Virology Journal

RESEARCH

Open Access

# Genetic and antigenic characteristics of genotype VII.1.1 Newcastle disease viruses currently circulating in Ethiopian chickens

Berihun Dires Mihiretu[1], Tatsufumi Usui[1], Tesfaye Rufael Chibssa[2] and Tsuyoshi Yamaguchi[1*]

## Abstract

**Background** Newcastle disease virus (NDV) is a causative agent of Newcastle disease (ND), a major infectious poultry disease associated with significant economic losses. Vaccination is usually effective at preventing the disease. However, in Ethiopia, ND is commonly detected in both unvaccinated and vaccinated chickens. In this study, we aimed to evaluate the pathogenicity of NDV isolated from both vaccinated and unvaccinated chickens, as well as to compare the antigenicity of the isolates with vaccine strains and genotyping by using the *F*-gene sequence.

**Methods** The partial *F* gene sequences of all isolates and the mean death times (MDTs) of representative isolates were used to determine genotype and pathogenicity of the isolates. Antigenicities were assayed with the hemagglutinin inhibition (HI) and virus neutralization (VN) tests using antiserum against the vaccine Hitchner B1 (HB1), which is the most commonly used NDV vaccine in Ethiopia. Thermostability was evaluated by incubating infected allantoic fluid at 56 °C.

**Results** Out of 231 samples tested, 10.8% (25/231) were positive for virus isolation. The *F* gene cleavage sites of all 25 isolates had $^{112}$RRQKRF$^{117}$, a characteristic of virulent NDVs. The MDTs of representative isolates were less than 60 h, indicating highly virulent (velogenic) pathotypes. The HI test revealed significant differences between our isolates and the HB1 vaccine strain, but the VN test showed no antigenic difference. Phylogenetic analysis based on the partial *F* gene sequences showed that all the isolates belonged to sub-genotype VII.1.1 of genotype VII, which is closely related to NDV strains from the Middle East and Eritrea. Thermostability test showed two of the 25 isolates were thermostable.

**Discussion** Although the HI test indicates antigenic differences between the velogenic Ethiopian isolates and the HB1 vaccine, the VN test showed that the vaccine could protect infections with these isolates. Phylogenetic analysis showed that all studied isolates belong to sub-genotype VII.1.1 of genotype VII, diverging from previously reported genotype XXI in Ethiopia.

**Conclusions** In Ethiopia, NDV genotype VII 1.1 is widely distributed. Since these viruses showed the same antigenicity as the HB1 vaccine in VN test, the occurrence of ND in vaccinated chickens may be due to vaccine failure caused by inadequate management or immunosuppression due to other infectious diseases.

**Keywords** Newcastle disease virus, Genotype, Antigenicity, Pathogenicity, Ethiopia

*Correspondence:
Tsuyoshi Yamaguchi
yamaguti@tottori-u.ac.jp

[1]Joint Graduate School of Veterinary Sciences, Tottori University, 4-101 Koyama-Minami, Tottori 680-8553, Japan
[2]Animal Health Institute, P.O.Box 04, Sebeta, Ethiopia

## Introduction

Newcastle disease virus (NDV), also known as avian paramyxovirus 1 (APMV 1), belongs to the family *Paramyxoviridae*, subfamily *Avulavirinae*, genus *Orthoavulavirus* [1]. Clinical signs observed in NDV-infected chickens vary depending on the virus pathotype, age, coinfection with other pathogens, stress, and immune status. Clinical signs include sudden death, depression, oedema of the head, twisting neck (torticollis), diarrhea, soft eggshell, and a drop in egg production [2]. Five NDV pathotypes have been identified based on clinical signs and pathological lesions: (1) viscerotropic velogenic with hemorrhagic intestinal lesions, (2) neurotropic velogenic resulting in high mortality with respiratory and neurological signs, (3) mesogenic (moderately virulent) with respiratory and some nervous signs, (4) lentogenic with mild or inapparent respiratory signs and (5) subclinical. Mortality may be as high as 50% with the mesogenic strains and up to 100% with the velogenic strains [2, 3]. Based on the mean death time (MDT) in embryonated chicken eggs (ECEs) [4], NDVs can also be classified into three pathotypes: (1) velogenic (highly virulent; MDT ≤ 60 h), (2) mesogenic (intermediately virulent; MDT between 60 and 90 h) and (3) lentogenic (low virulent; MDT > 90 h).

NDV has a single-stranded, non-segmented, negative-sense RNA genome of 15,186, 15,192, or 15,198 nucleotides in length [5, 6]. The genome encodes six structural proteins: NP (nucleoprotein), P (phosphoprotein), M (matrix), F (fusion), HN (haemagglutinin-neuraminidase), and L (RNA-dependent RNA polymerase) protein, as well as two nonstructural proteins, W and V, which are produced through specific RNA editing by nucleotide shifting of the P gene during transcription [7]. HN and F surface glycoproteins play vital roles in viral infection. HN protein is essential for attachment to the host cell, whereas F protein enables fusion of the virus envelope with the cell membrane [8]. The precursor fusion glycoprotein (F0) needs to be cleaved into F1 and F2 by host cell proteases for the virus to be infectious. Virulent NDV strains are characterized by a $^{112}$R/K-R-Q/K/R-K/R-R$^{116}$ sequence at the C-terminus of F2 protein, and a phenylalanine residue (F) at position 117 of F1 protein. F0 of these strains can be cleaved by proteases found in wide range of cells and tissues, thus allowing the virus to infect various organs. F0 of low virulent strains exhibits sequences in the same region as $^{112}$G/E-K/R-Q-G/E-R$^{116}$, with leucine residues (L) at position 117 that can be cleaved by proteases restricted to the respiratory and gastrointestinal tracts, resulting in local infection [2].

