EPIDEMIOLOGY AND EMERGENCE OF GENOTYPE XIII.2.2 OF CLASS II NEWCASTLE DISEASE VIRUS FROM VACCINATED FLOCKS IN KERALA

Abstract

Aims: To investigate the epidemiology and phylogenetic characteristics of Newcastle disease virus (NDV) outbreaks in poultry flocks in Kerala, India, during 2023-2024, with an emphasis on the emergence of new virulent genotypes and their implications for current vaccination strategies.

Study Design: This study was a field-based epidemiological and phylogenetic analysis of NDV in poultry flocks across various regions of Kerala.

Place and Duration of Study: The study was conducted across multiple poultry farms in Kerala from January 2023 to May 2024.

Methodology: Forty poultry flocks were surveyed for NDV infection. RT-PCR was employed to target the fusion protein gene, revealing the prevalence of NDV. Epidemiological data, including seasonal patterns of NDV occurrence, were collected. Phylogenetic analysis of the isolated NDV strains was performed to compare them with known vaccine strains.

Results: NDV was detected in 12.5% of the sampled poultry flocks, predominantly during the dry season from December to May. Phylogenetic analysis indicated that the prevalent genotype XIII.2.2, identified in the samples, differed significantly from the existing vaccine strains and emerged predominantly from vaccinated flocks.

Conclusion: The emergence of genotype XIII.2.2 in vaccinated flocks suggests that current vaccines may be insufficient for controlling NDV outbreaks in Kerala. There is a critical need for updated vaccine strains and ongoing epidemiological surveillance to enhance the region's NDV prevention and control measures.

Keywords: Newcastle disease, F gene, RT PCR, Kerala, genotype, Vaccination

INTRODUCTION

Newcastle disease, known as Ranikhet disease in India, is an acute viral disease affecting more than 700 host species, depending on age and sex, causing substantial economic loss due to its severe mortality and morbidity rate [1.2]. Newcastle disease virus (NDV), classified as Avian orthoavulavirus 1 (AOAV-1) and formerly known as Avian avulavirus 1 (AAvV-1) or Avian paramyxovirus 1 (APMV-1), belongs to the genus Orthoavulavirus, subfamily Avulavirinae, family Paramyxoviridae, and order Mononegavirales [3,4,5,6]. NDV

isolates are categorised into two classes based on *F gene* sequencing: Class 1, including genotype 1, and Class 2, encompassing 21 genotypes with a 10 percent nucleic acid variation. Class 1 viruses from waterfowl and shorebirds were avirulent in chickens, while Class 2 viruses, found in various birds, included both avirulent vaccine strains and virulent strains [7]. The Newcastle disease virus, pleomorphic and enveloped, measured 200-300 nm in diameter. Its envelope, derived from the host cell's plasma membrane, featured outward-facing spicules (HN glycoprotein and F protein) with an inner matrix protein. The viral genome, non-segmented RNA with negative polarity, ranged from 15,186 to 15,198 nucleotides, encoding six proteins, each from a single gene [8,9]. The fusion protein is one of the significant factors determining viral pathogenicity [10].

A new subtype of virulent genotype XIII has been identified as the cause of severe outbreaks in vaccinated commercial broiler farms in Tamil Nadu and Gujarat [11,12]. According to recent reports, there is a significant antigenic and phylogenetic difference between vaccines and circulating virulent NDV strains, which promoted the evolution of virulent NDV [13]. To track NDV evolution and genetic diversity, characterizing circulating field strains through sequencing and phylogenetic analysis was considered the preferred method [14]. In this study, we carried out the epidemiological analysis, molecular identification, and phylogenetic analysis of NDV from forty poultry flocks in Kerala that showed clinical signs. Moreover, genotype XIII 2.2 was recorded in vaccinated flocks in Kerala.

