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Epidemiology and Molecular Characterization of Genotype XIII.2.2 of Class II Newcastle Disease Virus from Vaccinated Flocks in Kerala, India

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Authors' contributions

This work was carried out in collaboration among all authors. Author GG designed the study, performed the statistical analysis, wrote the protocol, and wrote the first draft of the manuscript. Author DPM managed the analyses of the study. Author AJ, KCB corrected the draft of the manuscript. All authors read and approved the final manuscript.

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ABSTRACT

Aims: To investigate the epidemiology and phylogenetic characteristics of Newcastle Disease Virus (NDV) outbreaks in poultry flocks in Kerala, India, during 2023-2024, focusing on the emergence of new virulent genotypes. The emergence of these genotypes can increase disease severity in poultry, reduce the effectiveness of existing vaccines, and necessitate the development of updated vaccination strategies to control outbreaks effectively.
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 in Kerala from January f2023 to May 2024 in both layer and broiler farms.
Methodology: Forty samples from 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 5 out of 40 of the sampled poultry flocks (12.5%), 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 indicates that existing vaccines may not effectively control NDV outbreaks in Kerala. Future research should aim to characterize the genetic diversity of NDV strains and assess the efficacy of novel vaccine candidates. Practical recommendations include updating vaccine formulations to address emerging genotypes, strengthening surveillance systems, and reinforcing biosecurity measures to enhance NDV prevention and control in the region.

Keywords: Newcastle disease; F gene; RT PCR; Kerala; genotype; epidemiology.

1. INTRODUCTION

Newcastle disease (ND), also known as Ranikhet disease in India, is a critical viral infection affecting over 700 avian species and causing substantial economic losses due to its high mortality and morbidity rates [1,2]. The Newcastle disease virus (NDV), classified as Avian orthoavulavirus 1 (AOAV-1) and previously known as Avian avulavirus 1 (AAvV-1) or Avian paramyxovirus 1 (APMV-1), belongs to the genus Orthoavulavirus within the subfamily Avulavirinae, family Paramyxoviridae, and order Mononegavirales [3-6]. NDV isolates are divided into two primary classes based on F gene sequencing: Class 1, which includes genotype 1, and Class 2, encompassing 21 genotypes with a 10 percent variation in nucleic acid sequences. While Class 1 viruses are avirulent in chickens, Class 2 viruses, which include both avirulent vaccine strains and virulent strains, are found in various avian species [7]. The virus is pleomorphic and enveloped, with a diameter of 200-300 nm, and its genome is a non-segmented, negative-sense RNA that encodes six proteins [8,9]. The fusion protein is a major determinant of viral pathogenicity [10].

Recent studies have identified the emergence of genotype XIII.2.2, a new virulent NDV subtype, which has led to severe outbreaks in vaccinated commercial broiler farms in Tamil Nadu and Gujarat [11,12]. This development highlights a critical research gap: the current vaccines appear inadequate against evolving virulent NDV strains, creating a pressing need for updated vaccine formulations. Although previous research has characterized several NDV genotypes, there remains a lack of comprehensive understanding of the genetic divergence between vaccine strains and circulating virulent strains, particularly in regions such as Kerala.

This study aims to address these gaps by conducting an extensive epidemiological, molecular, and phylogenetic analysis of NDV in poultry flocks in Kerala that exhibit clinical signs. Specific objectives include (1) characterizing the genetic diversity of circulating NDV strains, (2) evaluating the phylogenetic relationships between these strains and existing vaccine strains, and (3) assessing the implications of genotype XIII.2.2 for vaccine efficacy and disease control strategies.

2. MATERIALS AND METHODS

2.1 Sample Collection and Epidemiological Data

Samples were collected from 40 poultry flocks across various districts in Kerala, including Wayanad, Kozhikode, Kannur, Thrissur, Malappuram, Palakkad, and Pathanamthitta, during 2023-2024. The flocks exhibited clinical signs consistent with Newcastle diseases, such as sudden death, respiratory distress, greenish diarrhea, conjunctivitis, ocular discharge, and neurological symptoms, including torticollis and paralysis [13,14]. Nasal and oropharyngeal swabs were obtained from live birds, while post-mortem samples included lung, trachea, intestine, cecal tonsil, proventriculus, spleen, liver, and brain tissues from birds showing neurological signs. All collected samples were preserved at -80°C until further analysis.

