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GENOMIC AND PHYLOGENETIC INSIGHTS INTO VELOGENIC NEWCASTLE DISEASE VIRUS GENOTYPE VII OUTBREAKS IN GENOTYPE II-VACCINATED POULTRY FLOCKS OF SOUTHERN PAKISTAN

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ABSTRACT

Newcastle Disease (ND), caused by Newcastle Disease Virus (NDV), remains a significant threat to the global poultry industry, particularly in endemic regions like Pakistan. Despite widespread vaccination programs, outbreaks of velogenic NDV continue to occur, raising concerns about vaccine efficacy and the circulation of novel or antigenically distinct viral strains. This study aimed to conduct a molecular characterization and phylogenetic analysis of velogenic NDV genotype VII strains isolated from recent outbreaks in commercial poultry flocks in the southern region of Pakistan, which were previously vaccinated with genotype II-based vaccines. Through reverse transcription-polymerase chain reaction (RT-PCR) and sequencing of key virulence-determining genes, the isolated strains were confirmed as velogenic NDV and assigned to Genotype VII, specifically sub-genotypes VIIh and VIIi. Phylogenetic analysis revealed that these strains clustered closely with other Genotype VII viruses circulating in the region and globally, suggesting ongoing evolution and potential transboundary movement. The findings highlight a significant antigenic mismatch between the circulating Genotype VII strains and the conventionally used Genotype II vaccines, contributing to vaccine failures and persistent outbreaks. This research underscores the critical need for updated vaccine strategies, potentially incorporating Genotype VII-specific strains, and enhanced surveillance to mitigate the economic impact of ND in Pakistan's poultry sector.

Keywords: Newcastle Disease; NDV; Genotype VII; Poultry; Pakistan; Molecular Characterization; Phylogenetic Analysis; Vaccine Efficacy; Outbreak.

INTRODUCTION

Imagine a disease so devastating it can wipe out entire poultry farms, leaving farmers with immense losses and threatening food supplies. That's the reality of Newcastle Disease (ND), a highly contagious and economically crippling viral illness that affects a wide range of birds, especially our domestic poultry [14, 20]. The culprit is the Newcastle Disease Virus (NDV), a tiny but mighty invader from the Orthoavulavirus genus, part of the Paramyxoviridae family [11, 15]. When NDV strikes, it can cause severe respiratory, nervous, and digestive problems, often leading to incredibly high rates of sickness and death in vulnerable birds [16, 26]. The financial toll of ND is truly staggering, causing significant setbacks for poultry production worldwide [13].

NDV strains are categorized by how deadly they are to chickens: velogenic (super virulent), mesogenic (moderately virulent), and lentogenic (mildly virulent) [27]. The key to NDV's power lies in a specific part of its fusion (F) protein – a tiny amino acid sequence at its cleavage site. This little detail dictates how easily the virus can replicate and spread throughout a bird's body [29, 30, 31, 32]. Velogenic strains, the most dangerous ones, have a special "multibasic" amino acid pattern at this site, allowing them to spread rapidly and cause widespread infection [29, 30].

From a genetic standpoint, NDV strains are grouped into many genotypes (currently I to XXI and beyond), with even more specific sub-genotypes, all identified by looking at parts or all of their F gene sequence [21, 24]. For a long time, Genotype II strains, like LaSota and B1, have been the go-to for live attenuated vaccines globally. They've offered broad protection against many NDV types [74, 75]. However, NDV is a master of evolution, constantly changing and giving rise to new genotypes and sub-genotypes. Genotype VII, in particular, has emerged as a major concern [33, 69]. First spotted in the late 1980s, this genotype has become deeply entrenched in many parts of Asia, Africa, and the Middle East, causing widespread outbreaks even in flocks that have been vaccinated [25, 53, 54, 55, 56, 64]. Its global spread and stubborn persistence make it a huge epidemiological challenge [58].

Pakistan's poultry industry, a cornerstone of its agricultural economy, is no stranger to ND outbreaks [36, 37, 38, 50]. Despite extensive vaccination programs using those traditional Genotype II-based vaccines, outbreaks keep happening. This suggests that something isn't quite right with the vaccine's effectiveness, perhaps due to the virus changing its "face" (antigenic drift) or the circulation

of field strains that are just too different from the vaccine strains [39, 76]. Past research has already shown that various NDV genotypes, including Genotype VII, are circulating in Pakistan [37, 52, 57, 59]. The southern region of Pakistan, with its rich and diverse populations of migratory wild birds [1, 60, 61, 62, 63], might even be acting as a hidden reservoir and transport route for NDV, making control efforts even more complicated [2, 4, 5].

This study was sparked by recent, severe ND outbreaks in commercial poultry farms across southern Pakistan, even though these farms were diligently vaccinating with Genotype II vaccines. Our main goal was to dive deep into the molecular characteristics and evolutionary history (phylogenetic analysis) of the NDV strains causing these outbreaks. We wanted to pinpoint the circulating genotypes, understand their potential for causing severe disease based on their F gene features, and map out their relationships with other global and regional strains. We believe these findings are absolutely vital for grasping the full picture of ND in Pakistan and for developing smarter, more effective strategies to control and prevent this disease, including the crucial possibility of needing vaccines that actually match the circulating genotypes.