MATERIALS AND METHODS

SAMPLE COLLECTION AND EPIDEMIOLOGICAL ANALYSIS

The samples were collected from 40 suspected flocks showing signs of Newcastle disease virus, including sudden death, respiratory distress, greenish diarrhoea, conjunctivitis, ocular discharge and neurological signs of torticollis, paralysis resulting in 80 percent mortality and 86 percent morbidity in flocks from different districts in Kerala including Wayanad, Kozhikode, Kannur, Thrissur, Malappuram, Palakkad and Pathanamthitta from a period of 2023 – 2024. Nasal and oropharyngeal swabs were collected from live birds, and tissue samples of lung, trachea, intestine, cecal tonsil, proventriculus, spleen, and liver from post-mortem along with brain from birds exhibiting nervous signs were also collected. The collected samples were preserved at ¯80°C until further processing.

MOLECULAR IDENTIFICATION OF NDV BY REVERSE TRANSCRIPTION POLYMERASE CHAIN REACTION (RT PCR)

The viral RNA from collected samples was extracted by Trizol method (Sigma-Aldrich, St. Louis, MO, USA) followed by reverse transcription of total RNA into cDNA using Verso cDNA synthesis kit (ThermoScientific, USA) according to manufacturer instructions. The F gene amplification was done by RT PCR using the forward primer 5'ACGGGTAGAAGATTCTGGATCC - 3' and reverse primer 5'CCARGTAGGTGGCACGCATATT -3'[15].

SEQUENCING AND ANALYSIS OF NUCLEOTIDE

The amplified 900bp product of RT PCR was sequenced at genes per kakkanad, Kochi, Kerala, and India by extraction and purification.

PHYLOGENETIC ANALYSIS OF THE F GENE FROM FIELD ISOLATES OF NDV

Two field isolates amplified partial F gene nucleotide sequences underwent phylogenetic analysis by the most recent NDV classification system [4]. Using MEGA11 software, a phylogenetic analysis of the sequences acquired in this work was performed [16]. Sequences of the F gene of NDV from India and other nations were retrieved from GenBank (www.ncbi.nlm.nih.gov/genbank/) for phylogenetic analysis. The downloaded sequences were aligned using the Clustal W software of MEGA11, and then they were trimmed to comply with the lengths of the sequences found in the current study. The neighbor-joining method and General Time Reversible with Gamma distribution model were used to infer evolutionary history. It had been presumed that the bootstrap consensus tree, derived from 1000 replicates, represented the sequences' evolutionary history. The determination of evolutionary distances was done using the Kimura 2-parameter method [17] for the *F gene* of NDV. Determination of evolutionary distances was done using the Tamura 3-parameter method [17] for the *F gene* of NDV.

RESULTS

The molecular detection of NDV of collected samples from 40 poultry flocks revealed five positive isolates giving a positivity rate of 12.5% in Kerala during 2023 – 2024 by RT PCR targeting *F gene* (fig. 1). Among the positive flocks, no age group specificity was detected, indicating the occurrence of Newcastle disease virus (NDV) across all ages of birds. Out of the positive flocks, it was observed that broiler birds were more infected with NDV than layers. However, all positive flocks and most suspected samples were obtained from December to May, corresponding to the dry season (fig 8). Of the flocks that tested positive for NDV, 57

percent had been vaccinated with the live LaSota vaccine, while 29 percent had received both the LaSota and R2B vaccines. Additionally, 14 percent of the positive flocks had not been vaccinated with either LaSota or R2B, indicating vaccination status is insignificant in current field NDV outbreaks in Kerala (fig 9).

The affected birds primarily exhibited symptoms of respiratory distress and gastrointestinal disorders. Neurological signs such as torticollis, ataxia, and tremors were observed infrequently. In the past, more hemorrhages were consistently observed across various internal organs, with notable occurrences in the trachea, cecal tonsils, proventricular papillae, and petechiae in the spleen. The lungs frequently showed signs of congestion. Segmental congestion was evident in the duodenal and ileal mucosa, with ulcers in the cecum. Intestinal contents appeared greenish to white. In one flock infected with NDV, hemorrhagic ovarian follicles, flaccid ovarian tissues, and egg yolk peritonitis were identified. Additionally, the liver was congested and friable, and the gall bladder was distended in all examined cases (fig 2 – 7). All flocks exhibited a high morbidity rate. Mortality rate varied from 18 to 61per cent and morbidity from 48 to 87 percent indicating the depth of economic loss due to NDV in field conditions (fig 10).

