6 ±42.69	35.4 ±4.67	633.6 ±33.32	123.6 ±6.6
0 (PBS)	<20	<10	<20	<10	<20	<10

Table 2. Duration of antibody responses in ducklings following immunization with CEF-adapted DEV, as measured by indirect ELISA (iELISA) and serum neutralization test (SNT) at different post-vaccination days.

	Day 0		Day 30		Day 60		Day 90		Day 120		Day 150	
	iELISA	**SNT**	**iELISA**	**SNT**	**iELISA**	SNT	iELISA	SNT	iELISA	SNT	iELISA	SNT
Virus	<20	<10	160	32	320	64	640	128	320	64	320	64
PBS	<20	<10	<20	<10	<20	<10	<20	<10	<20	<10	<20	<10

3.4. Challenge Study

Unvaccinated ducklings developed characteristic clinical signs within 3 days post-challenge and succumbed to infection within 6 days. Viral DNA was detected in cloacal and tracheal swabs, blood, and multiple visceral organs, indicating active viral replication and systemic dissemination. Gross pathological lesions observed in unvaccinated birds included hemorrhages, congestion, and tissue necrosis, consistent with classical DEV infection.

In contrast, ducklings vaccinated with CEF-adapted DEV at doses of 10^3, 10^4, and 10^5 $TCID_{50}$ remained clinically healthy throughout the observation period, and complete survival (100%) was recorded in all vaccinated groups following virulent challenge (Table 3). Viral DNA in vaccinated birds was either absent or detected only at minimal levels in sampled swabs and internal organs at the tested post-challenge time points, suggesting substantial restriction of viral replication and dissemination in vaccinated animals.

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Table 3. Clinical outcome, pathology scores, and survival of vaccinated and unvaccinated ducklings following challenge with virulent DEV.

Experimental group	Vaccine dose ($TCID_{50}$)	Clinical score at day post-infection				Pathology score	Survival (%)
		-3 to -1	0 to 2	3 to 5	6 to 8		
A	**$10^{3.0}$**	**0**	0	0	0	-	100.00
B	$10^{4.0}$	0	0	0	0	-	100.00
C	$10^{5.0}$	0	0	0	0	-	100.00
D	Unvaccinated	0	++	+++	Death	+++	0

– = no clinical signs; + = mild; ++ = moderate; +++ = severe clinical disease; Death = mortality due to infection.

3.5. Field Evaluation

Following a successful in-house evaluation, the vaccine was assessed under field conditions across Assam, Manipur, and Meghalaya, with over 9,000 ducklings vaccinated in both organized and backyard farming systems.

No pre-existing antibodies were detected prior to vaccination, except for low baseline titers at a single location. Post-vaccination, antibody titers increased progressively, with mean ELISA titers rising by day 30 and peaking at day 60, followed by a gradual decline up to day 180 (Figure 2).

A substantial proportion of birds achieved ELISA titers≥160 by day 30, which further increased by day 60, indicating the development of a strong humoral immune response under field conditions. Higher antibody titers were consistently observed in ducklings reared in unorganized farming systems compared to those reared on organized farms.

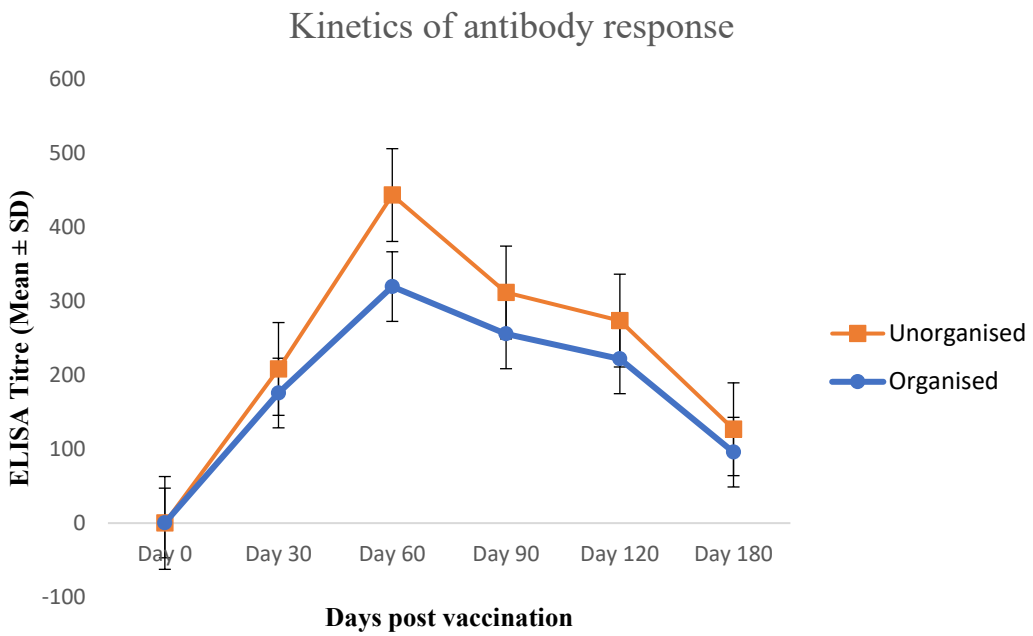

Figure 2. ELISA antibody response in ducklings following field vaccination with CEF-adapted DEV vaccine across organized and unorganized farming systems.