MATERIALS AND METHODS

Study Area

Our investigation focused on four key districts in the southern part of Sindh, Pakistan: Karachi, Hyderabad, Thatta, and Dadu. We picked these spots because they have a unique mix of geography and ecology that makes them especially vulnerable to bird diseases. This region boasts a vast coastline (Karachi and Hyderabad), important freshwater lakes like Khanjar and Manchar (in Thatta and Dadu, respectively), and the mighty Indus River system. This diverse landscape is a magnet for huge numbers of migratory birds, including various types of cormorants, ducks, and geese, particularly during the winter. This seasonal influx of wild birds creates a constant interaction with commercial poultry farms, sadly providing a perfect breeding ground for the incubation, spread, mutation, and transmission of bird pathogens, especially NDV, between different species [1, 60, 61, 62, 63]. The sheer concentration of both commercial poultry and wild bird habitats in these districts really highlights their critical role in the story of Newcastle Disease in Pakistan.

Sample Collection

We took a very thorough approach to sampling between 2023 and 2024. In total, we collected 877 serum samples and 370 tissue/swab samples from commercial poultry flocks across our chosen districts. The flocks we sampled included breeders, layers, and broilers of various ages, which is a pretty standard representation of the commercial poultry industry there. All the flocks we looked at had a history of regular NDV vaccination, following the usual local practices of giving multiple doses of live attenuated (like LaSota or B1) and/or inactivated NDV vaccines, sometimes even combined with other poultry vaccines [39].

We specifically collected samples from birds showing signs of ND, as well as from birds that had recently died or were so sick they had to be humanely euthanized. The clinical signs we observed included breathing difficulties (coughing, sneezing, gasping), discharge from the eyes and nose, swollen eyes and wattles, and nervous system issues (tremors, paralysis, twisted necks, and a dizzy look). We carefully collected tissue samples from vital organs like the lungs, intestines, trachea, and proventriculus, which are commonly affected by NDV. All samples were immediately placed in special transport media and kept chilled (2∘C–8∘C) during their journey to the lab to keep the virus intact.

Blood Sample Collection

For our serological tests, we meticulously collected blood samples from the wing veins of the affected birds. The process involved gently extending the bird's wing to make the brachial vein visible. Any feathers in the way were carefully plucked. Then, we disinfected the area where we'd draw blood with 70% ethanol. Using a 3 mL syringe with a sterile needle, we inserted it under the tendon and guided it along the wing vein, being very careful not to go too deep. We slowly pulled the plunger to draw the blood until we had enough. Right after collection, the blood was transferred to sterile vacutainer tubes. These tubes were then refrigerated at 2∘C–8∘C for about 12 hours to allow the serum to separate. Once separated, we carefully poured off the serum and heat-inactivated it by incubating it at 56∘C for 1 hour. This step helps to neutralize any remaining virus and other components. Finally, the heat-inactivated serum samples were stored at 2∘C–8∘C for immediate use or frozen at −80∘C for longer storage.

Necropsy and Collection of Tissue Sample

Before we performed necropsies, we first screened the birds based on their clinical signs, such as coughing, sneezing, watery eyes, and swollen eyes and wattles, as well as the overall sickness and death rates in the flock. Birds that were severely ill were separated and humanely euthanized. The necropsy began with placing the euthanized bird on its back. We did an initial external check for any obvious problems. Then, we thoroughly wet the belly area with soapy water to prevent feather contamination. We made an incision down the middle of the chest, carefully peeled back the skin to expose the muscles underneath. Using scissors, we cut through the breastbone and opened up the chest and abdominal cavities with forceps. We then systematically examined the internal organs for any signs of NDV infection. We paid close attention to the intestines, proventriculus, trachea, and lungs. What we often found were things like congested windpipes, tiny bleeding spots (petechial hemorrhages) in the intestine and proventriculus, swollen lungs, enlarged kidneys, and dead tissue spots (necrotic foci) in the spleen. These are classic signs of a highly virulent NDV infection.

We carefully collected tissue samples from these affected organs, placed them immediately in transport medium (containing 50% glycerol, 50% phosphate-buffered saline (PBS), and a mix of antibiotics like streptomycin, penicillin, and amphotericin B to keep bacteria and fungi out), and quickly transported them to the lab, keeping them cold.

Virus Isolation From Tissue Samples

Virus Isolation by Embryonated Eggs

To isolate the virus, we first finely minced the collected tissue samples to create a 10% (weight/volume) tissue homogenate in PBS. We then spun this mixture down in a centrifuge at 10,000×g for 5 minutes to separate out any cell debris. The liquid on top, which might contain virus particles, was then passed through a super-fine 0.45 µm membrane filter. This step removes any remaining bacteria or larger bits, giving us a sterile virus suspension. We then took 100 µL of this filtered liquid and carefully injected it into the allantoic cavity of 9-11-day-old specific pathogen-free (SPF) embryonated chicken eggs (ECEs). We followed the standard procedures outlined by the World Organisation for Animal Health (WOAH) [40, 41, 42]. After inoculation, we incubated the eggs at 37◦C for 48 hours, then chilled them at 4◦C for 24 hours. This chilling helps in collecting the allantoic fluid. Once chilled, we carefully removed the blunt end of each egg with sterilized scissors and harvested the allantoic fluid (AF). This fluid was then centrifuged again at 10,000×g for 5 minutes to get rid of any remaining cellular debris before we used it for our next tests.

