



ISOLATION AND MOLECULAR CHARACTERIZATION OF VERY VIRULENT INFECTIOUS BURSAL DISEASE VIRUS (vvIBDV) ISOLATES FROM BACKYARD POULTRY IN ASSAM, INDIA

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ABSTRACT

Infectious bursal disease (IBD), caused by the infectious bursal disease virus (IBDV), is a highly contagious viral infection of young chickens that causes immunosuppression and substantial economic losses in the poultry industry. This study aimed to isolate and characterize IBDV strains from backyard poultry in Assam, a major poultry-producing region of North-Eastern India. Cloacal swabs and bursal tissues were collected from chickens with clinical signs of IBD across four districts. Virus was isolated in chicken embryo fibroblast (CEF) cultures and propagated in Vero cells, and molecular characterization was performed by phylogenetic analysis of the VP2 gene. Affected flocks showed a cumulative mortality of approximately 5%, and viral titres reached $7.5 \log_{10}$ TCID₅₀ mL⁻¹ by the 15th Vero-cell passage. Necropsy findings were consistent with IBDV, including haemorrhages in breast and thigh muscles, a hyperaemic cloacal bursa, a necrotic and hyperaemic liver, and enlarged spleen and kidneys. Phylogenetic analysis based on partial VP2 gene placed both isolates within the lineage of previously reported very virulent IBDV (vvIBDV) strains from India. These findings indicate that vvIBDV-related strains circulate among unvaccinated backyard poultry in Assam and may serve as candidate strains for future antigenic and vaccine-related investigations.

Keywords: Assam, India, infectious bursal disease virus (IBDV), molecular characterization, Vero cells, VP2 gene

INTRODUCTION

The global poultry sector is central to the food security and agro-based economies. In India the industry has grown enormously making the country the world's 2nd largest egg producer (<https://pib.gov.in/PressReleasePage.aspx?PRID=2086052>). Viral pathogens, particularly infectious bursal disease (IBD), remain a major challenge to this growth. IBD predominantly affects chicks aged 3 to 6 weeks, coinciding with decline of maternal antibodies and colonization of immature B cells in the bursa, and causes considerable economic loss through mortality, stunted growth, reduced egg production, and veterinary costs (Jackwood and Sommer-Wagner, 2007). The disease remains widespread in India despite extensive vaccination (Agnihotri *et al.*, 2023). Its causative agent, IBD virus (IBDV), belongs to the family Birnaviridae, genus Avibirnavirus (Delmas *et al.*, 2019), and has

a bi-segmented RNA genome: segment A encodes VP2-5 and segment B encodes VP1. Serotype 1 IBDV, which includes all pathogenic strains, is further classified into avirulent, classic (cIBDV), variant (vaIBDV), and very virulent (vvIBDV) pathotypes (Bayliss *et al.*, 1990; Islam *et al.*, 2021); serotype 2 strains are nonpathogenic. VP2, the protein responsible for eliciting neutralizing antibodies and determining serotype specificity, contains a hypervariable region (amino acid positions 206-350) widely used in molecular epidemiology, comprising two major hydrophilic domains (A: 212-224, B: 314-324) (Schnitzler *et al.*, 1993; Vakharia *et al.*, 1994) and three minor domains (248-252, 279-290, 299-305) (Berg *et al.*, 1996), as well as a serine-rich heptapeptide (SWSASGS, 326-332) recognized as a virulence marker (Heine *et al.*, 1991). Analysis of this region is, therefore, central to distinguish virulent pathotypes and develop appropriate control strategies (Liu *et al.*, 1994).