NDVs are classified into two classes (I and II) based on their complete genome sequences [6]. The *F* gene sequence is used for NDV genotyping. Class I NDVs have a single genotype, whereas class II NDVs are subclassified into 21 genotypes [9]. Class II NDVs are responsible for several panzootics in poultry. Genotypes II, III and IV were responsible for the first NDV panzootics that happened from 1920 to 1960. The second panzootic was caused by genotype V in the late 1960s. Former sub-genotype VIb (current sub-genotype VI.1.1) resulted in the third panzootic in pigeons in the 1980s. The fourth panzootic, which began in 1985 and the most recent fifth panzootic were caused by genotype VII [10–12].

The poultry population in Ethiopia is about 57 million [13]. Ethiopian poultry production comprises backyard production (89% of the total chicken population), small-scale commercial farms (8%) and large-scale commercial farms (3%) [14]. Poultry production in Ethiopia is significant as a source of protein and income [15], although it is hampered by disease and poor management. One of the most important diseases affecting poultry production in Ethiopia is ND [16]. ND outbreaks were first reported in 1971 in Eritrea and in 1972 in Addis Ababa, Ethiopia [17]. Since then, the disease has spread to other parts of Ethiopia and became endemic with outbreaks reported every year [18]. NDV from backyard poultry farms where vaccination is not common has been reported in different parts of Ethiopia [16, 19–21]. Poor handling practices in live bird markets (LBMs) has also been reported to play a major role in the spread of the virus in Ethiopia and other countries [22–26]. Even though ND vaccination in commercial farms has been conducted since 1974 [27], there have been several reports of ND outbreaks in vaccinated commercial farms in Ethiopia [28–31]. However, the causes of ND outbreaks in vaccinated chickens have not been determined, possibly because of a difference in antigenicity between field and vaccine strains [32, 33]. Currently available ND vaccines in Ethiopia were derived from genotypes I and II. Genotype-matched ND vaccines have been reported to provide better protection than heterologous vaccines [34, 35]. Thus, knowing the currently circulating NDV genotypes is necessary. Ethiopia is a landlocked country close to the Red Sea (an important global trade route) and borders six African countries. Frequent movements of poultry and migrations of wild birds characterize this region. Therefore, the characterization of NDVs prevalent in Ethiopia will contribute to the development of effective strategies for controlling ND throughout East Africa.

## Materials and methods

### Sample collection

Oropharyngeal and cloacal swab samples were collected from commercial and backyard chickens, as well as LBM in five different locations in Ethiopia (Fig. 1). A total of 231 chickens were sampled, including 225 with NDV-related clinical signs (torticollis, respiratory distress, greenish diarrhea, ruffled feather and depression) from a farm visit, one from a suspected NDV outbreak, and

