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Identification of Newcastle disease virus (*Orthoavulavirus javaense*) genotype XIII in an outbreak among indigenous chickens

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Abstract
Newcastle disease (ND) is a highly contagious viral infection with huge economic impact on the poultry industry. ND is caused by *Orthoavulavirus javaense* (OAVJ) which belongs to the genus *Orthoavulavirus* of the family *Paramyxoviridae.* The present study reports an outbreak of ND in desi chicken. Sudden mortality accompanied by characteristic lesions such as haemorrhages in the proventriculus and caecal tonsils, were noticed during necropsy. Samples were processed and confirmed by polymerase chain reaction using in-house designed primers targeting 258 bp region of F gene of OAVJ. Virus isolation was carried out in 9 to 11 day old embryonated chicken eggs through allantoic route which showed diffuse haemorrhage in embryos. The gel purified PCR product was sequenced and phylogenetic analysis revealed that the isolate belonged to genotype XIII of OAVJ.

Keywords: Newcastle disease virus, Mortality, Polymerase chain reaction, Isolation, Phylogenetic analysis

1. Introduction
Newcastle disease (ND) is a highly contagious disease causing significant economic losses to the poultry industry worldwide [1]. It was first reported in 1926 from Java, Indonesia [2] and subsequently, in 1927, from Newcastle-upon-Tyne, England [3]. The first outbreak of ND in India occurred in 1927 at Ranikhet, Uttarakhand state [4]. hence, ND is also called as Ranikhet disease [5]. ND is caused by *Orthoavulavirus javaense* (OAVJ), also known as Newcastle disease virus (NDV) / Avian Paramyxovirus type 1, belonging to the genus *Orthoavulavirus* within the subfamily *Avulavirinae* of the family *Paramyxoviridae* [6]. Currently, 21 serotypes of avian paramyxoviruses have been identified, namely APMV-1 to APMV-21 [7]. OAVJ is classified into 22 distinct genotypes based on variations in the fusion (F) protein [8]. Based on disease severity in chicken, OAVJ strains are classified into five pathotypes namely viscerotropic-velogenic, neurotropic-velogenic, mesogenic, lentogenic and asymptomatic [9].
The OAVJ virion is an enveloped virus with a negative sense single stranded RNA genome of 15 kb that encodes for six structural proteins such as fusion protein (F), hemagglutinin-neuraminidase (HN), nucleoprotein (NP), phosphoprotein (P), matrix protein (M) and RNA-dependent RNA polymerase (L) [10]. OAVJ virulence is mainly determined by the fusion (F) protein, encoded by the F gene which possess a hypervariable fusion protein cleavage site (FPCS) crucial for pathogenicity [11]. OAVJ has been reported to infect more than 250 species of birds [12]. OAVJ affects a wide range of avian species including domestic poultry, wild birds and captive birds with chickens being highly susceptible hosts [13-17].
Transmission of OAVJ occurs through direct contact with infected birds or their respiratory secretions and droppings as well as via aerosols, contaminated fomites. Birds that recover from infection may shed the for up to four weeks [18-19]. Clinical manifestations of ND include depression, loss of appetite, greenish diarrhoea, sudden drop in egg production, gasping, coughing, sneezing, rales, tremors, torticollis, circling, and paralysis of the wings and legs [20].

Newcastle disease can be diagnosed based on history, clinical signs and characteristic lesions at necropsy but requires laboratory confirmation. Diagnostic assays like Reverse transcriptase PCR (RT-PCR), hemagglutination (HA) and hemagglutination inhibition (HI) test, enzyme-linked immunosorbent assay (ELISA) and virus isolation have been commonly used for diagnosis [19] and RT-PCR is the preferred test for Newcastle disease diagnosis [18]. Recent advancements such as real-time PCR, multiplex PCR and nucleotide sequencing enable rapid detection and differentiation of OAVJ pathotypes and genotypes [16, 21].

Therefore, this study aims to identify and characterize *Orthoavulavirus javaense* from a suspected outbreak in desi chickens using PCR targeting the *F* gene and phylogenetic analysis.

2. Materials and Methods

2.1 Sample Collection

A desi chicken farm with a flock strength of 52 birds experienced sudden mortality among 20 day old chicks, which were brought for post mortem examination. The chicks had been vaccinated previously against ND using the LaSota strain vaccine. Clinical signs reported included diarrhoea and torticollis. Post-mortem examination revealed characteristic lesions such as diphtheritic ulcers on the intestinal mucosa, pinpoint petechial haemorrhages in proventriculus, and haemorrhagic caecal tonsils. Tissue samples like proventriculus, intestine, spleen were collected and stored at –80°C for further analysis.

2.2 Extraction of RNA and cDNA synthesis

The tissue samples were homogenised and centrifuged at 5000 rpm for 5 minutes. RNA was extracted from the supernatant using the conventional method with RNAisoplus® reagent as described by Green and Sambrook [22]. The extracted RNA was converted into cDNA using the iScript cDNA synthesis kit (Bio-Rad, Cat # 1708891) following the manufacturer's protocol.