vvIBDV strains have caused numerous outbreaks worldwide since entering India in late 1992, with reports continuing across the country (Sah *et al.*, 1995; Kataria *et al.*, 2001; Ramadass *et al.*, 2003; Nandhakumar *et al.*, 2020; Shinde *et al.*, 2021; Agnihotri *et al.*, 2022; Agnihotri *et al.*, 2023). The disease remains a significant threat in North-Eastern regions like Assam, where poultry farming underpins rural livelihoods, yet the reports of vvIBDV involvement from this region remain limited (Morla *et al.*, 2016; Rajkhowa *et al.*, 2018; Yaqub *et al.*, 2026), and a few Indian studies have combined virus isolation with molecular characterization. Outbreaks in unvaccinated backyard chickens frequently go unreported, leaving a gap in understanding of which IBDV genotypes circulate in this production system in Assam, and whether local flocks harbour strains genetically similar to, or diverging from, those previously described in the region (Morla *et al.*, 2016; Rajkhowa *et al.*, 2018). This study, therefore, was aimed to isolate IBDV from unvaccinated backyard chickens in Assam and characterize the isolates by partial VP2 gene sequencing and phylogenetic analysis. A concurrent sero-surveillance study across 19 districts of Assam found 306 of 1,093 (27.99%) serum samples from apparently healthy local poultry ELISA-positive for IBDV antibody, with sero-prevalence ranging from 12.12% (Tinsukia) to 66.66% (Tezpur) and highest in birds aged 2-4 weeks (54.93%), indicating widespread IBDV exposure among unvaccinated backyard flocks and providing epidemiological context for the outbreak-based sampling.

MATERIALS AND METHODS

Sample collection

Clinical samples were collected from the unvaccinated local (indigenous) chickens during outbreak investigations conducted between 2015 and 2020 at the College of Veterinary Sciences, AAU, Guwahati, Assam, India. A total of 23 samples were obtained from chickens aged 3 to 6 weeks across four districts of Assam following disease reports from local veterinarians. The samples originated from Kamrup district (Hajo, Khanapara, Sonapur, Bijoynagar, and Chaigaon; $n = 14$), Darrang (Mongoldoi; $n = 3$), Nalbari (Mukalmuwa and Loharkhata; $n = 4$), and Jorhat ($n = 2$). Of the 23 samples, 12 (52.17%) yielded the expected 643 bp VP2 gene amplicon by RT-PCR and were, therefore, considered infectious Bursal disease virus (IBDV)-positive. Seven positive samples were selected for virus isolation. Bursa of Fabricius and spleen tissues were aseptically collected in pre-sterilized, ice-chilled transport medium and transported to the laboratory under cold-chain conditions. The tissues were homogenized to prepare a 10% (w/v) suspension in sterile normal saline. Following three freeze-thaw cycles, the homogenates were clarified by refrigerated centrifugation ($6000 \times g$ for 10 min), passed through 0.22 μm syringe filter, and stored at -80°C until further use.

RT-PCR

The total RNA was extracted from the 23 tissue samples using TRI reagent (Sigma, USA), dissolved in 20 μL DEPC-treated water, and quantified with a Nanodrop 1000 spectrophotometer (Thermo-Fisher, USA). cDNA was synthesized using the SuperScript III First-Strand Synthesis System

(Invitrogen), following the manufacturer's instructions. RNA (14 μ L) was reverse-transcribed using a primer cocktail of random hexamers and gene-specific primers, and 1 μ L of the resulting cDNA served as template for VP2 gene amplification using the primer pair of Liu *et al.* (1994)—forward 5'-TCACCGTCCTCAGCTTAC-3' and reverse 5'-TCAGGATTGTTGGGATCAGC-3', targeting nucleotides 587-604 and 1212-1229 of VP2 gene—yielding a 643 bp amplicon. PCR was performed in a 20 μ L reaction (10 $\times$ buffer 2.0 μ L, 25 mM MgCl₂, 1.5 μ L dNTPs, 0.4 μ L forward and reverse primers, 0.12 μ L each at 10 pmol μ L⁻¹ Taq DNA polymerase, 0.4 μ L cDNA template, 1.0 μ L nuclease-free water to volume) under the following cycling conditions: initial denaturation at 95°C for 5 min; 25 cycles of 95°C for 30 sec, 54°C for 30 sec (annealing), and 72°C for 1 min; final extension at 72°C for 10 min; and a 4°C hold. A commercially available intermediate live-type strain vaccine (Ventri Biologicals) served as positive control, and DEPC-treated water as negative control. PCR products were resolved on a 1.7% agarose gel in 1 $\times$ TAE buffer at 100 V for 60 min. The resulting products were verified through electrophoresis and recorded with a gel documentation system.