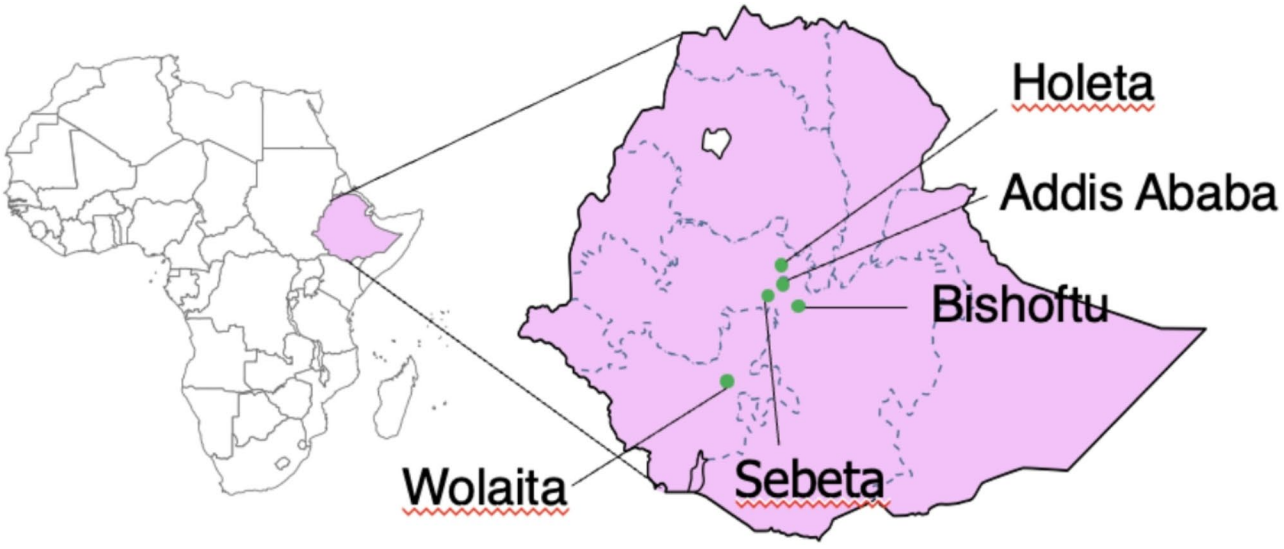


**Fig. 1** Location of sample collection sites

**Table 1** Sample collection sites, collection date and number of chickens sampled

| S/No. | Collection site | Collection date | Season | Sampled chickens | Production system | Vaccination history |
|---|---|---|---|---|---|---|
| 1. | Wolaita | 2023/01/31-2023/02/16 | Winter | 50 | Backyard | Unvaccinated |
| | | 2023/10/12-2023/11/05 | Autumn | 25 | Backyard | Unvaccinated |
| 2. | Bishoftu | 2023/02/09-2023/03/01 | Winter | 50 | Commercial | Vaccinated* |
| | | 2023/12/02-2023/12/29 | Autumn | 25 | Commercial | Vaccinated* |
| 3. | Holeta | 2023/01/09-2023/02/17 | Winter | 50 | Commercial | Vaccinated* |
| | | 2023/09/24-2023/10/17 | Autumn | 25 | Commercial | Vaccinated* |
| 4. | Addis Ababa | 2023/03/09 | Spring | 1 | Commercial | Vaccinated* |
| 5. | Sebeta | 2023/12/31 | Winter | 5 | LBM | Unknown |
| Total | | | | 231 | | |

*: vaccinated with HB1 and LaSota

five from live bird market (LBM) (Table 1). All samples were transported under cold chain conditions to the Animal Health Institute (AHI), Sebeta, Ethiopia for NDV screening.

#### NDV screening by real time reverse transcriptase polymerase reaction (RRT-PCR)

Individual swabs were centrifuged at 20,000×g for 5 min, then five swab suspensions from the same location (oropharyngeal with oropharyngeal and cloaca with cloacal) were pooled together and processed as one pool according to World Organization for Animal Health manual [2]. RNA extraction was conducted using MagExtractor™ Viral RNA kit (Toyobo, Osaka, Japan) from pooled samples and examined by RRT-PCR. The NDV screening was performed using primer targeting M gene, Forward M+4100 5′-AGTGATGTGCTCGGACCTTC-3′, Reverse M-4220 5′-CCTGAGGAGGCATTTGCTA-3′ and probe 5′- [FAM] TTCTCTAGCAGTGGGACAGCCTGC [TAMRA]-3` [36]. All NDV-positive pool samples in the RRT-PCR were subjected to RRT-PCR for each individual sample contained in the pool sample. All individual samples NDV-positive in the RRT-PCR were sent to the Avian Zoonosis Research Center, Tottori University, Japan, for further analysis.

#### Virus isolation and hemagglutination assay (HA) test

Virus isolation was conducted according to the procedure described by Alexander [4]. Briefly, 0.1 ml of all RRT-PCR positive swab samples were inoculated into the allantoic sac of 9–11 days old ECEs (Aoki Breeder Farm, Tochigi, Japan). The eggs were incubated at 37 °C for 48 h and viability of the eggs was checked every 24 h by candling. The eggs that died within 24 h of incubation were discarded. All eggs that either died after 24 h of incubation or survived the incubation period were chilled overnight at 4 °C. Then, allantoic fluids were harvested and tested for the presence of hemagglutinating virus using 0.6% chicken red blood cells. All HA negative samples were passaged in ECEs for the second time, when the second passage tested HA negative, the sample was considered negative for NDV. NDVs isolates in this study were used for antigenic, genotypic and phylogenetic characterization of currently circulating NDV strains in Ethiopia.

### Antigenicity test

NDV HB1 antiserum prepared by immunizing chickens with commercial Hitchner B1 (HB1) live vaccine (Nisseiken, Tokyo, Japan) according to the manufacturer's instruction was used to examine the difference in the antigenicity of Ethiopian field NDV strains against the commonly used HB1 vaccine. Infective allantoic fluid from the commercially available live NDV HB1 was prepared based on the above-mentioned method and used to compare the antigenicity against field strains with the hemagglutinin inhibition (HI) test and virus neutralization (VN) test. The HI test was performed according to the WOAH protocol [2]. The agglutination is assessed by tilting the plates. The highest dilution of serum causing complete inhibition of 4 HAU of antigen was considered as HI titer. VN test was conducted with slight modification of the method described in WHO Manual on Animal Influenza Diagnosis and Surveillance [37]. Briefly, HB1antiserum was heat inactivated at 56 °C for 30 min and twofold diluted antiserum was mixed with 100 $TCID_{50}$/50 µl of HB1 and field NDVs in quadruplicate. The virus-antiserum mixture was gently agitated and incubated at 37 °C with 5%$CO_2$ for 1 h. 100 µl of the virus-antiserum mixture was transferred to confluent DF-1 cells in 96 well plates. After 1 h of incubation, the virus-antiserum mixture was removed and 200 µl of D-MEM (+/-0.1 µg/ml TPCK trypsin) was added. The plates were incubated for 4 days at 37 °C with 5% $CO_2$ and observed daily for cytopathic effect (CPE). The highest dilution of the antiserum that protected CPE in 2 of the 4 wells was considered as the VN titer (VNT).

### Thermostability test

Thermostability test was conducted as described by Grund et al. [38]. Briefly, undiluted allantoic fluids were centrifuged at 100×g for 10 min, aliquoted in 1.5 ml microtube and incubated at 56 °C in a water bath for 5, 15, 30, 60 and 120 min and rapidly chilled. Thermostability of the isolates was examined by HA test. Strains showing stable HA activity after 15 min of incubation were considered as thermostable [39].