2.3. Polymerase chain reaction

Polymerase chain reaction was carried to identify *Orthoavulavirus javaense* (OAVJ) and Infectious Bursal Disease Virus (IBDV) using in-house designed primers. For OAVJ, primer pairs NDV-F 5'- AGG-AAG-GAG-ACA-GAA-ACG-CT 3' and NDV- R 5'- CTC-TCG-TGC-TGT-ATT-ATT-AAA-C 3' targeting a 258 bp region were used. For IBDV, primer pairs IBDV- F 5'- GCC-CAG-AGT-CTA-CAC-CAT-AA-3' and IBDV- R 5' TAG-GCT-CCC-ACT-TGC-TGA-C-3' were. The PCR reaction (10 µl total volume) consisted of 5 µl *Taq* DNA Polymerase 2x Master Mix RED (Ampliqon®, Denmark, Cat # 180303), 1 µl each of 10 pmol forward and reverse primers, 1 µl template cDNA and 2 µl of nuclease free water. Thermal cycling was carried out on a C1000, BioRad thermal cycler with initial denaturation at 94 °C for 4 minutes, followed by 35 cycles of 94 °C for 30 s, annealing at 53 °C for 30 s, extension at 72 °C at 30 s and a final extension at 72 °C for 7 minutes. PCR amplicons (5 µl) were electrophoresed on 1.5% agarose gel at 100 volts for 30 minutes using a Gel Doc™ (Bio-Rad, USA).

2.4. Virus isolation in embryonated chicken eggs and confirmation by PCR

Tissue homogenates were centrifuged and filtered through 0.22 µm syringe filter. Antibiotic solution at 10 µl/ml (Gibco, Thermofisher Scientific, USA, Cat # 15240096) was added and incubated at 37 ℃ for 1 hour. Then 0.2ml of the antibiotic-treated filtrate was inoculated into the allantoic cavity of 9 to 11 day old embryonated chicken eggs and incubated at 37 °C with relative humidity of 60% for 2-7 days. The allantoic fluid was harvested and the embryo was examined for characteristic lesions. Presence of OAVJ in harvested fluid was further confirmed by PCR. The isolate was designated as VMC/MVC/CHN/2025.

2.5 Sequencing of amplicons and phylogenetic analysis

The PCR product was gel purified, and sequenced by Sanger sequencing by outsourcing. The nucleotide sequences obtained were aligned using the BioEdit version 7.2 and compared with 48 sequences representing all 21 genotypes of OAVJ from the NCBI GenBank using ClustalW multiple sequence alignment. Phylogenetic analysis was performed using the maximum likelihood method with 1000 bootstrap replicates in MEGA XII (version 12.0.15) software.

3.Results and Discussion

The reported clinicals signs and post mortem lesions, such as haemorrhages in the proventriculus and caecal tonsils, were indicative of Newcastle disease and consistent with earlier reports [19, 13]. To rule out IBD co-infection in chicks, PCR targeting the *VP2* gene of IBDV was performed and found negative for IBDV. PCR specific for the *F* gene of OAVJ showed a distinct 258 bp band confirming the presence of *Orthoavulavirus javaense*. (Fig 1). The *F* gene is crucial role for NDV virulence and commonly used for pathotyping and genotyping [10].

Virus isolation was further carried out by inoculating PCR positive samples into 9 -11 day old embryonated chicken eggs through allantoic route. The embryos were died within 48 hours post-inoculation. The allantoic fluid was harvested from inoculated embryos and the embryos showed diffuse haemorrhages over the body surface (Fig 2). The embryonic lesions observed were similar to earlier findings [19, 28, 29].

The F gene fragment sequenced from the OAVJ isolate (VMC/MVC/CHN/2025) showed 99.08% homology with other OAVJ strains reported from India. Phylogenetic analysis was carried out with 48 partial *F* gene sequences representing 21 OAVJ genotypes from NCBI GenBank. The phylogram clustered the study isolate ((VMC/MVC/CHN/2025) with genotype XIII strains from India, Iran, and Pakistan (Fig 3). Genotype XIII is the predominant circulating strain in India [4, 23-27]. Previous studies have reported that genotype II vaccines currently in use often fail to protect against genotype XIII [23, 27]. The outbreak in chicks vaccinated with the LaSota (genotype II) vaccine, combined with the higher mortality caused by genotype XIII in younger flocks with fewer vaccinations, underscores the need for genotype-matched vaccines to control future ND outbreaks.

