RESEARCH ARTICLE

CYPRUS
JOURNAL OF MEDICAL SCIENCES

DOI: 10.4274/cjms.2025.2024-146
Cyprus J Med Sci 2025;10(Suppl 1):83-86

Molecular Characterization of Newcastle Disease Virus Circulating in Pakistan

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Abstract

BACKGROUND/AIMS: Newcastle disease (ND) is a highly contagious disease in poultry caused by the ND virus (NDV). In Pakistan, NDV epidemics occur frequently, leading to massive economic losses; however, the genetic diversity of NDV in Pakistan is not well known. This study aims to investigate the genetic variation between the vaccine and circulating strains of NDV among poultry in Pakistan.

MATERIALS AND METHODS: Three vaccine strains [ND-Lasota, ND-infectious bronchitis (IB), ND-clone] were analyzed alongside 67 field samples collected from ND outbreaks. NDV samples were inoculated into 9-10-day-old eggs. Allantoic fluid was harvested, followed by a hemagglutination test. RNA was extracted from positive HA samples. Complementary DNA was prepared using reverse transcriptase-polymerase chain reaction (PCR). The *fusion (F)* gene was amplified through PCR using specific primers, and PCR products, measured in base pairs (bp), were analyzed using gel electrophoresis.

RESULTS: Among 67 field samples, the HA tests were positive in 50 samples, accounting for 75%. On gel, the *F1* gene of field NDV showed variable band size (250 bp, 350 bp, 400 bp), while F2 and F3 had band sizes of 650 bp and 500 bp, respectively. The band sizes F1, F2, and F3 of ND-Lasota and ND-IB were each 100 bp. The band sizes of the *F1, F2,* and *F3* genes of ND-clone were 100 bp, 150 bp, and 150 bp, respectively.

CONCLUSION: Variability among *F1* gene sizes indicates the emergence of mutations or new subgenotypes. Sequencing-based studies are needed to monitor circulating NDV strains, to modify vaccines.

Keywords: Newcastle disease virus, hemagglutination test, RT-PCR, Gel electrophoresis

INTRODUCTION

Newcastle disease virus (NDV), also known as Avian paramyxovirus-1, causes ND when birds are infected with a virulent strain.[1] It is the most lethal among avulaviruses, infecting approximately 236 bird species globally and causing significant economic losses in poultry.[2] Between 2006 and 2009, countries reported 56 to 68 ND outbreaks. NDV consists of single-stranded, negative-sense RNA and six structural proteins. These proteins are the nucleocapsid, matrix, phosphoprotein, fusion (F), hemagglutinin-neuraminidase, and polymerase (L).[3]

In Pakistan, ND caused the death of 45 million broiler chickens during 2011-12, resulting in a loss of 6 billion PKR. In 2012, NDV outbreaks affected peacocks in Lahore and Sindh.[4] Despite strict biosecurity efforts, NDV remains prevalent, transmitted largely via wild birds. Symptoms include shivering, spasms, and paralysis, leading to death from inability to eat or drink. There are four NDV pathotypes: asymptomatic enteric, lentogenic, mesogenic, and velogenic strains. The virus is highly contagious, with an incubation period of 2-15 days, and there is no treatment available.[1] Genetic recombination drives NDV evolution, with chimeric sequences identified in its genome. An intrinsic error rate of RNA L and the lack of proofreading ability during viral replication

To cite this article: Siddique U, Ali I. Molecular characterization of newcastle disease virus circulating in Pakistan. Cyprus J Med Sci. 2025;10(Suppl 1):83-86

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Received: 03.12.2024
Accepted: 08.03.2025
Publication Date: 04.06.2025

are the primary reasons behind the diversity and evolution of NDV.[1] This study investigates genetic variation between NDV vaccine strains (VS) and circulating field strains (FS) in Pakistan, focusing on the *F* gene, which is critical for virulence. The circulating strains belong to genotypes XIIIb and VIIi, while vaccines are from genotype II, showing a 17% evolutionary distance.[4] There is a pressing need for vaccines that are both safe to use and compatible with the genotype of the circulating FS to effectively combat ND.[2]