Virus isolation in chicken embryo fibroblast (CEF) cells

Eight tissue samples showing a clear, discrete 643 bp band on agarose gel—distinguished from samples with faint, smeared, or absent amplicons—were chosen for virus isolation. Seven RT-PCR-positive samples (T-4 and T-5 from Hajo, Kamrup; T-7 from Hajo, Kamrup; T-8 and T-9 from Mongoldoi, Darrang; T-7 from Jorhat; and T-28 from Nalbari) were inoculated in parallel into confluent CEF and Vero cell monolayers (Vero cells: NCCS, Pune, India; CEF prepared in-house from 9-day-old SPF embryonated eggs). CEF cultures were prepared from 9-day-old embryonated eggs sourced from a local hatchery in Guwahati, following established protocols (Freshney, 2016). Tissues were minced and trypsinized at 37°C, and the resulting cells were resuspended in minimum essential medium (MEM, Sigma, USA) with 10% fetal bovine serum (Gibco BRL, USA), adjusted to 1×10^6 cells mL⁻¹, and seeded into 24-well plates incubated at 37°C with 5% CO₂ for 12 h to reach 80-85% confluence. Confluent monolayers were rinsed with phosphate-buffered saline and inoculated in duplicate with 50 μ L inoculum per well, with gentle mixing every 10 min for 1 h at 37°C. Two wells served as uninfected controls and two as vaccine-virus controls (Ventri-Plus, India). Maintenance medium was then added and cells were observed twice daily for cytopathic effects (CPE) with reproducible rounding, swelling, and detachment (relative to controls) scored as positive; CPE-negative harvests were passaged blindly. CPE first appeared at passage 2 (T-5, T-8) or passage 3 (T-4, T-9, T-28), progressing to nuclear extrusion and cell aggregation by passages 3-4 and cell lysis and detachment by passages 4-5. Two samples (T-7, T-7) showed neither CPE nor PCR-detectable virus through five passages and were considered non-isolatable. Virus in culture lysates was confirmed at each passage by integrated cell-culture RT-PCR (ICC-PCR) targeting VP2 gene. The five successfully adapted samples were ICC-PCR- and CPE-positive from passage 2-3 through passage 5, after which CEF-adapted virus was transferred to Vero cells for further propagation.

Virus adaptation in Vero cells

Confluent Vero cell monolayers were infected with CEF-adapted virus at a multiplicity of infection (MOI) of 0.01 and incubated at 37°C for 1 h with occasional shaking to aid adsorption. Then 10 mL of EMEM with 2% FBS was added and the flasks transferred to a 37°C, 5% CO₂ incubator. An uninfected monolayer raised under identical conditions served as a control. Cells were observed daily for six days for the emergence of cytopathic effects (CPE), after which infected cells were harvested, labelled, and stored at -80°C. Harvested virus underwent three freeze-thaw cycles (-80°C to +30°C) before inoculation onto a fresh Vero monolayer; this was repeated to the 15th passage (P15), with samples at each passage labelled and stored at -80°C.

Tissue culture infective dose 50 (TCID₅₀)

Viral titres were determined in Vero cells by the 50% endpoint method of Ramakrishnan (2016). Virus isolates were serially diluted tenfold in PBS (10⁻¹ to 10⁻¹⁰) and titrated in 96-well microtiter plates of confluent Vero cells, with four replicate wells per dilution (100 μ L well⁻¹) and the last two rows used

as cell controls. After 1 h adsorption period at 37°C, the medium was replaced and plates incubated at 37°C in a 5% CO₂ atmosphere. CPE was observed daily and read at 96-120 h post-inoculation, with titres calculated by the Ramakrishnan (2016) method. Titration was performed in three independent assays per isolate/passage and the mean titre reported.