4. Discussion

The present study demonstrates the successful adaptation of the Holland strain of duck enteritis virus (DEV) in chicken embryo fibroblast (CEF) cells and its potential as a safe and efficacious live attenuated vaccine candidate. Progressive viral propagation changes observed in chicken embryonated eggs, including hemorrhage, congestion, and thickening of the chorioallantoic membrane (CAM), are consistent with DEV adaptation and corroborate earlier reports (Dinh et al.,

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2004). The gradual increase in lesion severity across successive passages further indicates active viral replication and host adaptation, hallmarks of herpesvirus attenuation in a heterologous host system.

Following adaptation in the chicken embryo, the propagated DEV virus was further adapted into CEF cells. The appearance of CPE in CEF cells from the third passage onwards, characterized by syncytia formation, cytoplasmic granulation, and vacuolation, aligns with previous descriptions of DEV-induced cellular pathology [3,6,16]. The earlier onset of CPE in subsequent passages (from 48 hpi to 24 hpi) reflects progressive viral adaptation to the fibroblast cell system, which is critical for efficient virus propagation and vaccine production [17,18]. Variations in the timing of CPE reported in earlier studies may be attributed to differences in viral strains, inoculum concentrations, and receptor interactions. The DVE virus is likely to initially interact to cell-surface heparan sulfate proteoglycans (HSPGs), followed by interaction with specific entry receptors, as reported for herpesviruses [19,20]. The virus's ability to adapt efficiently to chicken fibroblastic cells suggests the presence of conserved receptor-mediated entry mechanisms across avian species [21].

The high viral titer ($10^{6.88}$ $TCID_{50}$/mL) achieved at passage 60 further supports the suitability of primary CEF cells for DEV propagation. Comparable titers have been reported in earlier studies [3,6,11]. Primary cells often yield higher viral titers than continuous cell lines, likely due to enhanced cellular permissiveness, retention of native receptor expression, and reduced antiviral responses [22,23]. From a practical standpoint, achieving high titers is essential for large-scale vaccine production, particularly in endemic regions with high duck density.

Safety evaluation in seronegative ducklings revealed no adverse clinical signs, indicating successful viral attenuation after 60 passages in a heterologous host. Importantly, minimal to undetectable viral excretion was observed in vaccinated ducks under the conditions tested, suggesting that the attenuated strain does not readily disseminate into the environment, thereby supporting its biosafety profile.

The vaccine elicited strong humoral immune responses, as demonstrated by both indirect ELISA (iELISA) and serum neutralization test (SNT). The dose-dependent increase in ELISA titers, particularly at higher doses, suggests enhanced antigenic stimulation, although neutralizing antibody titers remained relatively consistent across doses. This indicates that even lower doses (10^3 $TCID_{50}$) may be sufficient to induce protective immunity, with important implications for dose optimization and cost-effective vaccine deployment. Live attenuated vaccines undergo limited replication within host cells, enabling antigen presentation through both major histocompatibility complex (MHC) class I and II pathways, thereby activating $CD8^+$ cytotoxic T cells, $CD4^+$ helper T cells, and B cells, resulting in both cellular and humoral immune responses with durable immune memory [24,25]. However, the contribution of cell-mediated immunity was not directly evaluated in the present study and warrants further investigation.

The antibody response peaked at day 90, followed by a gradual decline, consistent with the immunological profile of live attenuated vaccines. Sustained antibody levels up to five months post-immunization indicate the potential for long-term protection. However, the observed decline beyond peak titers suggests that booster immunization may be required to maintain adequate herd immunity under field conditions. Notably, a strong positive correlation was observed between iELISA and SNT titers ($r = 0.9944$, $p < 0.0001$), indicating a close association between binding and functional neutralizing antibody responses (Figure 3). Given the simplicity, rapidity, and scalability of ELISA compared to SNT, these findings support its use as a practical surrogate for assessing vaccine-induced immune responses, particularly in large-scale and field-based studies. While a protective SNT titer is generally considered ≥1:16, the present study maintained titers of 1:64 for up to 150 days post-vaccination, indicating a strong neutralizing response. However, a definitive correlate of protection for DEV remains to be established.

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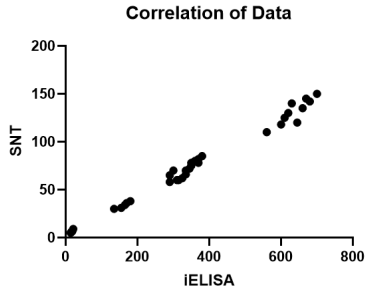

Figure 3. Correlation between iELISA and SNT titers. Scatter plot showing the relationship between indirect ELISA (iELISA) and serum neutralization test (SNT) titers across different time points. A strong positive correlation was observed (Pearson's r = 0.9944, $p < 0.0001$), indicating a close association between binding and neutralizing antibody responses.