MATERIALS AND METHODS

This study was conducted in the Molecular Virology Lab at COMSATS University, Islamabad. Three VSs [ND-infectious bronchitis (IB), ND Lasota, and ND clone] commonly utilized in Pakistan poultry vaccination programs were selected for this study. A total of 67 samples were analyzed. Among 67 field samples, 42 were collected from poultry farms located in Lahore, Sargodha, Islamabad, and Rawalpindi, while 25 samples were obtained from the outbreak-affected farms in Lahore and Sargodha. Field samples included the liver, trachea, and lungs. Embryonated eggs, aged nine to ten days, were collected, and candling was performed. Vaccine and field samples were prepared and inoculated in the eggs. After 24 and 48 hours, allantoic fluid was harvested, and the Hemagglutination test was conducted to detect the presence of NDV. Two methodologies were employed for RNA extraction: guanidinium thiocyanate (manual method) and the nucleic acid extraction kit (Tianlong, Xi'an, China). RNA quantification was performed by a Nanophotometer. Complementary DNA (cDNA) was synthesized through reverse transcriptase-polymerase chain reaction (RT-PCR), and the quantity was measured by a Nanophotometer. The *F* gene was amplified through PCR, and the resulting products were analyzed on the agarose gel. Ethical approval for this study was granted by the Ethics Review Board of COMSATS University Islamabad, Pakistan.

Statistical Analysis

No statistical analysis is needed for this study.

RESULTS

Viral Inoculation and Harvesting

All vaccine and field samples were inoculated in 9-10 embryonated eggs. After harvesting allantoic fluid (Figure 1a), the effects on the chicks of the VS and FS were meticulously observed. Notably, chicks exposed to FS exhibited hemorrhaging, resulting in mortality, while those exposed to VS remained healthy with no observable lesions, as depicted in Figure 1b.

Hemagglutination Test

The hemagglutination (HA) test was employed to detect viral presence in the samples. All 3 VS tested positive for HA. Out of 67 field samples, 50 were confirmed positive for the NDV strain, while the remaining 17 samples gave negative results. Viral titer in positive samples was calculated following standard protocols, based on the last hazy well observed before bead formation.

RNA Extraction and Quantification

RNA extraction was conducted on the HA-positive samples utilizing 2 methods: the guanidinium thiocyanate method and a nucleic acid extraction kit. The ND Lasota VS and ND-IB VS along with 20 field samples of NDV were processed using the nucleic acid extraction kit. Conversely, RNA extraction of ND clone and the remaining 30 NDV field samples was performed via the guanidinium thiocyanate method. RNA concentration was quantified using a nanophotometer.

Complementary DNA Preparation and Quantification

cDNA was synthesized using RT-PCR, followed by quantification using a Nanophotometer.

Fusion Gene Amplification

Amplification of the *F* gene was conducted using three sets of primers, as outlined in Table 1.[5] Each set of primers was applied sequentially for both vaccine and FS samples. The resultant PCR products were subjected to gel electrophoresis for band size analysis.

Newcastle Disease Virus Vaccine Samples

Using the F1, F2, and F3 sets of primers, 100 base pairs (bp) bands were successfully observed at different temperatures ranging from 61 °C to 70 °C in both the Lasota and ND-IB samples, as depicted in Figure 2a. In ND Clone, the *F1* and *F2* gene primers yielded 100 bp PCR products, while the *F3* gene primer generated a 150 bp product, as depicted in Figure 2b. These results were consistent across repeated experimental trials.

Figure 1. Harvesting of allantoic fluid and samples effects on the embryo.

Table 1. Primers of fusion (F) gene used[5]

Primer names	Sequence of primers
NDV-F330	5'-AGGAAGGAGACAAAAACGTTTTATAGG-3'
NDV-R700	5'-TCAGCTGAGTTAATGCAGGGGAGG-3'
NDV-F640	5'-CTAACTGAATTAACTACAGTATTCGGG-3'
NDV-R1290	5'-GTCTAATGATAAGACATTGCACGAATG-3'
NDV-F1200	5'-ATGTACAGACCCTCCTGGTATCATATC-3'
NDV-R1740	5'-CTTAAGTCTTATACTTGACAGGTTATC-3'

Newcastle disease forward primer = NDV-F, Newcastle disease reverse primer = NDV-R.

Newcastle Disease Virus Field Samples

In most field samples, band visibility was limited due to low concentration; however, in some instances, bands of varying bp were detected.

Utilizing *F1* gene primers, 350 bp bands were identified in three distinct samples (Figure 3a). PCR product of different sizes of field NDV strains is observed in Figure 3b. Left to right, Lane 1 has a 100 bp ladder. Lane 2 shows F1 fragment of 250 bp at 67.5 °C, lane 3 depicts F1 fragment of 400 bp at 67.5 °C, lane 4 shows F2 fragment of 650 bp at 68.3 °C and lane 5 shows F2 fragment of 650 bp at 68.3 °C (Figure 3b).

Notably, four of these samples were collected from a poultry farm in Islamabad, while three originated from a poultry farm in Sargodha. A single sample exhibited a 400 bp band, as illustrated in Figure 3b.