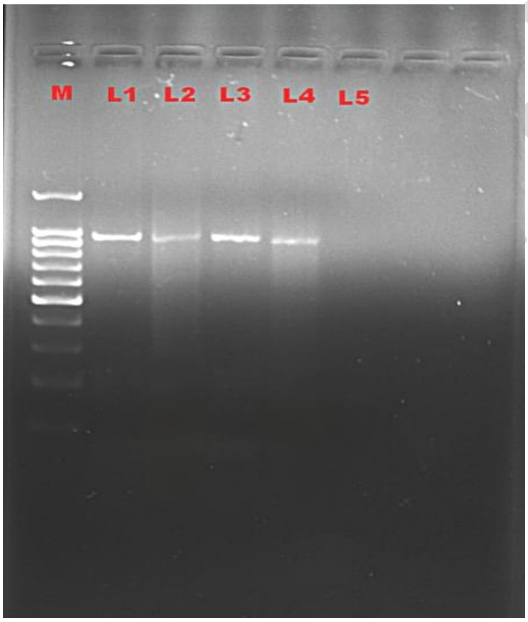

Fig 1. **Agarose gel electrophoresis of *F gene NDV***
M : Marker (100 bp)
Lanes 1 to 4 : Positive Samples (900bp)
Lane 5 : Negative control