Virus Titration by Hemagglutination (HA) Reaction

To confirm the presence of NDV in the allantoic fluid we collected, we used a test called the hemagglutination (HA) assay, following the WOAH guidelines [40, 47]. Here's how we did it: First, we put 100 µL of saline into every well of a U-shaped microtiter plate. Then, we added 100 µL of our allantoic fluid to the first column of wells and performed a two-fold serial dilution across the plate, going all the way to the 11th column. The 12th column was our negative control, just containing saline. After diluting, we added 100 µL of a 0.5% chicken red blood cell (RBC) suspension to every well. We gently mixed the plate and incubated it at 37◦C for 30 minutes. After incubation, we looked at the wells. If NDV was present, the red blood cells would form a diffuse mat at the bottom of the well (hemagglutination). If there was no virus, the red blood cells would settle into a compact button. We expressed the HA titer as the reciprocal of the highest dilution of allantoic fluid that caused complete hemagglutination, usually reported in log2 units. Any samples with an HA titer of 1/16 or higher (meaning ≥24) were considered positive for NDV and were frozen at −80◦C for further molecular analysis.

Serological Detection of NDV

Detection of NDV Exposure by ELISA

To figure out if the poultry flocks had been exposed to NDV and to check their antibody levels, we performed an Enzyme-Linked Immunosorbent Assay (ELISA) using a commercial IDEXX NDV ELISA kit. We followed the manufacturer's instructions very carefully.

Here's a step-by-step breakdown of the ELISA procedure:

1. Serum Dilution: We started by diluting each serum sample. For every 1 µL of serum, we added 499 µL of dilution buffer, making a 1:500 dilution.

2. Plate Preparation: We took an NDV antigen-coated microtiter plate from its packaging and removed the seal.

3. Control Addition: We added 100 µL of both the negative and positive control sera (provided with the kit) into their designated wells, making sure to do each in duplicate.

4. Sample Addition: Then, we added 100 µL of our pre-diluted serum samples into every well, except for the control wells.

5. First Incubation: The plate was then incubated for 30 minutes (give or take 2 minutes) at a temperature between 18◦C and 26◦C.

6. Washing Step 1: After incubation, we aspirated the liquid from each well and washed the plate five times with 350 µL of distilled water per well. We used an automatic plate washer to ensure a thorough rinse.

7. Conjugate Addition: Before the plate could dry completely, we added 100 µL of the conjugate solution (an anti-chicken antibody linked to horseradish peroxidase) to each well.

8. Second Incubation: We incubated the plate again for another 30 minutes (±2 minutes) at 18◦C−26◦C.

9. Washing Step 2: Once again, we removed the liquid and washed the plate five times with 350 µL of distilled water per well.

10. Substrate Addition: Next, we added 100 µL of the substrate solution (like TMB) to each well.

11. Third Incubation: The plate was incubated for 15 minutes (±1 minute) at 18◦C−26◦C in the dark, allowing the color to develop.

12. Stop Solution Addition: To stop the reaction and fix the color, we added 100 µL of stop solution (such as sulfuric acid) to each well.

13. Absorbance Reading: Finally, we immediately read the absorbance (optical density, OD) of each well at 650 nm using an ELISA plate reader.

We determined the antibody level in each sample by calculating the sample-to-positive (S/P) ratio using this formula:

S/P=Positive Control mean OD−Negative Control mean

ODSample mean OD−Negative Control mean OD

Here, S/P is your sample's ratio compared to the positive control. PCx is the average OD of your positive control, and NCx is the average OD of your negative control.

We then calculated the endpoint titer using the kit's specific equation:

log10Titer=1.09(log10S/P)+3.36

For interpreting the results, an S/P ratio of 0.20 or less meant the sample was negative, while anything above 0.20 indicated a positive result for NDV antibodies. We also compared our observed ELISA titers with the known vaccine titer limits for different ages and types of flocks. If an ELISA titer was significantly higher than what we'd expect from vaccination alone, it strongly suggested an active field infection.

Biological Characterization

Intracerebral Pathogenicity Index (ICPI) Assessment

To understand how pathogenic (disease-causing) our isolated NDV strains truly were, we performed the Intracerebral Pathogenicity Index (ICPI) test on day-old chicks, strictly following WOAH guidelines [41]. First, we took the allantoic fluid that tested positive for HA (meaning it had an HA titer of 1/16 or more) and diluted it 1:10 in sterile phosphate-buffered saline (PBS). Then, we injected a small amount, 0.05 mL, of this diluted virus directly into the brain (intracerebrally) of each of 10 one-day-old SPF (specific pathogen-free) chicks. We then watched these chicks very closely, checking them every 24 hours for 8 days straight. At each check, we scored their clinical status: 0 for looking perfectly normal, 1 for being sick (showing any signs like being depressed, having ruffled feathers, breathing hard, or showing nervous symptoms), and 2 for being dead. The ICPI score itself was calculated as the average score per chick per observation over those 8 days. If an ICPI score was 0.70 or higher, we considered that strain to be pathogenic (either mesogenic or velogenic).

Mean Death Time (MDT) of the Minimum Lethal Dose (MLD)

We also determined the Mean Death Time (MDT) of the Minimum Lethal Dose (MLD) using 10-day-old SPF embryonated chicken eggs, following the WOAH protocols [42, 43]. We prepared serial ten-fold dilutions of the HA-positive allantoic fluid from each of our virus isolates (for example, 10−6,10−7,10−8,and 10−9). For each dilution, we inoculated 0.1 mL into the allantoic cavity of a group of SPF embryonated eggs. We then incubated these eggs at 37∘C and checked them twice a day for any embryo deaths. We carefully noted the exact time of death for each embryo. The MLD was defined as the highest virus dilution that still managed to kill all the inoculated embryos. The MDT was then calculated as the average time (in hours) it took for the embryos to die at that MLD. Typically, highly virulent (velogenic) strains cause death in less than 60 hours, moderately virulent (mesogenic) strains take between 60 and 90 hours, and mildly virulent (lentogenic) strains take more than 90 hours.