For the *F2* gene, a 650 bp band was also observed in 2 NDV samples (Figure 3b).

For the *F3* gene, a 500 bp band was detected in 1 NDV sample (Figure 4).

DISCUSSION

In Pakistan, NDV outbreaks occur two-to-three times a year, making it the top fatal disease in poultry. This study examined three ND vaccines (ND Lasota, ND IB, and ND clone) in order to isolate, characterize, and compare the *F* gene of vaccine and FS from various regions. The *F* gene is vital for assessing the virulence of NDV, and significant variability was found, consistent with previous research, indicating a notable genetic distance among NDV strains.[5] ND prevalence peaks in January, February, June, July, and September in both Pakistan and Egypt.[5] Analysis revealed discrepancies in the band sizes in the *F1* gene products when compared to the results of other studies, likely due to the high mutation rates of RNA viruses.[6,7] These results indicated that there is a large genetic distance between the two NDV strains. In Pakistan, new subgenotypes of NDV keep emerging, especially VII subgenotypes, due to the uncontrolled vaccination programs.[8-10]

Current vaccines are insufficient against contemporary strains, highlighting a mismatch between available vaccines and circulating variants.[9,10] Despite the historical success of ND vaccines developed during the 1930s-1970s, they have proven inadequate against novel NDV variants emerging in recent years.[11] Given the historical success of ND vaccines, there is an urgent need for updated formulations that

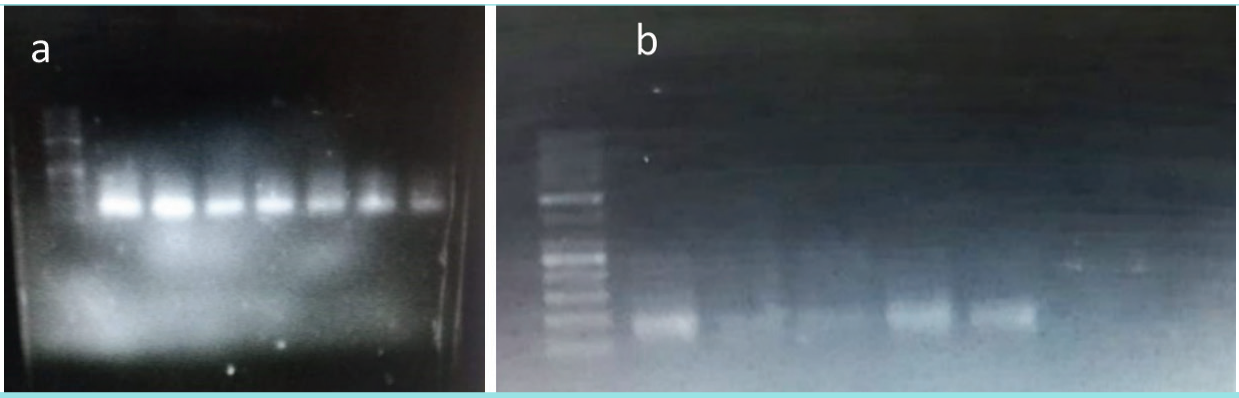

Figure 2. Gradient PCR results showing optimization of the *fusion* gene.
PCR: Polymerase chain reaction.

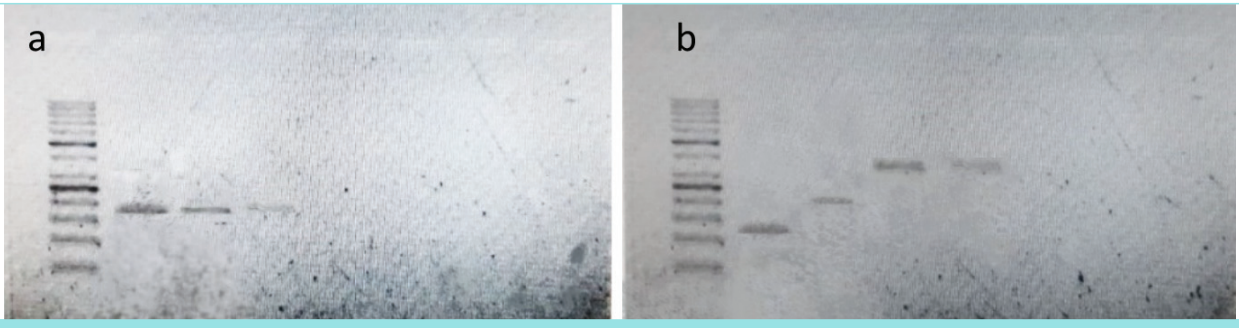

Figure 3. PCR products of different sizes.
PCR: Polymerase chain reaction.