Sequencing and phylogenetic analysis

RT-PCR products spanning the VP2 hypervariable region were generated from cell-culture fluid harvested at 5th Vero-cell passage. Since sequencing was performed on cell-culture-adapted rather than unpassaged field virus, therefore, some of the observed amino-acid substitutions may reflect cell-culture adaptation rather than field-circulating genotype. PCR products were purified by gel electrophoresis and sequenced bidirectionally by a commercial service. Sequences were aligned with reference strains available in GenBank using DNASTAR software (Lasergene). A phylogenetic tree was constructed using Neighbor-joining method in MEGA 11 software (Tamura *et al.*, 2021) to evaluate genetic relationships with strains from India and elsewhere.

RESULTS AND DISCUSSION

IBD causes considerable financial losses to the poultry sector so necessitates continuous monitoring due to its frequent genetic and antigenic variation. This study documents the outbreaks in unvaccinated backyard poultry, underscoring the continued circulation of harmful strains. The observed cumulative flock mortality of approximately 5% is consistent with earlier reports (Mor *et al.*, 2010; Agnihotri *et al.*, 2023). It is notably lower, however, than the flock mortalities of 15.25-60.18% reported in Central and Western India for outbreaks confirmed as vvIBDV (Agnihotri *et al.*, 2023). Mortality alone should, therefore, not be interpreted as supporting a very virulent designation, and the discrepancy may reflect differences in flock management, concurrent infections, or partial immunity in backyard birds, discussed further below. Necropsy results showed typical IBDV infection lesions, such as hemorrhages in thigh and abdominal muscles, a hemorrhagic cloacal bursa, a necrotic liver with hyperemia, and enlarged spleen and kidneys (Fig. 1A-F).

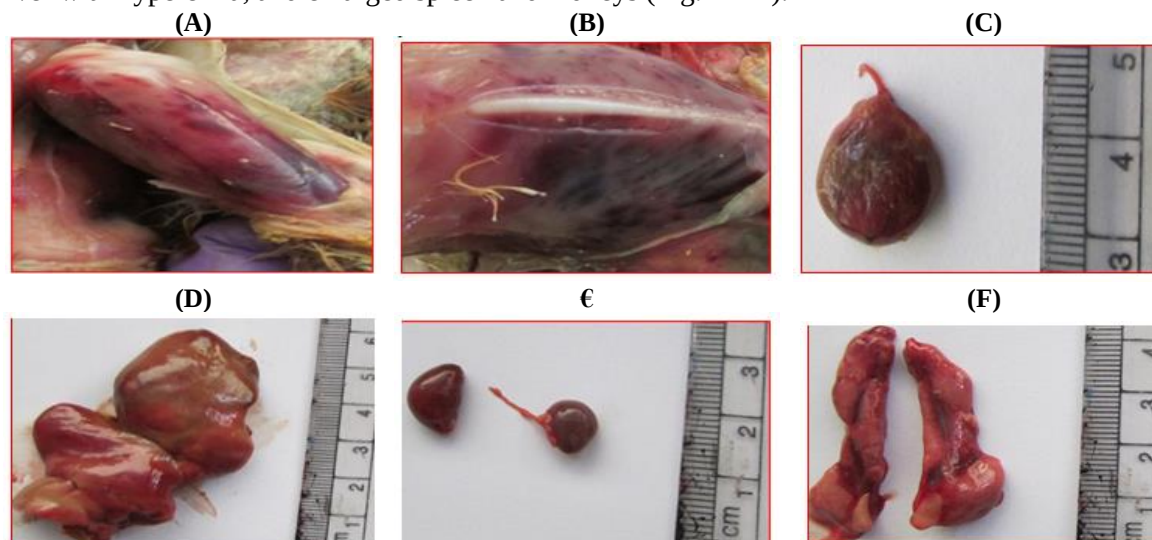


Fig. 1: Postmortem observations in local chickens affected by IBD. A & B) Hemorrhages ranging from petechial to ecchymotic in the thigh and abdominal skeletal muscles. C) Cloacal bursa showing edema and hemorrhage. D) Liver exhibiting necrosis. E & F) Spleen and kidneys that are hyperemic and enlarged.