MOLECULAR DETECTION

RNA Extraction

After we confirmed the presence of NDV in the allantoic fluid (AF) through hemagglutination, we moved on to extract the viral RNA. This was done from AF samples collected after 48 hours of incubation at 37∘C. First, we spun the AF in a centrifuge at 8000×g for 5 minutes to get rid of any cell bits or larger particles. Then, we extracted the NDV RNA using a TRIzol RNA extraction kit (Life Technologies, Catalog Numbers 15596026 and 15596018), following the manufacturer's detailed instructions.

Here are the specific steps we followed for RNA extraction using TRIzol:

1. Homogenization: We thoroughly mixed 250 µL of the HA-positive allantoic fluid with 750 µL of TRIzol reagent (a 1:3 ratio) in a sterile 1.5 mL microcentrifuge tube.

2. Phase Separation (TRIzol incubation): We let this mixture sit at room temperature for 10 minutes. This step is crucial for breaking down the nucleoprotein complexes.

3. First Centrifugation: After incubation, we spun the mixture at 12,000×g for 10 minutes at 4∘C. This separates the mixture into three layers: a lower red, organic phase (phenol-chloroform), an interphase, and an upper aqueous phase.

4. Aqueous Phase Transfer: We carefully transferred the upper aqueous phase, which contains the RNA, to a fresh 1.5 mL microcentrifuge tube, making sure to avoid taking any of the interphase or organic phase.

5. Chloroform Addition: We then added 250 µL of chloroform to the aqueous phase and shook the mixture vigorously for 15 seconds.

6. Second Centrifugation: After a 10-minute incubation at room temperature, we centrifuged the mixture again at 12,000×g for 15 minutes at 4∘C. This further cleans up the aqueous phase.

7. RNA Precipitation: We transferred the clean aqueous phase to a new tube and added 350 µL of chilled isopropanol to make the RNA precipitate out of the solution. We let it sit at room temperature for 10 minutes.

8. Third Centrifugation: To pellet the RNA, we centrifuged it for 10 minutes at 10,000×g at 4∘C. A visible RNA pellet should form at the bottom.

9. Ethanol Wash: We carefully discarded the liquid on top and washed the RNA pellet three times with 750 µL of chilled 75% ethanol. Each wash involved centrifuging for 5 minutes at 7500×g to ensure all contaminants were

removed.

10. RNA Dissolution: After the washes, we briefly air-dried the RNA pellet to evaporate any leftover ethanol, then dissolved it in an appropriate amount of RNase-free water (Promega).

11. Quantification: Finally, we measured the concentration and purity of our extracted RNA using a NanoDrop spectrophotometer. The purified RNA was then stored at −80∘C until we were ready for reverse transcription-polymerase chain reaction (RT-PCR).

RT-PCR

For the molecular identification and characterization of NDV, we used RT-PCR, specifically focusing on a conserved region of the Fusion (F) gene. This gene is super important because it's key to determining how virulent the virus is and for understanding its evolutionary relationships.

Here's how we prepared our RT-PCR reaction mixture:

Each 50 µL RT-PCR reaction mix contained:

- 10 µL of AMV/Tft 5x reaction buffer
- 0.2 mM/µL of dNTPs (the building blocks of DNA)
- 1 mM Magnesium Sulfate (MgSO4)
- 0.1 µM of the forward primer (4306F): 5'-GACCGCTGACCACGAGGTTA-3'
- 0.1 µM of the reverse primer (5005R): 5'-AGTCGGAGGATGTTGGCAGC-3'
- 0.1 U/µL of AMV reverse transcriptase
- Tft DNA polymerase
- 8.42 ng/µL of our RNA template (the viral RNA we extracted)

We then topped up the volume to 50 µL with RNase-free water.

And here are the RT-PCR conditions we used in the thermal cycler [44]:

1. Reverse Transcription: We first converted the viral RNA into cDNA at 45∘C for 60 minutes.

2. Initial Denaturation: A single step at 95∘C for 1 minute to get the DNA polymerase ready and unwind the cDNA.

3. Cycling (30 cycles):

   ○ Denaturation: 94∘C for 1 minute (separating the DNA strands).

   ○ Annealing: 50∘C for 1 minute (allowing the primers to stick to the target DNA).

   ○ Extension: 72∘C for 3 minutes (where the DNA polymerase builds new DNA strands).

4. Final Extension: A last step at 72∘C for 10 minutes to make sure all DNA products were fully synthesized.

After the PCR, we ran the products on a 2% agarose gel, stained with ethidium bromide, and visualized them under UV light. We expected to see a clear band around 700 bp, which confirmed that we had successfully amplified the NDV F gene.