### cDNA synthesis, partial *F* gene amplification and sequencing

RNA was extracted from all HA positive allantoic fluids using QIAamp viral RNA mini kit (Qiagen, Hilden, Germany) in accordance with the manufacturer's instructions. cDNA was synthesized from the RNA using a Superscript III reverse transcriptase (Invitrogen, CA, USA) and random hexamer primers. Partial *F* gene (489 bp), was then amplified from the cDNA with forward primer 5′-ATGGGCYCCAGACYCTTCTAC-3′ and reverse primer 5′-CTGCCACTGCTAGTTGTGA TAATCC-3′ [40]. The amplified products were run on 1% gel electrophoresis and extracted using a MinElute Gel Extraction kit (Qiagen, Hilden, Germany). The PCR products were cycle sequenced using BigDye Terminator v3.1 cycle sequencing kit (Thermo Fisher, MA, USA) and sequenced by Sanger method sequencer (FASMAC, Kanagawa, Japan). The sequence products were edited and aligned by CodonCode Aligner version 10.0.1 (CodonCode, Dedham, MA, USA).

### Selection of representative isolates, pathogenicity of NDV isolates and MDT test

Seven representative isolates were selected for the MDT and VN test by considering the sample collection sites and seasons. Accordingly, two samples from Wolaita (NDV/chicken/Eth-SZ1T2/2023 and NDV/chicken/Eth-WS10C7/2023), two samples from Holeta (NDV/chicken/Eth-HT2T1/2023 and NDV/chicken/Eth-HT9C15/2023), one sample each from Bishoftu, Addis Ababa and Sebeta (NDV/chicken/Eth-BS2T14/2023, NDV/chicken/Eth-AA3T1/2023 and NDV/chicken/Eth-SLBMC1/2023), respectively, were selected.

The pathogenicity of NDV isolates was determined by the deduced amino acid sequence of the *F* gene cleavage site and MDT test. MDT was conducted to differentiate velogenic, mesogenic and lentogenic strains as per the standard procedure described by Alexander [4]. Fresh infective allantoic fluids of seven representative isolates were diluted from $10^{-6}$ to $10^{-9}$. 0.1 ml of each dilution was inoculated into five 10-day-old specific pathogen free (SPF) eggs (KM Biologics Co., Ltd., Kumamoto, Japan) and incubated at 37 °C. The embryos were examined every 12 h for seven days and the time of death was recorded. The highest dilution that kills all eggs is the minimum lethal dose and the MDT is the mean time in hours for the minimum lethal dose to kill the embryos. Isolates were considered velogenic: with an MDT of less than 60 h, mesogenic: MDT between 60 and 90 h and lentogenic with MDT of more than 90 h. $EID_{50}$ (the amount of infectious virus required to infect 50% of inoculated embryonated eggs) and $ELD_{50}$ (the amount of infectious virus required to cause mortality of 50% of inoculated eggs) were determined by the Reed and Muench method [41] from the same SPF eggs used to test MDT.

### Phylogenetic analysis

Partial *F* gene sequences of all the isolates were used to determine their genotype according to the previous reports [28, 42, 43]. The sequences were subjected to Basic Local Alignment Search Tool (BLAST) (https://blast.ncbi.nlm.nih.gov/Blast.cgi) at first to retrieve closely related sequences from the GenBank. The sequences and the pilot dataset based on the NDV *F* gene sequence proposed by Dimitrov et al. [9] were aligned using a Multiple Sequence Comparison by log expectation (MUSCLE)

in Molecular Evolutionary Genetic Analysis Version X (MEGA X) [44]. Subsequently, phylogenetic trees from the aligned sequences were constructed using the maximum likelihood method with the general time-reversible model and 1,000 bootstrap. The genotypes of the isolates were determined based on the *F* gene sequences from the pilot data set reported previously [9].

## Results

### NDV detection and isolation

Of 231 chickens that were examined, 29 (12.6%) tested positive for NDV by RRT-PCR. NDV was successfully isolated from 25 of these chickens. The detection and isolation rates of NDV from backyard chickens (Wolaita) with no vaccination history were 13 out of 75 (17.3%) and 12 out of 75 (16%), respectively. These rates were relatively higher than those for vaccinated chickens from Holeta, Bishoftu and Addis Ababa with 15 out of 151 (9.9%) for detection and 12 out of 151 (7.9%) for virus isolation. NDV was detected and isolated from one of the five chickens (20%) sampled in the LBM (Table 2).

NDVs were most frequently detected from oropharyngeal swabs only, with 15 chickens (51.7%) testing positive from Wolaita (6), Holeta (7) and Bishoftu (2), followed by 7 chickens (24.1%) with positive cloacal swabs only from Wolaita (4) and Holeta (3), and 7 chickens (24.1%) with positive results from both oropharyngeal and cloacal swabs from Wolaita (3), Holeta (2), Addis Ababa (1) and Sebeta (1). The virus isolation and RRT-PCR detection rates showed similar patterns, in which the virus was isolated from oropharyngeal swabs only in 13 chickens (52%) from Wolaita (7), Holeta (4) and Bishoftu (2), followed by cloacal swabs only 7 (28%) from Wolaita (4) and Holeta (3), and both oropharyngeal and cloacal swabs from 5 chickens (20%) from Wolaita (1), Holeta (2), Addis Ababa (1) and Sebeta (1) (Table 2).