Following virus confirmation via RT-PCR (Fig. 2A), two virus isolates—designated Nalbari and Hajo—were successfully cultured through seven passages in CEF cells and a further eight passages in Vero cells. They showed CPE from the 3rd passage in CEF cells (Fig. 2B-C) and the 10th passage in Vero cells (Fig. 2D-E). The viral titers for both strains rose from 5.5 log₁₀ TCID₅₀ mL⁻¹ at passage 9 to 7.5 log₁₀ TCID₅₀ mL⁻¹ by passage 15 in Vero cells. By passage 12, Nalbari strain reached a titer of 7.0 log₁₀ TCID₅₀ mL⁻¹ and Hajo strain 6.5 log₁₀ TCID₅₀ mL⁻¹, with both converging at 7.5 log₁₀ TCID₅₀ mL⁻¹ by passage 15 (Fig. 3). This steady rise in titre with repeated passage in Vero cells is consistent with earlier findings on LC-75 strain (Kebede *et al.*, 2021), and is confirmatory to the reports that Vero cells are a cost-effective and efficient alternative to primary CEF cultures for avian virus propagation (Hussain and Rasool, 2005; Hassan, 2015; Kebede *et al.*, 2021). However, high titre in cell culture does not, by itself, establish vaccine suitability, immunogenicity, protective efficacy, genetic stability, attenuation, safety or freedom from reversion to virulence. Further research is required to evaluate the immunogenicity and protective efficacy of these Vero-adapted strains before any claim of suitability for vaccine production can be made.