Molecular Characterization

Nucleotide Sequencing

Once we had successfully amplified the F gene using RT-PCR, we carefully cut out the target bands (around 700 bp) from the agarose gel. We then purified these DNA fragments using the Promega purification kit, following the manufacturer's instructions to the letter. After purification, we sent our PCR products for Sanger sequencing (a method that uses dideoxy nucleotides) to a commercial sequencing facility (like Macrogen Inc. in South Korea). We made sure to sequence both the forward and reverse strands to guarantee high accuracy and reliability of the genetic information we obtained. All the sequence data we generated was then submitted to the GenBank database, where each isolate received its own unique accession number. The key isolates we characterized in this study were:

1. Isolate Chicken/Pakistan/691, NDV accession No. MN096605.

2. Isolate Chicken/Pakistan/633, NDV accession No. MN096604.

3. Isolate Chicken/Pakistan/697, NDV accession No. MN096603.

4. Isolate Chicken/Pakistan/688, NDV accession No. MN096606.

Phylogenetic Analysis

Once we had our nucleotide sequences, we meticulously assembled, edited, and analyzed them using various bioinformatics software. We started with programs like Geneious Prime or DNASTAR Lasergene for initial editing and assembly. To find out how closely related our NDV strains were to others, we performed homology searches using the Basic Local Alignment Search Tool (BLAST) against the NCBI GenBank database (which you can find at http://www.ncbi.nlm.nih.gov). This helped us confirm the identity of our isolates.

For our phylogenetic analysis, we gathered a comprehensive collection of previously characterized NDV reference strains from GenBank, covering various genotypes (I-IX) from different parts of the world, including Asia and the Middle East. We then aligned our Pakistani F gene sequences with these reference sequences using ClustalW, a widely used program for multiple sequence alignment. The aligned file was then imported into GeneDOC for further adjustments and a visual check of the alignment.

To understand the evolutionary relationships among our

NDV isolates, we built phylogenetic trees using two main methods: the Neighbor-Joining method [46] and the Maximum Likelihood method [45, 46]. Both of these were done using MEGA11 (Molecular Evolutionary Genetics Analysis, version 11) software [45]. We calculated the evolutionary distances between sequences using the Kimura 2-parameter model [46], which accounts for different types of DNA changes. To make sure our tree branches were statistically sound, we performed a bootstrap analysis with 1000 replicates [45]. We assigned genotypes and sub-genotypes to our local isolates based on how they clustered with the established reference strains, following the latest unified classification system for Newcastle disease virus [21]. The resulting phylogenetic tree clearly showed the genetic connections and diversity among the NDV strains we analyzed.

Determination of Amino Acid Sequence at Cleavage Site of Fusion Protein

To understand the molecular "pathotype" (how disease-causing) our NDV isolates were, we translated the nucleotide sequences of the F gene cleavage site into amino acid sequences using MEGA Version 11 software. We then pinpointed the amino acid sequence at the crucial F protein cleavage site (positions 112-117) for each of our local isolates. We rigorously compared these sequences with amino acid sequences from previously published reference isolates that we found in the GenBank database. We determined the pathotype by looking for specific molecular "pathogenic factors" at this cleavage site. For instance, if we found a "polybasic" amino acid motif, like R, R, Q, R, R, and F at position 117 (for example, 112RRQRR↓F117), that was a clear sign of a highly virulent (velogenic) NDV strain. On the other hand, if we saw a "monobasic" amino acid sequence (like 112GRQGR↓L117), it indicated a less virulent (lentogenic) pathotype. This molecular analysis gave us a quick and reliable way to figure out how deadly the circulating NDV strains were.

RESULTS

Clinical Observations and Sample Details

When we examined the poultry flocks experiencing outbreaks in southern Pakistan, we consistently saw a pattern of severe Newcastle Disease. The affected birds showed a range of classic symptoms affecting their breathing, nervous system, and digestion. This included obvious difficulty breathing, coughing, sneezing, and nasal discharge. Their eyes were often swollen, and their heads were puffy. We also saw prominent nervous system issues like tremors, paralysis in their wings and legs, muscle weakness, and a dizzy appearance, all pointing to a virus that attacks the nervous system. Digestive problems included watery greenish diarrhea. In broiler flocks, a shocking 25% to 40% of birds died, while in layer flocks, mortality was between 10% and 16%, and in breeder flocks, it ranged from 8% to 12%. Typically, birds would die within a week of showing these clinical signs. What was particularly concerning was that despite these severe outcomes, the affected birds had received multiple doses of NDV vaccines, and the farmers reported that their vaccine titers (antibody levels) were within the expected range: 4–6 log2 in broilers, 7–8.5 log2 in layers, and 7–8.3 log2 in breeders. This mismatch between vaccination status and the severity of the disease strongly suggested that highly virulent, antigenically different field strains were circulating and causing these problems.

Postmortem Findings

When we performed necropsies (post-mortem examinations) on the birds that had died or were euthanized, we found severe gross lesions that were unmistakably consistent with velogenic NDV infection. We commonly observed significant congestion in the trachea, a clear sign of severe inflammation in the windpipe. There were also often tiny hemorrhages (petechial hemorrhages) in the intestine and proventriculus, indicating widespread damage to blood vessels. Other notable findings included congested lungs, swollen kidneys, and small areas of dead tissue (necrotic foci) in the spleen. These pathological changes are classic hallmarks of highly virulent NDV strains, and they directly explain the high mortality rates we saw in the field. Out of 141 broiler birds we examined, 72 (51.1%) showed clear post-mortem lesions. Similarly, among 122 layer birds, 61 (50%) had consistent lesions, and out of 107 breeder birds, 38 (35.5%) presented with these characteristic post-mortem findings. The presence of such severe lesions, even in flocks that reportedly had good vaccine titers, further supported our suspicion that a highly pathogenic circulating virus was overcoming the vaccine-induced immunity.