### Antigenicity and thermostability

The HI titers of anti-HB1 antiserum for the 25 isolates ranged from 40 to 80, while the HI titer for the live NDV vaccine HB1 was 320, indicating a significant difference. However, the HB1 antiserum neutralized live HB1 at a 1:640 dilution, with all seven representative samples in this study showing similar VNT ranging from 640 to 1280. HA activity from 23 of the 25 isolates was destroyed by only 5 min at 56 °C, while the other two still showed HA activity after 15 min. These two isolates were determined as thermostable. (Table 3).

### Comparison of nucleotide sequences of *F* genes

The nucleotide similarity of the partial *F* gene sequence (489 bp) among the 24 isolates in this study ranged from 97 to 100%. Only the isolate from the LBM was relatively different, with similarities from 95.8 to 97.2%. The strains in GenBank that are most closely related to our isolates were Eritrean (6_19 isolate, Accession OQ466555.1) and Iranian (CHRT40IR2011 isolate, ON184061.1) with 97-98.4% and 96.4–97.4% nucleotide identities, respectively. The current NDV isolates differed considerably from the ND strains isolated in Ethiopia between 2011 and 2014 that belongs to genotype XXI and VII.1.1, with nucleotide identities ranging from 65.9 to 95.3%. All partial *F* gene nucleotide sequences of the current study were deposited in NCBI GenBank/EMBL/DDBJ (Table 3).

### Pathogenicity of NDV isolates

All isolates had identical multiple basic amino acids and $F^{117}$ at the F protein cleavage site (Table 3), indicative of virulent strains according to the WOAH Terrestrial Manual [2]. In addition, the MDTs of all seven representative isolates were less than 60 h, which is typical of velogenic strains. The mean infectious dose ($EID_{50}$) and mean lethal dose ($ELD_{50}$) were identical for all isolates ranging from $10^{7.16}$ to $10^{8.83}$/0.1 ml (Table 3).

**Table 2** NDV screening by RRT-PCR and virus isolation by ECEs

| Sample collection sites | Number of chickens | Vaccination history | Number of NDV positive chickens | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | RRT-PCR | Virus isolation | Only from oropharyngeal swab | | Only from cloacal swab | | From both oropharyngeal and cloacal swabs | |
| | | | | | RRT-PCR | Isolated virus | RRT-PCR | Isolated virus | RRT-PCR | Isolated virus |
| Wolaita | 75 | Unvaccinated | 13 | 12 | 6 | 7 | 4 | 4 | 3 | 1 |
| Holeta | 75 | Vaccinated* | 12 | 9 | 7 | 4 | 3 | 3 | 2 | 2 |
| Bishoftu | 75 | Vaccinated* | 2 | 2 | 2 | 2 | - | - | - | - |
| Addis Ababa | 1 | Vaccinated* | 1 | 1 | - | - | - | - | 1 | 1 |
| Sebeta LBM | 5 | Unknown | 1 | 1 | - | - | - | - | 1 | 1 |
| Total | 231 | | 29 | 25 | 15 | 13 | 7 | 7 | 7 | 5 |