2.2 Molecular Identification of NDV

Viral RNA was extracted from the samples using the Trizol method (Sigma-Aldrich, St. Louis, MO, USA). Reverse transcription of the total RNA into complementary DNA (cDNA) was performed using the Verso cDNA synthesis kit (ThermoScientific, USA) as per the manufacturer's protocol. The F gene of NDV was amplified by reverse transcription polymerase chain reaction (RT-PCR) using specific primers (see Table 1).

2.3 Sequencing and Phylogenetic Analysis

The 900 bp RT-PCR product was sequenced by Genes Per Kakkanad, Kochi, Kerala, India. Sequence extraction and purification were performed prior to analysis. Phylogenetic analysis of the partial F gene sequences from field isolates was conducted using MEGA11 software [16]. Sequences from the F gene of NDV, including those from India and other countries, were retrieved from GenBank (www.ncbi.nlm.nih.gov/genbank/). These sequences were aligned using the Clustal W tool in MEGA11. The alignment was trimmed to match the lengths of the sequences in the current study. The neighbor-joining method with the General Time Reversible model and Gamma distribution was employed to infer the evolutionary relationships. Bootstrap consensus trees were constructed from 1000 replicates to estimate the evolutionary history. Evolutionary distances were calculated using the Kimura 2-parameter and Tamura 3-parameter methods [17].

3. RESULTS

The molecular detection of NDV in collected samples from 40 poultry flocks revealed five positive cases, giving a positivity rate of 12.5% in Kerala during 2023 – 2024 by RT PCR targeting the F gene (Fig. 1). Among the positive flocks, no age group specificity was detected, indicating the occurrence of 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 (Table 2). 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 mesogenic Ranikhet disease (R2B strain) 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 (Table 3).

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 (Figs. 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 (Table 4).

Table 1. Primer sequence used for RT-PCR targeting F gene of NDV

Target gene	Primer sequence	Band size (bp)	Reference
F gene	Forward-5'ACGGGTAGAAGATTCTGGATCC - 3' Reverse-5'CCARGTAGGTGGCACGCATATT -3'	900	Rajasekhar et al. [15]

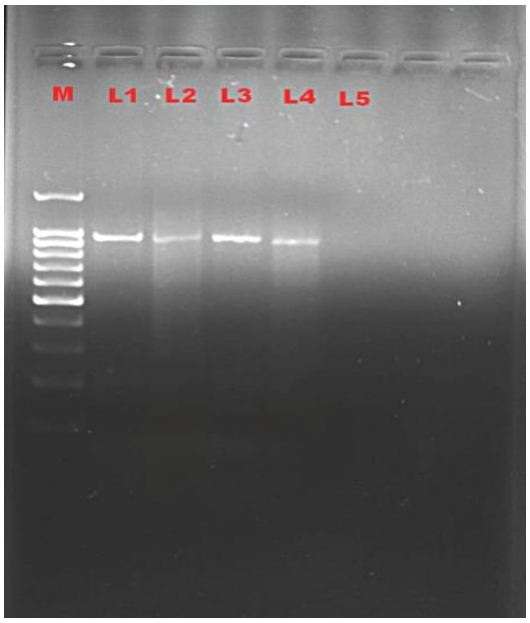

Fig. 1. Agarose gel electrophoresis of F gene NDV
M: DNA ladder (100 bp)
Lane 1 Positive control(900bp)
Lanes 2 to 4; positive samples
Lane 5: Negative control