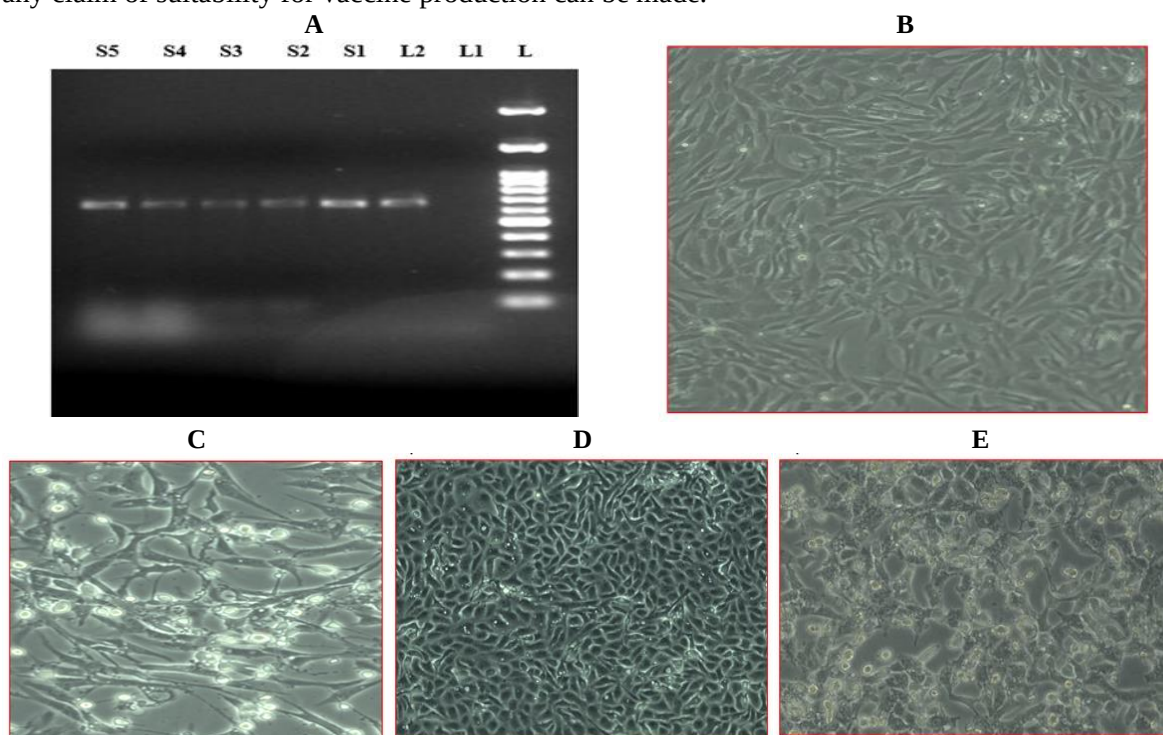


Fig. 2: A) RT-PCR amplification of a segment of the VP2 gene from clinical samples. L: 100 bp DNA ladder, L1: Negative control, L2: Positive control, S1-S5: Representative clinical samples showing an amplified product of about 640 bp for the VP2 gene. B): A confluent monolayer of chicken embryo fibroblast (CEF) cells. C): CEF cells exhibiting typical cytopathic effects (CPE) such as rounding and swelling at the 7th passage under 20X magnification. D): A confluent monolayer of Vero cells. E): Vero cells displaying typical CPE at the 15th passage.

To achieve molecular characterization, a 643 bp segment of VP2 gene was amplified and sequenced from both isolates and submitted to GenBank (Accession No. Nalbari-OR933579 and Hajo-OR933580). Both isolates showed over 99% sequence similarity with previously identified vvIBDV strains from India, particularly those from the North-Eastern regions (Morla *et al.*, 2016; Rajkhowa *et al.*, 2018). The Nalbari strain retained P at position 69, consistent with other classical strains (Morla *et al.*, 2016; Rajkhowa *et al.*, 2018; Nandhakumar *et al.*, 2020). Both strains also carried the serine-containing heptapeptide SWSASGS (site: 326-332) characteristic of vvIBDVs

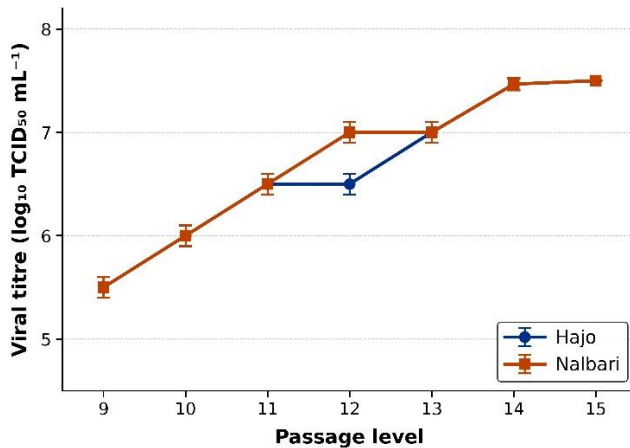


Fig. 3: The log TCID₅₀ mL⁻¹ values for Hajo and Nalbari strains from passages 9 to 15 in Vero cells. The virus titers were assessed using Ramakrishnan method. Both strains showed a steady titer increase, reaching 7.5 log TCID₅₀ mL⁻¹ by passage 15. Hajo strain showed a slower increase between passages 11 and 12, while Nalbari strain reached 7.0 log TCID₅₀ mL⁻¹ at passage 12.