Fig 2: Conjunctivitis

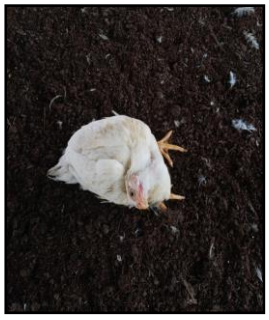

Fig 3: Bird showing

Fig 4: Greenish diarrhea and pasty vent

Fig 5: Haemorrhagic spleen

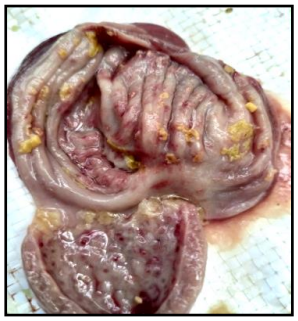

Fig 6: Intestinal and proventricular papillae hemorrhage

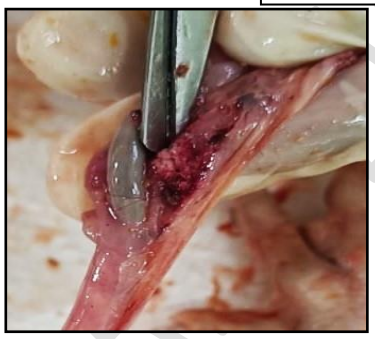

Fig 7: Cecal tonsil

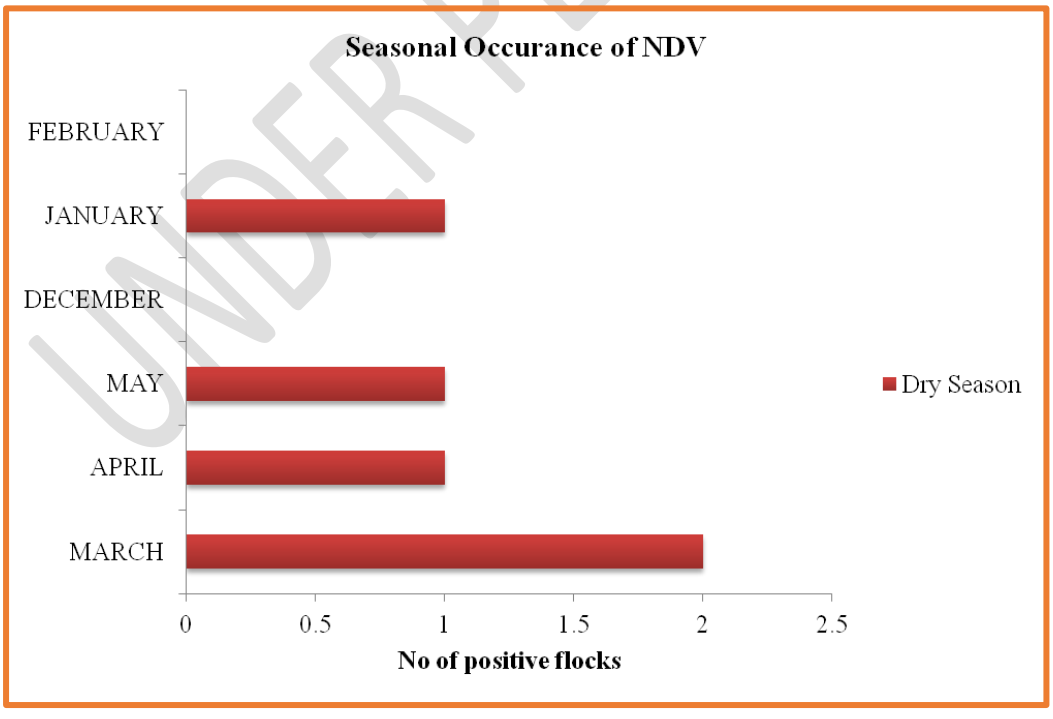

Fig 8: **Seasonal distribution of flocks positive for NDV**

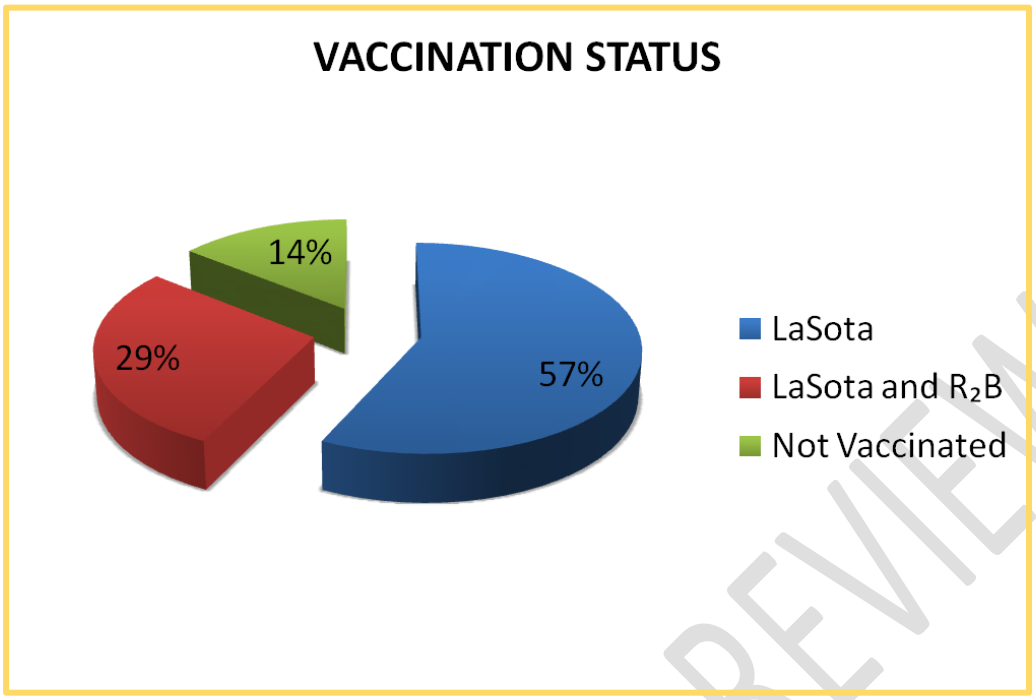

Fig 9: **Vaccination status in flocks positive for NDV**

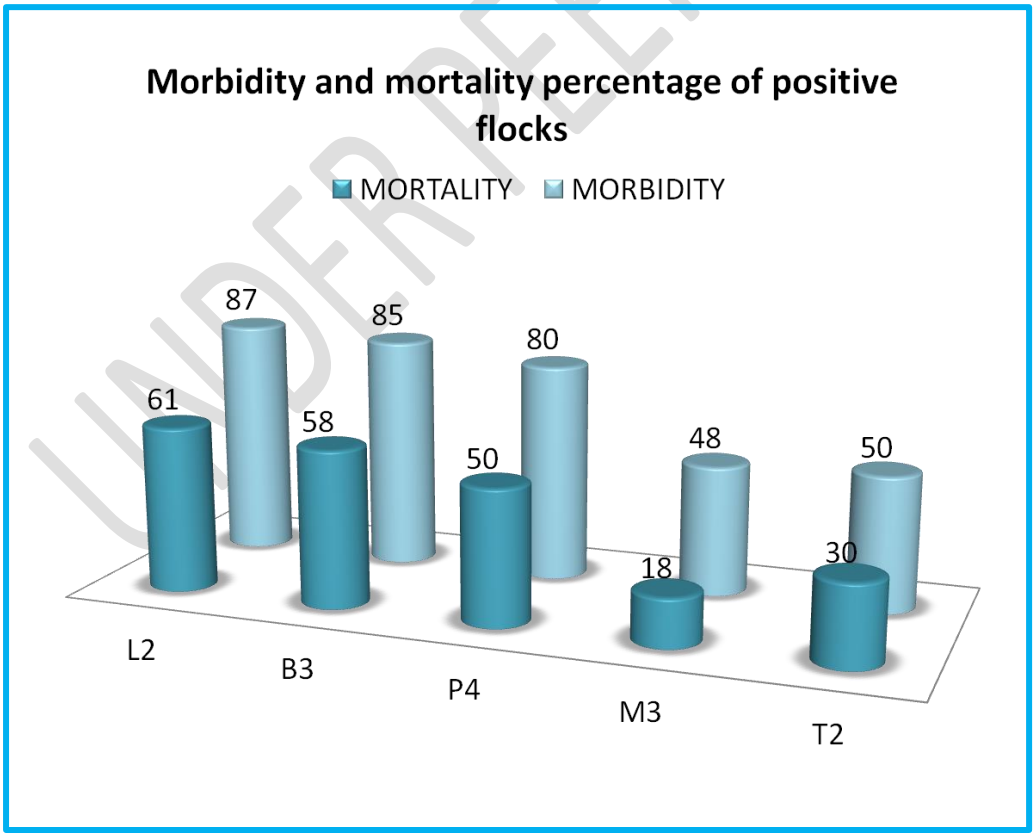

Fig 10: **Morbidity and mortality rate in flocks positive for NDV**

SEQUENCING OF PCR PRODUCTS FOR CONFIRMATION OF NDV

Chromatogram analysis was performed to ensure data accuracy of obtained sequences, and only high-quality sequences were included in the subsequent analysis. BLAST analysis confirmed the identity of NDV. The sequences of the two NDV isolates were deposited in GenBank, and accession numbers were obtained as PP936174 for L2/MIB/PKD/23 and PP936175 for B3/MIB/PKD/23. The isolate PP936174 was vaccinated with LaSota and R2B, whereas PP936175 was vaccinated with LaSota only.

ANALYSIS OF NUCLEOTIDE SEQUENCES

BLAST analysis of the 900 bp partial *F gene* of NDV revealed 99.17 percent to 99.15 percent nucleotide similarity with the Indian isolate (OK149201), 93.28 percent to 93.05 percent similarity with the Pakistani isolate (MH392223), 92.76 percent to 92.67 percent similarity with the Iranian isolate (JQ267585), and 92.65 percent to 92.55 percent similarity with the Japanese isolate (LC650538).

PHYLOGENETIC ANALYSIS OF GENE SEQUENCES

A phylogenetic tree was constructed based on the partial *F gene* 900 bp of the NDV sequences of the present isolate and the sequences retrieved from the GenBank database representing isolates from India, Pakistan, Iran, Japan, China, South Africa, Korea, and Indonesia. In phylogenetic analysis of the *F gene* sequence, L2/MIB/PKD/23 (PP936174) and B3/MIB/PKD/23 (PP936175) showed a close relationship with the Kerala isolate (OP086232), Indian isolates (MT178234, OK149201, KM056349, KT734767) which were coming under genotype XIII.2.2 (fig 11).