Serological Detection of NDV Infection

ELISA Titer Greater Than the Limit of Vaccine Titer

Our serological investigation using the IDEXX NDV ELISA kit revealed that the vaccinated flocks had widespread exposure to NDV, with antibody titers actually exceeding what we'd expect from just vaccine-induced immunity. Out of 877 serum samples from sick birds, a significant 368 (41.9%) tested positive by ELISA, which clearly indicated an active NDV infection. The high ELISA titers and Coefficient of Variation (CV%) values we observed were strong evidence of this field exposure.

To be more specific: in breeder flocks, we saw a maximum ELISA titer of 25,020, with CV% values ranging from 36.3% to 42.1%. Both these high numbers strongly pointed to significant NDV exposure. For layer flocks, the maximum ELISA titer hit 20,732, with CV% values from 39.8% to 52.3%, again, clearly suggesting NDV infection. In broiler flocks, the highest ELISA titer was 4,173, with CV% values between 33.4% and 37.3%. When we compared these values to the established vaccine titer limits for different age groups, it became very clear that these

antibody responses were a result of active field infection, not just routine vaccination. The overall prevalence of NDV infection detected by ELISA was 27% in breeder flocks, 45% in layer flocks, and a striking 62% in broiler flocks, truly highlighting how widespread these outbreaks were.

Detection of Higher ELISA Titer in the Affected Flock

A closer look at the ELISA titers across different age groups within the affected flocks further confirmed the presence of active field infection. For breeder, layer, and broiler flocks, the ELISA titers we observed at 5-6 weeks of age were consistently higher than the vaccine titers reported by the manufacturers (for example, breeder flocks usually had less than 8000, layer flocks less than 5000, and broiler flocks less than 4000). Similarly, in breeder and layer flocks, the ELISA titers at 10-12 weeks and 22-25 weeks were significantly higher than their respective vaccine titers (e.g., breeder flocks typically below 25,000 and layer flocks below 20,000). What's more, even in older birds (over 55 weeks for breeders and layers), the ELISA titers remained elevated (e.g., breeder flocks below 25,000; layer flocks below 1900), suggesting ongoing exposure or repeated infections. These consistently high antibody titers across various age groups and flock types strongly indicated that the immune responses we were seeing were due to natural infection with wild NDV strains, rather than simply the result of the regular vaccination schedule.

MOLECULAR DIAGNOSIS

Molecular Diagnosis of NDV by RT-PCR

To directly detect and confirm NDV at the molecular level, we used reverse transcription-polymerase chain reaction (RT-PCR). Our RT-PCR results, targeting the Fusion (F) gene, showed a high presence of NDV in the areas we investigated. Out of 167 allantoic fluid (AF) samples that tested positive for hemagglutination (HA), 66 were confirmed positive for NDV by RT-PCR. We consistently saw a clear band of the expected size, about 700 bp, on our 2% agarose gel. This successful amplification gave us solid molecular proof of NDV infection in the affected flocks and paved the way for us to further characterize the virus by sequencing its genes.

Prevalence of NDV Detected by RT-PCR

Our RT-PCR tests, specifically looking at the F gene, showed varying percentages of NDV presence across the different types of flocks that had been previously vaccinated multiple times. Broiler flocks had the highest prevalence, with 21% of the tested samples coming back positive. Layer flocks showed a 17% prevalence, and breeder flocks had 15%. These molecular detection rates, combined with what we saw clinically and serologically, really emphasized that NDV was circulating significantly in the vaccinated poultry populations in southern Pakistan.

Phylogenetic Analysis of NDV Based on Fusion Gene

Our phylogenetic analysis, which looked at the partial Fusion gene sequences (around 700 bp) from our local NDV isolates, revealed a striking genetic diversity when compared to the Genotype II vaccine strains commonly used in southern Pakistan. All the local isolates we selected, including Chicken/Pakistan/691 NDV (Accession No. MN096605), Chicken/Pakistan/633 NDV (Accession No. MN096604), and Chicken/Pakistan/688 NDV (Accession No. MN096606), consistently grouped very closely with reference strains belonging to Newcastle Disease Virus Genotype VII. This clear grouping confirmed that these field isolates were indeed Genotype VII, which is widely known as a velogenic (highly virulent) type of NDV.

Interestingly, one specific isolate, Chicken/Pakistan/697/2017 (Accession No. MN096603), grouped distinctly with reference strains of NDV Genotype II. This tells us that both Genotype VII and Genotype II strains are circulating in the region, though Genotype VII seemed to be the main cause of outbreaks in vaccinated flocks. The phylogenetic tree clearly showed a significant genetic difference between the circulating Genotype VII field strains and the Genotype II vaccine strains (like LaSota and B1), which formed a separate, more distantly related group. This genetic distance is a crucial finding because it strongly suggests that the vaccines aren't a perfect match for the viruses currently in circulation, providing a molecular explanation for why we're seeing vaccine failures and ongoing outbreaks.

Pathotyping of NDV

Detection of Biological Factors Involved in Pathogenicity

To understand the real-world impact of our isolated NDV strains, we performed biological characterization on 66 isolates from various districts in Sindh (Karachi, Hyderabad, Thatta, and Dadu). We used two key tests: the Intracerebral Pathogenicity Index (ICPI) in day-old chicks and the Mean Death Time (MDT) in 10-day-old embryonated eggs.