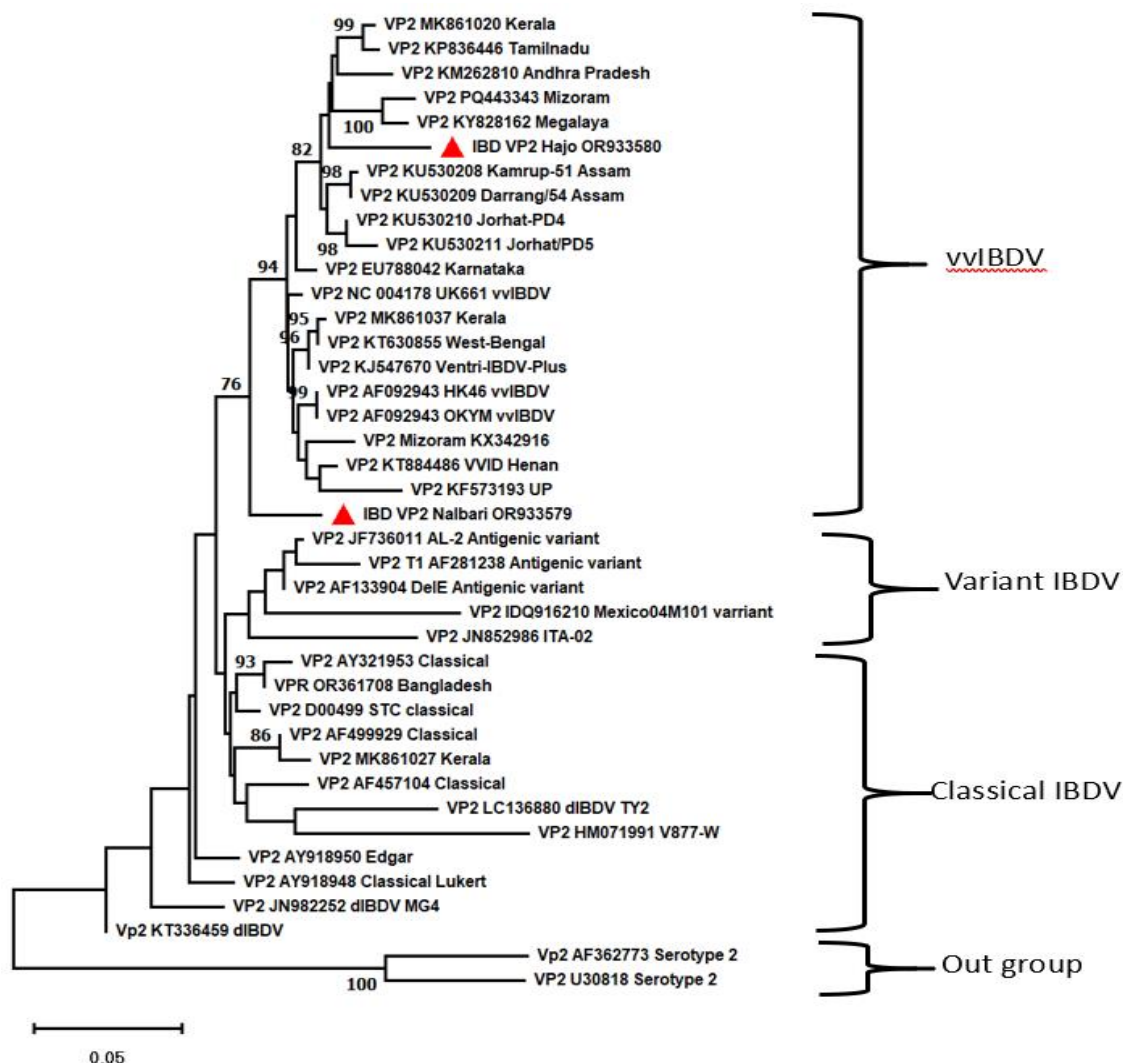


Fig. 4: Phylogenetic analysis of infectious bursal disease virus (IBDV) isolates based on the VP2 gene. The two strains examined in this research are indicated with a triangle. The phylogenetic tree was constructed using the Neighbor-Joining method, incorporating a bootstrap analysis with 1,000 replicates via MEGA 11. Bootstrap values are displayed at significant branch points.