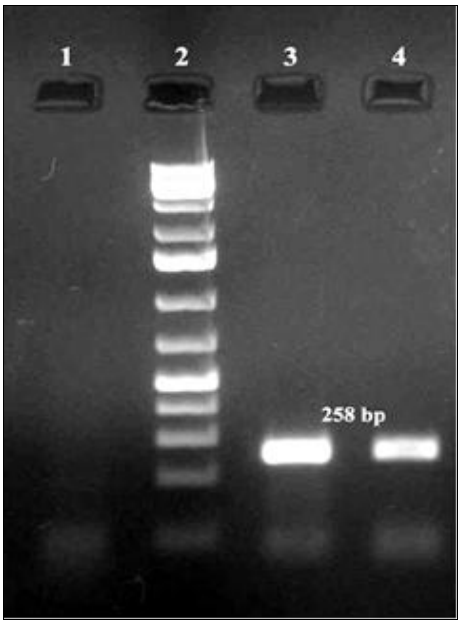

Fig 1: Agarose gel electrophoresis of F gene PCR amplicons showing 258 bp specific products in samples, Lane 1: Negative control; Lane 2: 1 kb plus ladder; Lane 3: Sample 1; Lane 4: sample 2

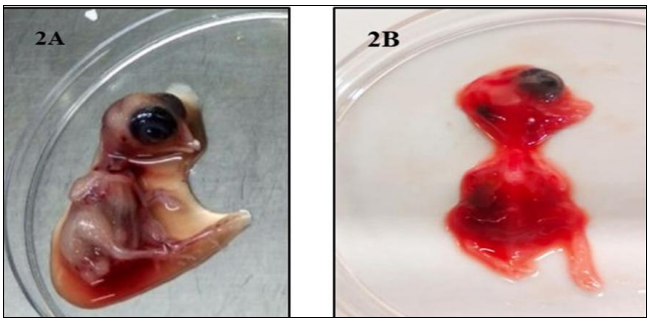

Fig 2: Embryonic lesions observed in chicken embryos inoculated with *Orthoavulavirus javaense* (OAVJ) samples those were found positive in PCR showing diffuse haemorrhages over the body surface 2A -Control; 2B – OAVJ inoculated

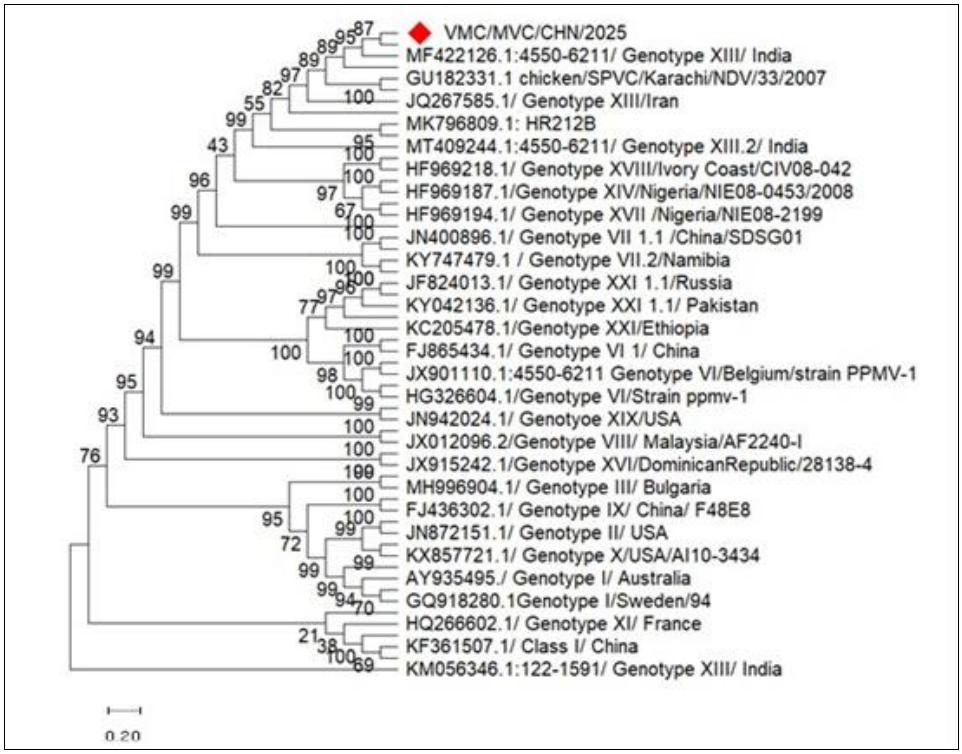

Fig 3: Phylogram based on partial F gene sequences using Maximum likelihood method with 1000 boot strap replicates clustered study sequence with genotype XIII

4. Conclusion

The present study confirmed an outbreak of Newcastle disease in desi chickens by PCR targeting the F gene, virus isolation in embryonated chicken eggs and sequencing. Phylogenetic analysis of partial *F* gene identified the isolate as Genotype XIII which is a predominant genotype circulating in India. This finding emphasizes the continuous threat posed by virulent strains in indigenous poultry. Effective control of Newcastle disease outbreaks requires regular disease surveillance, implementation of vaccination strategies using genotype-matched vaccines, and proper flock management.

Conflict of Interest

Not available

Financial Support

Not available

Reference

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