Fig. 2. Conjunctivitis

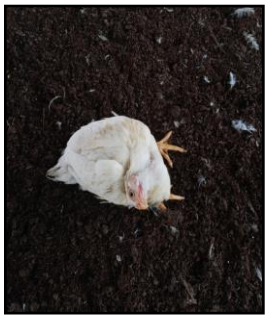

Fig. 3. Torticollis

Fig. 4. Greenish diarrhea and pasty vent

Fig. 5. Haemorrhagic spleen

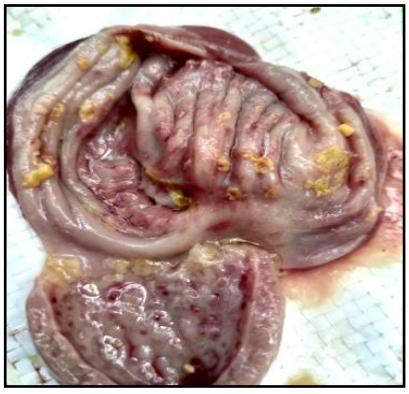

Fig. 6. Intestinal and proventricular papillae hemorrhage

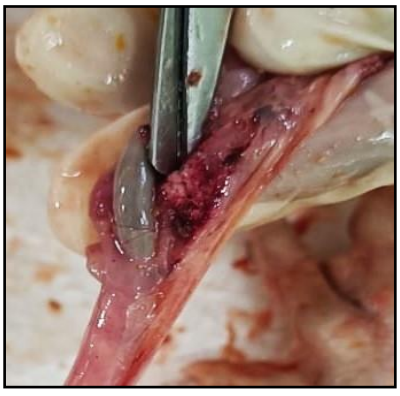

Fig. 7. Cecal tonsil haemorrhage

Table 2. Distribution of NDV in flocks by district, season, and bird type

	Type of bird	Positive no of flock	Positive Sample ID	District	Season
Broiler	Pre starter (1-7 days)	0			
	Starter (8-21 days)	1	B3/MIB/PKD/23	Kozhikode	Dry
	Finisher (>22 days)	1	T2/MIB/PKD/24	Thrissur	Dry
		1	P4/MIB/PKD/23	Pathanamthitta	Dry
Layer	Chick (0 - 8 wks)	1	L2/MIB/PKD/23	Wayanad	Dry
	Grower (9 - 20wks)	1	M3/MIB/PKD/24	Malappuram	Dry
	Layer Phase 1 (21- 45wks)	0			
	Layer phase 2 (46-72 wks)	0			

Table 3. Vaccination coverage in flocks positive for NDV

Vaccination	Percentage (%)
LaSota	3/5(57)
R_2B and lasota	2/5 (29)
Not Vaccinated	1/5(14)

Table 4. Morbidity and mortality rate of flocks positive for ND

Flocks	Flock size	Mortality	Mortality %	morbidity	Morbidity %
1	250	153	61	218	87
2	450	261	58	383	85
3	800	400	50	640	80
4	500	90	18	240	48
5	1000	300	30	500	50

3.1 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 (Fig. 8). The sequences of the 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.

3.2 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) (Table 4).

3.3 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. 9).