Figure 4. A gradient PCR result for the optimization of the F3 gene.
PCR: Polymerase chain reaction.

align with current strains. Regular surveillance and strain identification are essential for developing more effective vaccines. Improved strategies are crucial to mitigate the economic impact of ND and address the ongoing evolution of the virus, emphasizing the importance of sequencing and phylogenetic analysis in future studies. Reducing genetic diversity between vaccine and FS may aid in the control and eventual elimination of ND.

Study Limitations

Sequencing and phylogenetic analysis could not be performed because of time limitations. Future studies, must be performed to identify NDV FS. According to recent findings, a new vaccine should be formulated immediately to combat the NDV situation.

CONCLUSION

In conclusion, our study revealed large genetic differences between the VS and the FS. Furthermore, variability was observed among F1 gene sizes indicating mutations or evolutions of new subgenotypes. Future studies must be performed regarding the identification of NDV FS. According to which new vaccines should be formulated immediately to combat the NDV situation.

MAIN POINTS

- Large genetic distance observed between the field strain (FS) and vaccine strains (VS).
- VS are lentogenic while FS are velogenic.
- Variability among *F1* gene sizes indicates mutations or the emergence of new subgenotypes.

ETHICS

Ethics Committee Approval: Ethical approval for this study was granted by the Ethics Review Board of COMSATS University Islamabad, Pakistan. (approval number: CUI/Bio/ERB/10-2024/1, date: 01.01.2024).

Informed Consent: Not available.

Footnotes

Authorship Contributions

Surgical and Medical Practices: U.S., Concept: I.A., Design: U.S., Data Collection and/or Processing: U.S., Analysis and/or Interpretation: U.S., Literature Search: U.S., Writing: U.S.

DISCLOSURES

Conflict of Interest: No conflict of interest was declared by the authors.

Financial Disclosure: The authors declared that this study had received no financial support.

REFERENCES

1. Suarez DL, Miller PJ, Koch G, Mundt E, Rautenschlein S. Newcastle disease, other avian paramyxoviruses, and avian metapneumovirus infections. Diseases of poultry. 2020 Jan 13:109-66.
2. Hu Z, He X, Deng J, Hu J, Liu X. Current situation and future direction of Newcastle disease vaccines. Vet Res. 2022; 53(1): 99.
3. Qiu X, Jia Y, Zhang Z, Fo X, Wang W. Characterization of chicken-derived genotype VII Newcastle Disease Virus isolates from Northwest China. J Poult Sci. 2023; 60(2): 2023010.
4. Tanveer S, Malik HA, Shahid N, Salisu IB, Ahmed N, Latif A, et al. Production of proinflammatory cytokines by expressing Newcastle disease vaccine candidates in corn. J King Saud Univ Sci. 2023; 35(3): 102537.
5. Selim KM, Selim A, Arafa A, Hussein HA, Elsanousi AA. Molecular characterization of full fusion protein (F) of Newcastle disease virus genotype VIId isolated from Egypt during 2012-2016. Vet World. 2018; 11(7): 930-8.
6. Sarcheshmei M, Dadras H, Mosleh N, Mehrabanpour MJ. Comparative evaluation of the protective efficacy of different vaccination programs against a virulent field strain of the Newcastle disease virus in broilers. Braz J Poult Sci. 2016; 18: 363-70.
7. Roohani K, Tan SW, Yeap SK, Ideris A, Bejo MH, Omar AR. Characterization of genotype VII Newcastle disease virus (NDV) isolated from NDV vaccinated chickens, and the efficacy of LaSota and recombinant genotype VII vaccines against challenge with velogenic NDV. J Vet Sci. 2015; 16(4): 447-57.
8. Shabbir MZ, Abbas M, Yaqub T, Mukhtar N, Subhani A, Habib H, et al. Genetic analysis of Newcastle disease virus from Punjab, Pakistan. Virus Genes. 2013; 46(2): 309-15.
9. Siddique N, Naeem K, Abbas MA, Malik AA, Rashid F, Rafique S, et al. Sequence and phylogenetic analysis of virulent Newcastle disease virus isolates from Pakistan during 2009-2013 reveals circulation of new subgenotype. Virology. 2013; 444(1-2): 37-40.
10. Munir MT, Chowdhury MR, Ahmed Z. Emergence of new sub-genotypes of Newcastle disease virus in Pakistan. J Avian Res. 2016; 2(1): 1-7.
11. Mehmood A, Rasool MH, Anjum AA, Zahoor MA, Shafique M. pathotyping and genetic characterization of a highly virulent newcastle disease virus strain isolated from recent outbreak in district Okara, Pakistan. Pak Vet J. 2019; 39(3) 353-8.