**Table 3** Biological characteristics of the 25 NDV isolates

| Isolates | Collection site | Thermo stability in minutes | HI titer | F gene cleavage site | MDT (hours) | $EID_{50}$/0.1 ml | $ELD_{50}$/0.1 ml | Accession number | Pathotype |
|---|---|---|---|---|---|---|---|---|---|
| NDV/chicken/Eth-SZ1T2/2023 | Wolaita | 5 | 40 | $^{112}$RRQKR↓F$^{117}$ | < 60 | $10^{7.63}$ | $10^{7.63}$ | LC846624 | Velogenic |
| NDV/chicken/Eth-ST2T7/2023 | Wolaita | < 5 | 40 | $^{112}$RRQKR↓F$^{117}$ | - | - | - | LC846621 | Meso/Velo |
| NDV/chicken/Eth-ST2T8/2023 | Wolaita | < 5 | 40 | $^{112}$RRQKR↓F$^{117}$ | - | - | - | LC846622 | Meso/Velo |
| NDV/chicken/Eth-ST2T10/2023 | Wolaita | < 5 | 40 | $^{112}$RRQKR↓F$^{117}$ | - | - | - | LC846623 | Meso/Velo |
| NDV/chicken/Eth-ST2C9/2023 | Wolaita | 5 | 40 | $^{112}$RRQKR↓F$^{117}$ | - | - | - | LC846619 | Meso/Velo |
| NDV/chicken/Eth-ST2C11/2023 | Wolaita | 5 | 40 | $^{112}$RRQKR↓F$^{117}$ | - | - | - | LC846620 | Meso/Velo |
| NDV/chicken/Eth-WG2T4/2023 | Wolaita | 5 | 40 | $^{112}$RRQKR↓F$^{117}$ | - | - | - | LC846626 | Meso/Velo |
| NDV/chicken/Eth-WGT9/2023 | Wolaita | 5 | 40 | $^{112}$RRQKR↓F$^{117}$ | - | - | - | LC846627 | Meso/Velo |
| NDV/chicken/Eth-WG2T10/2023 | Wolaita | < 5 | 40 | $^{112}$RRQKR↓F$^{117}$ | - | - | - | LC846628 | Meso/Velo |
| NDV/chicken/Eth-WG2C5/2023 | Wolaita | 5 | 40 | $^{112}$RRQKR↓F$^{117}$ | - | - | - | LC846625 | Meso/Velo |
| NDV/chicken/Eth-WS10C7/2023 | Wolaita | < 5 | 40 | $^{112}$RRQKR↓F$^{117}$ | < 60 | $10^{8.53}$ | $10^{8.53}$ | LC846629 | Velogenic |
| NDV/chicken/Eth-WS10T8/2023 | Wolaita | < 5 | 40 | $^{112}$RRQKR↓F$^{117}$ | - | - | - | LC846630 | Meso/Velo |
| NDV/chicken/Eth-HT2T1/2023 | Holeta | < 5 | 80 | $^{112}$RRQKR↓F$^{117}$ | < 60 | $10^{7.16}$ | $10^{7.16}$ | LC846610 | Velogenic |
| NDV/chicken/Eth-HT2T2/2023 | Holeta | < 5 | 40 | $^{112}$RRQKR↓F$^{117}$ | - | - | - | LC846611 | Meso/Velo |
| NDV/chicken/Eth-HT2T3/2023 | Holeta | < 5 | 40 | $^{112}$RRQKR↓F$^{117}$ | - | - | - | LC846612 | Meso/Velo |
| NDV/chicken/Eth-HT2T4/2023 | Holeta | < 5 | 40 | $^{112}$RRQKR↓F$^{117}$ | - | - | - | LC846613 | Meso/Velo |
| NDV/chicken/Eth-HT2T5/2023 | Holeta | < 5 | 80 | $^{112}$RRQKR↓F$^{117}$ | - | - | - | LC846614 | Meso/Velo |
| NDV/chicken/Eth-HT9T11/2023 | Holeta | 5 | 40 | $^{112}$RRQKR↓F$^{117}$ | - | - | - | LC846617 | Meso/Velo |
| NDV/chicken/Eth-HT9C8/2023 | Holeta | 5 | 40 | $^{112}$RRQKR↓F$^{117}$ | - | - | - | LC846615 | Meso/Velo |
| NDV/chicken/Eth-HT9C13/2023 | Holeta | 15 | 40 | $^{112}$RRQKR↓F$^{117}$ | - | - | - | LC846616 | Meso/Velo |
| NDV/chicken/Eth-HT9C15/2023 | Holeta | 15 | 40 | $^{112}$RRQKR↓F$^{117}$ | < 60 | $10^{8.5}$ | $10^{8.5}$ | LC846614 | Velogenic |
| NDV/chicken/Eth-BS2T13/2023 | Bishoftu | < 5 | 40 | $^{112}$RRQKR↓F$^{117}$ | - | - | - | LC846608 | Meso/Velo |
| NDV/chicken/Eth-BS2T14/2023 | Bishoftu | 5 | 40 | $^{112}$RRQKR↓F$^{117}$ | < 60 | $10^{7.83}$ | $10^{7.83}$ | LC846609 | Velogenic |
| NDV/chicken/Eth-AA3/T1/2023 | Addis Ababa | < 5 | 80 | $^{112}$RRQKR↓F$^{117}$ | < 60 | $10^{8.83}$ | $10^{8.83}$ | LC846607 | Velogenic |
| NDV/chicken/Eth-SLBM12C1/2023 | Sebeta LBM | < 5 | 40 | $^{112}$RRQKR↓F$^{117}$ | < 60 | $10^{7.5}$ | $10^{7.5}$ | LC846618 | Velogenic |

↓: $F_0$ cleavage site; -: test not conducted; Meso/Velo: Mesogenic or Velogenic

### Phylogeny of NDV isolates

A phylogenetic tree based on the partial *F* gene nucleotide sequences showed that all this study isolates belong to genotype VII sub-genotype VII.1.1 and cluster into two distinct groups. The isolate from the LBM (NDV/chicken/Eth_SLBM12C1/2023) clustered separately from the other 24 isolates. All of the current isolates clustered with Eritrean and Middle East strains. Isolates from Holeta clustered into two separate branches. Those collected during the winter season (NDV/chicken/Eth_HT2T1-T5/2023) clustered with an isolate from Wolaita collected during the same season. Meanwhile, isolates from Holeta collected in the autumn season clustered with samples of Bishoftu (NDV/chicken/Eth_BS2T13 and 14/2023) collected during winter. Except for the one sample, all Wolaita isolates clustered into one branch with an isolate from Addis Ababa (NDV/chicken/Eth_AA3T1/2023) irrespective of sample collection season (Fig. 2).