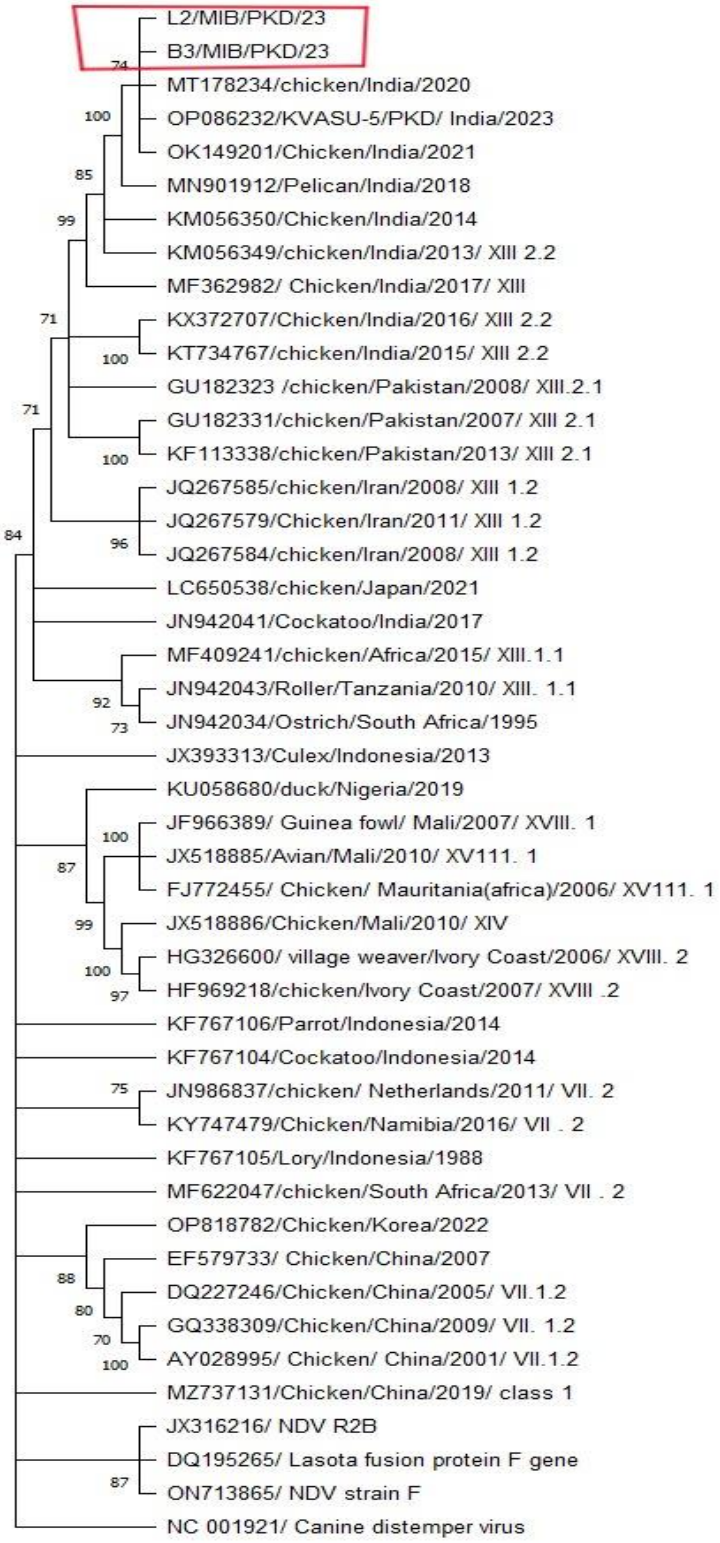

Fig. 8. Phylogenetic analysis of F gene of NDV isolates by maximum likelihood method