A large majority of our isolates, 57 out of 66 (86.4%), were classified as velogenic, meaning highly virulent, based on these tests. These velogenic isolates consistently showed high ICPI scores, ranging from 1.7 to 2.0, and a quick MDT, between 48 and 53 hours. These numbers are standard indicators for highly virulent NDV strains [47].

On the other hand, the remaining 9 isolates (13.6%) had much lower ICPI scores, between 0.4 and 0.5, and a much longer MDT, greater than 90 hours. These were classified as lentogenic (low pathogenic) types. It's worth noting that all 9 of these low pathogenic strains came specifically from Hyderabad. This biological assessment strongly matched what we saw clinically – high mortality and severe disease in the field were primarily caused by these velogenic strains.

Detection of Molecular Factors Involved in Pathogenicity

To understand the genetic basis of the NDV's deadliness, we analyzed the amino acid sequence at the Fusion (F) protein cleavage site. This particular region, from positions 112-117, is a major determinant of how virulent NDV is.

All the field isolates that we biologically classified as velogenic (like Chicken/Pakistan/691 NDV, Chicken/Pakistan/633 NDV, and Chicken/Pakistan/688 NDV) showed a very specific "polybasic" amino acid pattern at their cleavage site: 112RRQKRF117. The presence of four basic amino acid residues (R, R, Q, R) right before the cleavage site and a phenylalanine (F) at position 117 is a well-known molecular signature of highly virulent, velogenic NDV strains [48, 49]. This specific pattern allows the F protein to be easily cut by common proteases in the host, which then enables the virus to spread throughout the body and cause severe disease.

In contrast, the single isolate, Chicken/Pakistan/697 (Accession No. MN096603), which we found to be lentogenic in our biological tests, had a "monobasic" amino acid sequence at its cleavage site: 112GRQGR↓L117. This monobasic pattern is typical of avirulent NDV strains; they need very specific trypsin-like enzymes to be cleaved, which limits their ability to replicate and spread within the host. The strong agreement between our molecular findings (the F protein cleavage site pattern) and our biological assessments (ICPI and MDT) really solidified our understanding of how deadly the circulating NDV strains were in southern Pakistan.

Amino Acid Substitution in Fusion Protein

When we analyzed the amino acid sequence of the Fusion (F) protein, we discovered specific changes (substitution mutations) in several of the Pakistani NDV isolates, which tells us that the virus is continuously evolving. We found a unique substitution at positions 105 and 107 in the F protein of isolates Chicken/Pakistan/633 NDV (MN096604) and Chicken/Pakistan/691 NDV (MN096605). In these specific isolates, the amino acid serine (S) at position 105 was replaced by proline (P), and serine/threonine (S/T) at position 107 was replaced by alanine (A). This is different from most other reference isolates, especially the less virulent ones, which typically have S at position 105 and S/T at position 107.

Another isolate we looked at, Chicken/Pakistan/688 NDV (MN096606), also showed a substitution at position 107 (S/T replaced by A), but it didn't have the change at position 105. Interestingly, only one isolate, Chicken/Pakistan/697 NDV (MN096603), which we identified as Genotype II and was lentogenic, had an amino acid pattern similar to other avirulent NDVs, meaning it lacked these specific substitutions. These observed amino acid changes, especially at positions 105 and 107, suggest that there's genetic diversity among the circulating velogenic Genotype VII strains. While we need more research to fully understand how these specific mutations affect the virus's deadliness or its ability to escape vaccines, such changes in the F protein have been previously linked to altered viral infectivity and potentially increased virulence or reduced vaccine effectiveness [83, 84].

Distribution of NDV Genotypes in the South Region

Mapping out where the different NDV genotypes were found in the southern districts of Sindh, Pakistan, showed us that Genotype VII was the dominant one, with the exception of Hyderabad district. Newcastle Disease Virus Genotype VII was highly prevalent in Karachi, causing 30% of the detected infections there. In Dadu district, Genotype VII was found in 21% of cases, and in Thatta district, it was even higher, at 35%. This clearly indicates that Genotype VII is widespread and deeply rooted across these important poultry farming areas and wild bird habitats. On the other hand, NDV Genotype II was found to be the main genotype specifically in Hyderabad district, accounting for 13% of the cases we detected. This geographical pattern paints a picture of a complex NDV situation in Southern Pakistan, where different genotypes might be circulating at the same time, but Genotype VII seems to be the primary driver of outbreaks in most of the region.

DISCUSSION

Newcastle Disease Virus (NDV) continues to be a massive headache for Pakistan's poultry industry. We keep seeing outbreaks, even in flocks that have been vaccinated, leading to huge financial losses [50]. The alarmingly high death rates we observed in our study's vaccinated poultry flocks strongly suggest that highly virulent field strains are circulating. To truly tackle this problem, we absolutely need to characterize these prevalent strains and understand their genetic makeup.

Our molecular characterization and phylogenetic analysis clearly showed that velogenic NDV Genotype VII (specifically sub-genotypes VIIh and VIIi) is the main culprit behind the recent outbreaks in southern Pakistan. This lines up with what we've seen globally: Genotype VII is widespread and deeply entrenched, especially in Asia, Africa, and the Middle East [25, 53, 54, 55, 56, 58, 64]. The fact that we found the distinctive polybasic amino acid motif (112RRQKRF117) at the F protein cleavage site in all our virulent isolates further confirmed how deadly they are. This directly explains the severe clinical signs and devastating mortality we witnessed in the affected poultry flocks [29, 30, 81]. This molecular signature is a reliable indicator of widespread infection and severe disease in birds [40].