## Discussion

ND poses a significant threat to poultry production worldwide, particularly in developing countries like Ethiopia, where outbreaks continue to cause severe economic losses [18]. In this study, the antigenicity, genotype and pathogenicity of currently circulating NDV strains in Ethiopia were assessed. These analyses revealed that all isolates in this study were velogenic and they belonged to genotype VII 1.1, which is different form the vaccine. The HI test revealed significant 8-fold antigenic differences between the HB1 vaccine and the 25 Ethiopian strains. However, it has been reported that the HI test does not always reflect neutralizing antibodies [48] that are effective in preventing the virus infection, and in this study, the VN test showed no significant difference between the vaccine strain and the field strains, indicating that the HB1 vaccine can protect effectively against these field strains infection regardless of genotype differences. On the other hand, it remains unclear why many ND outbreaks were reported in vaccinated chicken flocks. In general, it is believed that genotype-matched vaccines are suitable for better protection than genotype-mismatched vaccines [34, 35]. Since the HI test showed significant differences between their antigenicity, vaccines that are genetically and antigenically identical to the field strains even in HI test, may be necessary to achieve more effective prevention of the disease. The other possible reasons are the vaccine failure, immunosuppression due to other

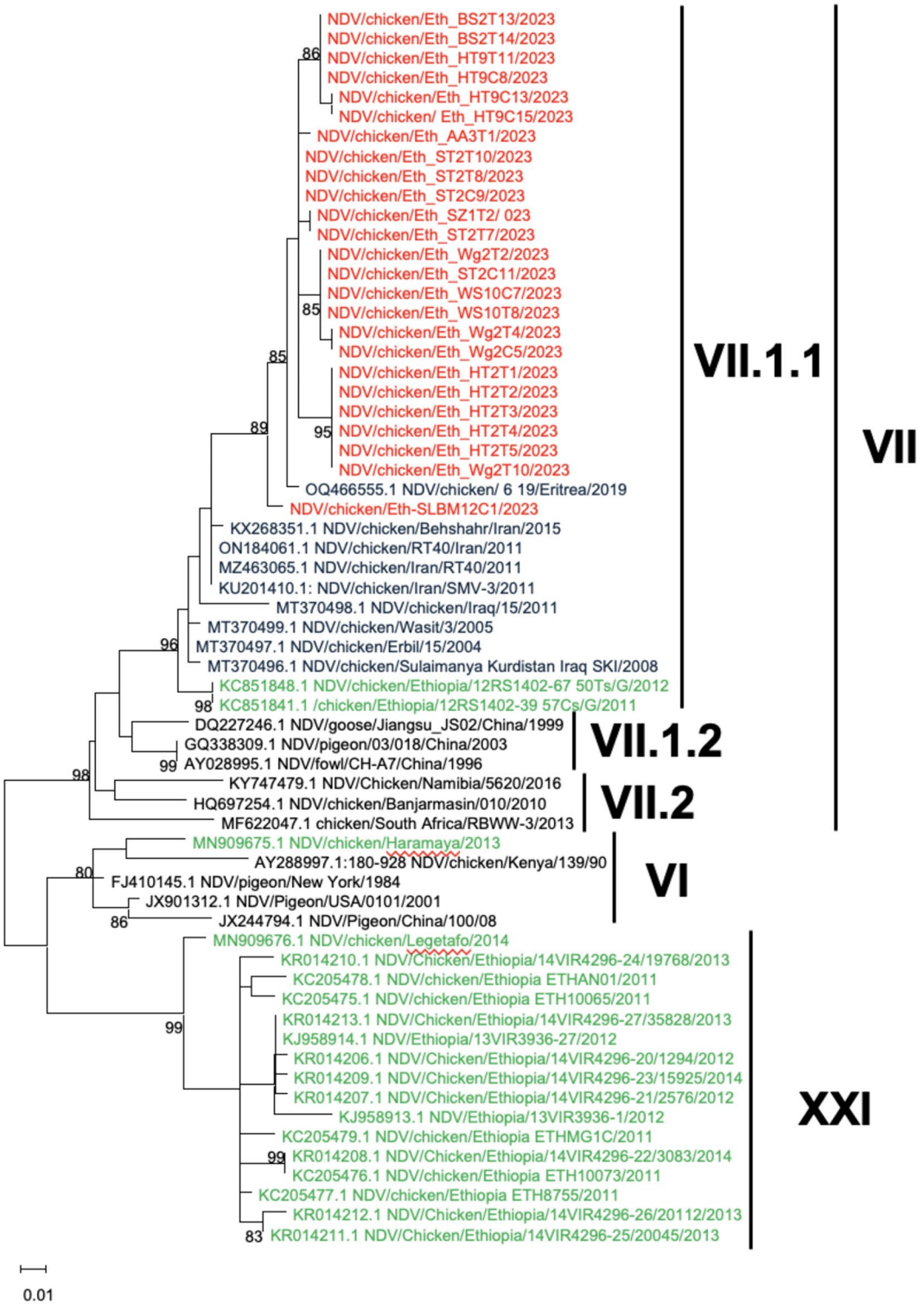


**Fig. 2** Phylogenetic tree based on partial *F* gene sequences (aligned 218 nucleotides from all strains). The tree was constructed using the maximum likelihood method with the general time-reversible model and 1000 bootstrap replicates. Bootstrap values ≥ 80% are shown at the nodes. Red: Ethiopian NDV isolates in this study; Green: Previous Ethiopian NDV strains; Black: Top hit NDV strains from other countries closely related to Ethiopian strains selected from the 1000 sequences retrieved by BLAST search and reference sequences for genotyping reported by Dimitrov et al. [9] for genotype VII.1.2, VII.2 and VI.

infectious diseases such as infectious bursal disease, or poor hygiene management. To establish more effective control measures for this disease in future, further investigation is required.

Only two of the 25 strains in this study remained active after 15 min at 56 °C, which is less than that reported in a previous study, in which 6 of 10 strains remained active after 1 to 2 h at 56 °C [29]. Because thermostable strains can survive for long periods in the environment [45], they present a greater risk for virus transmission in tropical countries like Ethiopia.