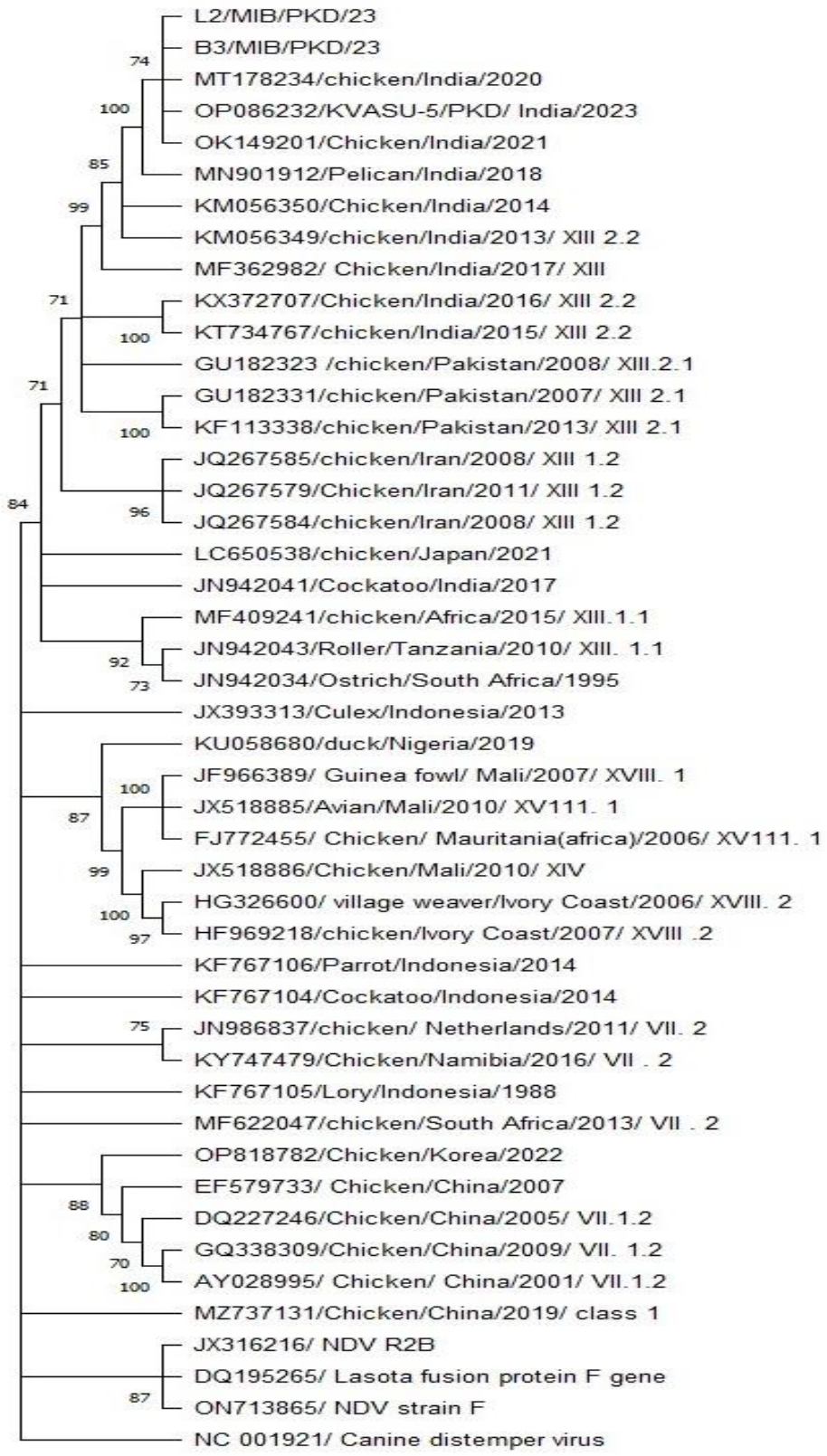

Fig 11: Phylogenetic analysis of *F gene* of NDV isolates by maximum likelihood method