Our phylogenetic analysis also revealed that the Pakistani Genotype VII isolates were genetically very close to other Genotype VII strains previously reported in Pakistan [37, 57, 59], as well as in neighboring countries like Iran [3, 9], China [8], and Egypt [25, 28, 64], and other parts of Africa [10, 19]. This strong genetic connection suggests that these virulent strains are constantly moving within the

region and potentially even across borders. NDV is always evolving, and new genotypes and sub-genotypes are constantly emerging, which creates ongoing challenges for disease control worldwide [33, 66, 69]. The fact that we found Genotype VII so prevalent in Karachi, Dadu, and Thatta, alongside a significant presence of Genotype II in Hyderabad, paints a complex picture of NDV epidemiology in Southern Pakistan.

A crucial takeaway from this study is that the widely used Genotype II-based vaccines (like LaSota and B1) don't seem to be offering enough protection against the circulating Genotype VII strains. Even though flocks were vaccinated multiple times and reportedly had good antibody levels, the outbreaks kept happening with high mortality. This "vaccine breakdown" is a growing concern in many areas where NDV genotypes that are antigenically different are common [74, 75, 76, 77]. While Genotype II vaccines have historically provided broad protection, there's increasing evidence that antigenic differences between vaccine strains and current field strains, especially Genotype VII, can lead to reduced cross-protection. Studies have shown that antibodies produced by Genotype II vaccines might not effectively neutralize these antigenically distinct Genotype VII viruses, leading to more viral shedding and transmission, even in vaccinated birds [75, 76, 78, 80]. This antigenic mismatch is a major reason for the vaccine failures and persistent ND outbreaks in the region. Our findings strongly support the growing consensus among researchers that we urgently need genotype-matched vaccines – vaccines that use strains genetically similar to the viruses actually circulating – to better protect poultry and control ND effectively [77, 79, 84].

Southern Pakistan's unique geography and environment, with its vast coastlines, numerous lakes (like Khanjar and Manchar), and river systems, make it a crucial hub for migratory wild birds [1, 60, 61, 62, 63]. These wild bird populations, including cormorants and other waterfowl, are known to be natural carriers and potential spreaders of NDV, introducing diverse viral types into domestic poultry [2, 4, 5, 22, 73]. The reported cases of virulent NDV in wild peacocks in the southern region [36] further highlight how wild birds could be spreading these deadly strains to backyard and commercial poultry. This means we need to do more research into the role of water birds in NDV epidemiology and develop strategies to minimize contact between wild and domestic birds.

The amino acid changes we saw in the Fusion protein, specifically at positions 105 (Serine to Proline) and 107 (Serine/Threonine to Alanine) in some of the velogenic Genotype VII isolates, are important. While we need more research to fully understand what these specific mutations do, changes in the F protein can significantly impact how infectious the virus is, how it fuses with cells, and its antigenic properties. This could potentially lead to the virus becoming even more deadly or better at evading vaccines [83, 84]. These genetic variations remind us that NDV is constantly under evolutionary pressure, and we need ongoing molecular surveillance to keep track of new variants as they emerge.

The ongoing circulation of velogenic Genotype VII NDV and its impact on vaccinated flocks clearly show the limitations of our current ND control strategies in Pakistan. We need a comprehensive approach that goes beyond just routine vaccination. This includes strict biosecurity measures on farms, quick detection systems, and rapid response protocols to contain outbreaks. Continuous molecular surveillance and characterization of circulating NDV strains are absolutely essential to monitor how the virus is evolving, identify any new genotypes or variants, and guide the development of more effective vaccine strategies. The huge financial burden these repeated outbreaks place on poultry farmers in Pakistan [13] emphasizes just how urgent it is to implement updated and comprehensive disease control programs.

Of course, every study has its limitations. In our case, we primarily relied on molecular characterization to assess how deadly the virus was, rather than performing direct in-vivo tests like the Intracerebral Pathogenicity Index (ICPI) and Mean Death Time (MDT) for all isolates. While the F gene cleavage site motif is a very reliable indicator of virulence, conducting direct pathogenicity tests on a wider range of isolates would give us an even more complete picture of their biological deadliness. Future research should definitely include extensive in-vivo pathogenicity tests and, importantly, detailed antigenic characterization (for example, using cross-hemagglutination inhibition tests) to directly measure the antigenic differences between the current Genotype II vaccine strains and the prevalent Genotype VII field isolates. Furthermore, sequencing the complete genome of these circulating strains would offer a much deeper understanding of their genetic makeup, evolutionary history, and any potential recombination events, which could ultimately help us develop the next generation of vaccines and antiviral strategies [49].

CONCLUSION

This study offers strong proof that highly virulent Newcastle Disease Virus Genotype VII is actively circulating and causing major outbreaks in poultry flocks in southern Pakistan, even though these flocks were vaccinated with Genotype II vaccines. Our molecular tests confirmed how deadly these strains are, and our phylogenetic analysis showed their close genetic ties to other Genotype VII lineages both regionally and globally. The findings clearly point to a significant mismatch between the current Genotype II vaccines and the prevalent Genotype VII field strains. This mismatch is leading to vaccine failures and substantial financial losses. To effectively control ND in Pakistan, we urgently need to rethink our current vaccination strategies, possibly by including vaccine candidates that match Genotype VII. Enhanced surveillance, strict biosecurity, and continuous molecular monitoring of circulating NDV strains are

absolutely vital to protect the poultry industry and ensure food security in the region.

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