(Heine *et al.*, 1991). These substitutions and SWSASGS motif were consistent with previously strains, and carried substitutions I273T (matching the West Bengal strain and the Ventrivirus vaccine virus) and G319D (resembling antigenic variant strains). Hajo strain carried Q250K and G255S (also present in antigenic variant strains) and A278E and L290P (closely resembling Southern Indian described vvIBDV and antigenic-variant strains (Morla *et al.*, 2016; Rajkhowa *et al.*, 2018; Nandhakumar *et al.*, 2020). None of these markers, singly or together, is by itself definitive proof of virulence phenotype, and they are presented here as supportive sequence-level observations rather than as confirmation of biological virulence.

Phylogenetic analysis of two isolates revealed that the Nalbari and Hajo strains are part of the vvIBDV lineage prevalent in India. The Hajo strain clustered with strains from Assam, Mizoram, Meghalaya, Andhra Pradesh, Kerala, and Tamil Nadu, indicating widespread circulation of vvIBDVs in these regions (Morla *et al.*, 2016; Rajkhowa *et al.*, 2018; Nandhakumar *et al.*, 2020), while Nalbari strain formed a distinct sub-clade, possibly reflecting localized evolutionary processes (Fig. 4). These findings highlight the presence of vvIBDV-related strains with regional amino-acid variation among local chickens in Assam; the identified substitutions suggest evolutionary pressures that warrant further investigation, and the genetic resemblance to strains from other parts of the country underscores the value of continued regional molecular surveillance.

Limitations

This study has several limitations that need attention while interpreting the findings. First, pathotype assignment is based on partial VP2 (segment A) sequence and phylogenetic clustering alone—segment B (VP1) was not analysed, no reassortment or recombination analysis was performed (Legnardi *et al.*, 2023; Pikuła *et al.*, 2023), and no biological or challenge-based virulence assessment was undertaken—so the isolates are best described as belonging to a VP2-defined vvIBDV-associated lineage rather than as being definitively confirmed as vvIBDV by comprehensive genotypic and phenotypic criteria. Second, only a partial (~643 bp), rather than complete, VP2 or segment A sequence was analysed, which limits conclusions about whole-genome diversity and evolutionary relationships. Third, sequencing was performed on cell-culture-adapted rather than unpassaged field virus, so some observed amino-acid substitutions may reflect cell-culture adaptation rather than field-circulating genotype. Fourth, the modest number of isolates obtained (two, from eight samples selected out of 23 collected) limits the epidemiological generalizability of the findings to backyard poultry in Assam as a whole. Finally, no immunogenicity, challenge, cross-protection or safety data are available for either isolate, and the isolates should therefore be regarded as candidates for future antigenic and vaccine-related investigation rather than as validated vaccine strains. Comprehensive bisegment or whole-genome sequencing, genotype assignment following the unified classification framework (Islam *et al.*, 2021), and biological virulence characterization are planned as follow-up work and were beyond the scope of the present regional surveillance report.

Conclusion: The study documents the isolation and molecular characterization of two IBDV strains, designated Nalbari and Hajo, from unvaccinated backyard poultry in Assam, India. Both isolates were successfully adapted to CEF and subsequently Vero cells, reaching titres of $7.5 \log_{10} \text{TCID}_{50} \text{ mL}^{-1}$ by passage 15. Partial VP2 sequence analysis and phylogenetic reconstruction placed the isolates within the Indian vvIBDV-associated lineage and revealed several amino-acid substitutions that warrant further studies, indicating the circulation of genetically variable IBDV-related strains in backyard poultry in Assam and underscoring the importance of continued molecular surveillance. Comprehensive assessment of the biological virulence, antigenic properties, genetic stability and protective efficacy of these isolates is required before their suitability for vaccine development can be established.

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