Evolutionary analysis by Maximum Likelihood method

The evolutionary history was inferred by using the Maximum Likelihood method and Tamura 3-parameter model [1]. The tree with the highest log likelihood (-7561.35) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Tamura 3 parameter model, and then selecting the topology with superior log likelihood value. A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories (+*G*, parameter = 1.0589)). This analysis involved 46 nucleotide sequences. There were a total of 819 positions in the final dataset. Evolutionary analyses were conducted in MEGA11 [2]

DISCUSSION

Newcastle disease (ND) threatens global poultry production substantially due to its high infectivity and significant economic repercussions. Despite implementing vaccination programs utilizing lentogenic and mesogenic strains of the Newcastle disease virus (NDV), recent outbreaks involving emergent virulent genotypes have been reported in India. Studies have documented the occurrence of highly virulent ND outbreaks even among vaccinated flocks in northeastern states of India [14] Previous studies by [18] have demonstrated that virulent NDV isolates could be effectively identified through reverse transcription polymerase chain reaction (RT-PCR) targeting the *F gene* encoding the fusion protein cleavage site sequence of tissues and feces from infected birds followed by nucleotide sequencing. In the present study, the molecular detection of the virulent Newcastle disease virus was carried out by targeting the *F gene* by RT PCR [19]. The rationale for employing *F gene* primers, as elucidated [20], is based on their specificity for identifying mesogenic or velogenic strains of NDV. Unlike *M gene* primers, which predominantly detect lentogenic vaccine strains, *F gene* primers provide a more accurate assessment of the prevalence of virulent NDV strains.

The incidence of Newcastle disease virus (NDV) in poultry flocks in Kerala during 2023-2024 was determined to be 12.5 percent based on RT-PCR targeting the *F gene* from samples collected from suspected flocks. This finding is consistent with the study [20], which reported a 14.5 percent prevalence rate of NDV among 893 samples collected from diseased flocks in Kerala. Similarly, [21] identified an 8.9 percent positivity rate for NDV in commercial and backyard poultry in Haryana through *F gene*-targeted screening. [22] reported an overall prevalence of 11.7 percent for ND in chickens in Odisha, with 8.1 percent attributed to virulent strains. In contrast, [23] found a 23.9 percent prevalence of NDV in Assam. The observed variations in NDV prevalence rates across different regions could be attributed to several factors, including new genotypes, mutations arising from vaccine strains, inadequate

vaccination coverage, and reduced bird immunity. Studies [24,25,26] have highlighted these factors as critical contributors to the dynamic epidemiology of NDV.

The flocks that tested positive for NDV were distributed across various age groups, indicating that the disease occurs in birds regardless of age. This finding corroborates the statement [27] that NDV affects domestic poultry regardless of age and sex.

Among the positively tested flocks, both broiler and layer flocks were included in a ratio of 3:2. This finding aligns with the observations of [20,28], who reported that the risk of Newcastle Disease (ND) in broilers is nearly five times higher than in layers or backyard birds. This increased risk is attributed to the challenges of disease control in small, rural, extensive poultry flocks in developing countries despite these birds being reared under intensive systems [29].

Despite the collection period spanning from March 2023 to April 2024, all positive cases were observed during the dry season (December to May). This finding is in alignment with studies by [30] in Ethiopia and [31] in Nigeria, who reported elevated Newcastle Disease (ND) prevalence (86.6%) during the dry season. However, this contrasts with [32], who documented a higher incidence of ND during the winter months in Tamil Nadu. This discrepancy might be attributed to geographical and climatic variations. Additionally, [33] found significantly higher ND mortality rates during the rainy season compared to winter and autumn in Bangladesh, attributed to increased humidity, heavy rainfall, and compromised biosecurity during the rainy season. The observed higher prevalence of ND outbreaks during the dry season might be related to increased wind speed, dust, and heat stress, as noted [31].