Validation of the challenge model was achieved through pathological and molecular analyses. Unvaccinated birds exhibited classical gross lesions, including hemorrhages, congestion, and tissue damage, consistent with DEV pathology [14,26]. Detection of viral genome in these lesions and in internal organs confirmed that the observed pathology was directly associated with viral replication. Moreover, in birds that succumbed to infection, the presence of viral DNA in multiple organs established that mortality was due to successful replication and systemic spread of the challenge virus. These observations collectively validate the challenge model and confirm that the challenge virus retained its pathogenicity.

In the post-vaccination challenge study, of the 50 birds, a subset (n = 10) was sacrificed for detailed virological and pathological evaluation, while the remaining birds were monitored for immune responses and clinical protection. The absence of viral genome in the internal organs of vaccinated birds further reinforces the conclusion of effective viral neutralization.

Challenge studies demonstrated complete (100%) protection in vaccinated ducklings, even at the minimum dose of 10^3 $TCID_{50}$. These findings are consistent with earlier reports demonstrating the effectiveness of CEF-adapted DEV vaccines [3,6,16]. A critical observation from the challenge study was the absence of detectable viral excretion in vaccinated birds, as determined by cloacal and tracheal swab analysis. In contrast, unvaccinated birds exhibited viral shedding as early as 48 hours post-challenge, along with detectable viral genome in internal organs, confirming active viral replication and systemic dissemination. These findings indicate that the vaccine induces strong immunity, effectively restricting viral replication and reducing the likelihood of transmission. The comparable protection observed across different doses suggests that a threshold level of immune response is sufficient for protection, beyond which increasing antigen dose may not proportionally improve protective outcomes. Optimizing the vaccine dose is therefore critical not only for ensuring consistent efficacy but also for improving cost-efficiency and scalability, particularly in large-scale field applications.

Recent studies have reported the development of CEF-adapted live attenuated DEV vaccines with promising outcomes under experimental conditions. For instance, high viral titers (~$10^{7.5}$ $TCID_{50}$/mL) and complete protection under laboratory conditions [7] . The findings of the present study are in close agreement, as the CEF-adapted strain demonstrated high viral titers ($10^{6.88}$ $TCID_{50}$/mL), strong immunogenicity, and complete protection. However, a key distinction of the present study lies in its translational scope, extending beyond laboratory evaluation to large-scale field validation.

The field evaluation, involving over 9,000 ducklings across multiple locations in Northeast India, provides critical evidence of vaccine performance under diverse agro-climatic and management conditions. Including both organized and unorganized farming systems enabled a comprehensive assessment of vaccine efficacy under real-world conditions. Higher antibody titers observed in

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unorganized farming systems may be attributed to natural immune boosting from repeated environmental exposure to circulating viruses. Despite these differences, the overall antibody kinetics remained comparable, reinforcing the vaccine's robustness and adaptability across diverse conditions. The findings also suggest that a neutralization titer of approximately 1:121 is associated with protection; however, further studies are required to determine the exact antibody and neutralization thresholds required for protection against DEV infection.

While peak antibody titers were observed at day 90 in controlled in-house conditions, the earlier peak observed under field conditions may reflect additional antigenic stimulation. A gradual decline in antibody levels by day 180 was observed in both systems, consistent with the expected waning of humoral immunity over time. These findings highlight the potential need for booster immunization to sustain long-term protection and ensure effective herd immunity.

From a broader perspective, the vaccine's ability to limit detectable viral shedding has important epidemiological implications, as it may help reduce transmission within flocks. In addition, reduced detection of viral DNA in vaccinated birds may have implications for the future development of DIVA-compatible surveillance approaches, although dedicated marker-based studies would be required to establish this capability.

Despite these promising findings, certain limitations should be acknowledged. The study primarily focused on humoral immune responses, and the role of cell-mediated immunity was not evaluated. Additionally, the genetic stability of the attenuated virus and the potential risk of reversion to virulence were not assessed and should be investigated in future studies. Field-based evaluations inherently involve biological and environmental variability, which may influence immune responses under real-world conditions. Furthermore, the absence of neutralization data in large-scale field trials limits direct assessment of functional immunity. Addressing these aspects in future studies will further strengthen the evaluation of this vaccine.

5. Conclusions

The present study demonstrates that a CEF-adapted live attenuated duck viral enteritis vaccine is safe, immunogenic, and capable of conferring complete protection under experimental conditions. The successful large-scale field evaluation further highlights its robustness and applicability under diverse farming systems. These findings support the potential of this vaccine as a scalable and cost-effective strategy for controlling duck viral enteritis in endemic regions. Further studies on long-term immunity, genetic stability, and field-level optimization will strengthen its application in widespread vaccination programs.

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