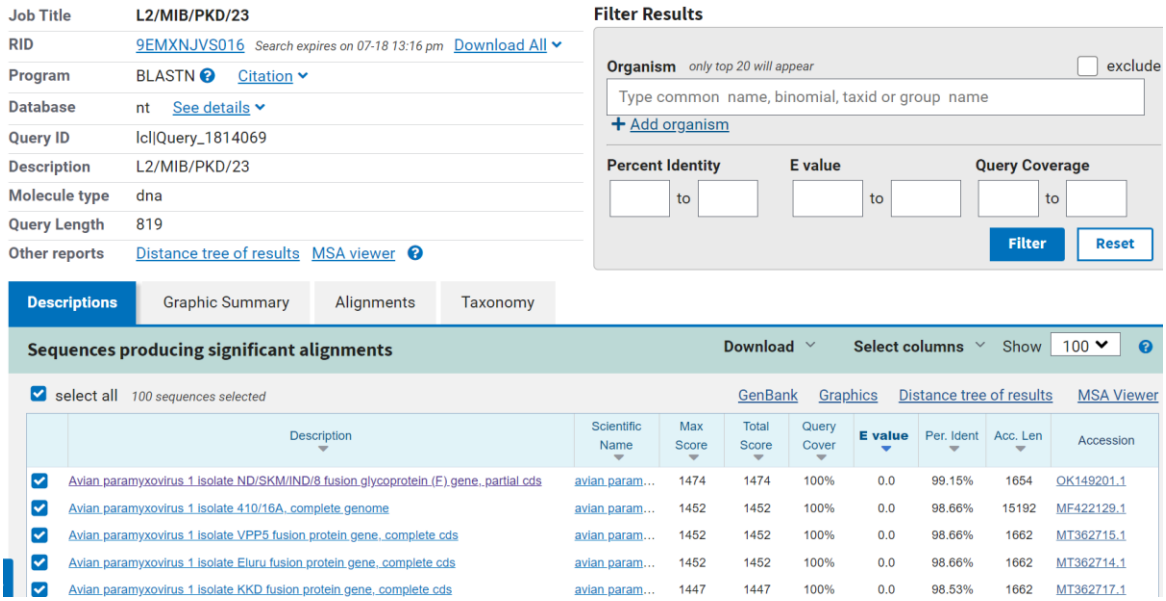

Fig. 9. Blast analysis of NDV isolate

Table 5. Nucleotide similarity of NDV F gene with isolates from India, Pakistan, Iran, and Japan

S. No.	Sequence ID of isolates in the present study	Nucleotide identity with Indian isolate (OK149201)	Nucleotide identity with Pakistan isolate (MH392223)	Nucleotide identity with Iran isolate (JQ267585)	Nucleotide identity with Japan isolate (LC650538)
1.	L2/MIB/PKD/23	99.15	93.28	92.67	92.55
2.	B3/MIB/PKD/23	99.17	93.05	92.76	92.65

3.4 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].

4. 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 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 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 [15,19]. 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 NDV in poultry flocks in Kerala during 2023-2024 was determined to be 12.5% 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% prevalence rate of NDV among the 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% for ND in chickens in Odisha, with 8.1 percent attributed to virulent strains. In contrast, [23] found a 23.9% 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 [24-26] 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, 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 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 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%. Despite these variations, the vaccination status did not significantly correlate with the incidence of 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 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 NDV-affected birds primarily exhibited symptoms of respiratory distress and gastrointestinal disorders, including greenish diarrhea and neurological signs. The post-mortem examination revealed hemorrhagic lesions predominantly in the trachea, spleen, proventriculus, caecal tonsils, ovarian follicles, and intestines. These findings are consistent with previously documented symptoms of greenish-yellow diarrhea, head and wattle edema, depression, neurological signs such as torticollis and paralysis, and respiratory distress, as [34-36].

In flocks affected by NDV, the morbidity rate exceeded the mortality rate, ranging from 48 % to 87%, and mortality from 18% to 61%. These findings align with previous reports, including a maximum mortality rate of 50 % with an average of 21.21 percent in Gujarat [37], an average mortality of 79.5% among layer flocks in Uttar Pradesh [38], and an up to 75% 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%. In contrast, velogenic

strains can cause up to 100 % 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 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 [11,41]. 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 % incidence of genotype XIII in unvaccinated backyard poultry. Subgenotype XIIIc has been identified in northeastern India through whole-genome sequencing and phylogenetic analysis [42]. 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.

The limitations of the study include a small sample size, restricted geographic and seasonal scope, and reliance on RT-PCR which may not capture all genetic variations. Additionally, the study did not directly assess the impact of genotype XIII.2.2 on vaccine efficacy.

This manuscript holds significant value for the scientific community as it highlights the emergence of a new virulent genotype of NDV, genotype XIII.2.2, in vaccinated poultry flocks in Kerala. The findings are particularly noteworthy because they reveal the insufficiency of current vaccines in addressing evolving NDV strains, underscoring the urgent need for developing new, more effective vaccines. By integrating epidemiological, molecular, and phylogenetic analyses, this study offers valuable insights into the challenges of NDV control.

5. CONCLUSION

This study reveals the emergence of the virulent genotype XIII.2.2 of 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.

DISCLAIMER (ARTIFICIAL INTELLIGENCE)

Author(s) hereby declare that NO generative AI technologies such as Large Language Models (ChatGPT, COPILOT, etc) and text-to-image generators have been used during writing or editing of manuscripts.

ETHICAL APPROVAL

Animal Ethic committee approval has been collected and preserved by the author(s).

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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