Most positive flocks were vaccinated with LaSota (57%), compared to 14% in non-vaccinated flocks. Flocks that received a vaccination regimen of R2B, followed by LaSota, accounted for only 29 percent. Despite these variations, the vaccination status did not significantly correlate with the incidence of Newcastle Disease (ND) in the positive cases. This finding is consistent with [20], who reported that in Kerala, the vaccination strategy of administering the lentogenic strain at one week of age followed by the mesogenic R2B strain at two months did not significantly impact ND prevalence. Additionally, [13] highlights that the high genetic diversity among circulating Newcastle Disease Virus (NDV) strains and existing vaccine strains could result in persistent viral shedding even in vaccinated birds, potentially explaining the lack of significant difference in infection risk based on vaccination status.

The Newcastle Disease Virus (NDV) affected birds primarily exhibited symptoms of respiratory distress, gastrointestinal disorders including greenish diarrhoea, and neurological signs. The post-mortem examination revealed haemorrhagic lesions predominantly in the

trachea, spleen, proventriculus, caecal tonsils, ovarian follicles, and intestines. These findings are consistent with previously documented symptoms of greenish-yellow diarrhoea, head and wattle oedema, depression, neurological signs such as torticollis and paralysis, and respiratory distress, as [34,35,36].

In flocks affected by Newcastle Disease Virus (NDV), the morbidity rate exceeded the mortality rate, ranging from 48 percent to 87 percent, and mortality from 18 percent to 61per percent. These findings align with previous reports, including a maximum mortality rate of 50 percent with an average of 21.21 percent in Gujarat [37], an average mortality of 79.50 percent among layer flocks in Uttar Pradesh [38], and an up to 75 percent mortality in commercial broilers in Egypt [39]. The impact of morbidity in ND-infected birds often surpasses mortality, with mesogenic strains of NDV causing mortality rates up to 50 percent. In contrast, velogenic strains can cause up to 100 percent mortality (Sharma *et al.*, 2023). The variability in mortality and morbidity rates can be attributed to factors such as the NDV strain, its pathogenicity, tropism, host species, age, immune status, concurrent diseases, and environmental conditions [40].

Sequencing of two representative PCR products followed by BLAST analysis of obtained nucleotide sequences revealed 99.17 to 99.15 identity towards Indian isolate of NDV with accession number OK149201 obtained from Sikkim.

Phylogenetic analysis of the *F gene* sequences from two isolates revealed high nucleotide sequence homology with the Newcastle Disease Virus (NDV).

The identified genotype XIII.2.2 from these isolates has previously been linked to severe outbreaks in vaccinated broiler farms in Tamil Nadu, as reported [11]. Genotype XIII (sub-genotype XIII.2.2.) has been recognized as a predominant strain causing outbreaks in vaccinated flocks in India, as documented [12,42]. This observation contrasts with the findings of [23], who reported a 23.89 percent incidence of genotype XIII in unvaccinated backyard poultry. Subgenotype XIIIc has been identified in northeastern India through whole-genome sequencing and phylogenetic analysis [43]. All the NDV viruses belonging to genotype XIII were found to be virulent and mostly isolated from chicken [14]. This is the first circulating genotype XIII.2.2 report among vaccinated Kerala flocks. This suggests significant genetic divergence between emerging and existing vaccine strains, highlighting the need to reassess current vaccination protocols.

This approach underscores the critical role of the *F gene* in the molecular detection of NDV. It highlights the necessity of precise diagnostic techniques in the surveillance and management of Newcastle disease in poultry populations. This study provides a crucial insight into the

incidence of NDV in Kerala, highlighting the ongoing challenge posed by this pathogen despite vaccination efforts. Continuous surveillance and molecular characterization of NDV are imperative for understanding its epidemiology and developing effective control strategies to mitigate the impact of Newcastle disease on poultry populations.

CONCLUSION

This study reveals the emergence of the virulent genotype XIII.2.2 of Newcastle Disease Virus (NDV) in vaccinated poultry flocks in Kerala during 2023-2024, emphasizing the need for continuous surveillance and updated vaccination strategies. The findings underscore the challenge posed by NDV evolution, necessitating the development of more effective vaccines and re-evaluation of current protocols to better control this economically significant disease in the region.

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