Phylogenetic analysis based on the partial *F* gene nucleotide sequence revealed that all of the Ethiopian NDVs isolated in this study belong to genotype VII sub-genotype VII.1.1. Although all isolates in this study belong to the same sub-genotype VII.1.1 due to less than 0.05 genetic distance [9], the isolates from the same locations did not end up in the same branch, suggesting slight differences among NDVs circulating in Ethiopia. Similar to our findings, genotype VII was detected recently in the central part and mid rift-valley in Ethiopia [21, 31], In contrast, most of the previous NDV isolates belonged to genotype XXI (Fig. 2).

As of May 30, 2024, GenBank had only 19 (7 full and 12 partial) *F* gene sequences of virulent NDV strains from Ethiopian chickens. All except two strains were classified as previous genotype VI [22, 28, 29], which has more recently been divided into two genotypes (VI and XXI). Only one of the previous Ethiopian strains belongs to the current genotype VI, while the other 16 belong to the new genotype XXI (Fig. 2). Only the remaining two previous NDV strains were clustered with the current genotype VII.1.1 [46], suggesting that there has been a shift in the most prevalent genotype of NDVs in Ethiopia which could be due to vaccination effect or host related factors [47, 48].

Genetic analysis showed that the current NDV strains isolated from Ethiopian chicken farms were genetically close to strains detected in the Middle East (Iran and Iraq) and Eritrea. Outbreaks in these countries have been attributed to the same genotype [49, 50]. Genotype VII.1.1 was also detected in wild birds and chickens in Egypt [51, 52]. Since all these countries are located close to the Red Sea, a global trade route [48], the virus might have been brought to Ethiopia through poultry trade in the region.

In the present study, NDV was detected more frequently in backyard chickens with no vaccination history than in commercial chickens, agreeing with recently reported data from Ethiopia's central part and Mid rift-valley [21]. Together, these studies show that NDV vaccines are partly effective. Since a small number of LBM chicken samples were tested, we cannot determine the prevalence of NDV in this study. However, Delesa et al. [22] found a high prevalence of NDV in chickens in central Ethiopia (44 of 146 (30.1%)) of which 29 (19.7%) were virulent strains from LBM in Addis Ababa. Similarly, in North Gondar (Northwest Ethiopia), NDV was detected in 39.2% (188/480) of LBM chickens among which 39/95 (41.1%) were virulent strains [24]. Detection of virulent NDVs with such high prevalence from LBMs demonstrates the importance of LBMs as a hotspot for the spread of NDV and other poultry diseases, as chickens from different areas gather there and are either returned back or transferred to other locations at the end of the market day.

The F proteins of all isolates had an $^{112}$RRQKR↓F$^{117}$cleavage site, which is a characteristic of mesogenic/velogenic strains [2]. Additionally, an in vivo study of representative isolates confirmed that all were velogenic pathotypes with MDTs less than 60 h. Velogenic NDV strains have also been reported in several parts of Ethiopia [19, 21, 28, 29, 31, 46], suggesting that there is a need to implement effective control measures.

## Conclusions

All of the virulent NDV strains isolated in this study belong to genotype VII.1.1. The VN test confirmed that the classical vaccine available in the country provides protection despite antigenic differences between field and vaccine strains, as indicated by the HI test. Therefore, the cause of NDV outbreaks in vaccinated chickens in Ethiopia requires further investigation. Due to Ethiopia's geographical location, virulent NDVs can easily spread to and from Ethiopia, the Middle East, and other neighboring countries through the movement of fomites, poultry, and poultry products. Thus, studies of the genotypes and antigenicity of NDVs in Ethiopia should help to design control measures for ND in the region.

### Abbreviations

| | |
|---|---|
| NDV | Newcastle disease virus |
| ND | Newcastle disease |
| MDT | Mean death time |
| HI | Hemagglutinin inhibition |
| HB1 | Hitchner B1 |
| ECE | Embryonated chicken egg |
| LBM | Live bird market |
| AHI | Animal Health Institute |
| $EID_{50}$ | 50% egg infectious dose |
| $ELD_{50}$ | 50% egg lethal dose |
| MEGA | Molecular Evolutionary Genetic Analysis |
| TPCK trypsin | N-tosyl-L-phenylalanine chloromethyl ketone |
| VN | Virus neutralization |
| VNT | Virus neutralization titer |
| CPE | cytopathic effect |

### Acknowledgements

The first author (BDM) would like to express gratitude to Ministry of Education, Culture, Sports, Science and Technology (MEXT) for providing a scholarship during the study.

**Author contributions**
Conceptualization: TY, TU, TRC, BDM; Data acquisition: BDM; Resource acquisition: TY, TU, TRC; Data analysis, BDM, TY; Original draft preparation, BDM; Review and editing TY, TU, TRC. Supervision: TY. All authors read and approved the final manuscript.

**Funding**
The present study was supported by International Platform for Dryland Research and Education (IPDRE). The funding body had no role in the conceptualization, design, data collection, analysis, decision to publish, or preparation of the manuscript.

**Data availability**
The sequence datasets generated during the current study are available in the GenBank/EMBL/DDBJ database. Accession number (LC846607- LC846631).

## Declarations

**Ethics approval and consent to participate**
Not Applicable.

**Consent for publication**
Not Applicable.

**Competing interests**
The authors declare no competing interests.

Received: 13 November 2024 / Accepted: 28 February 2025
Published online: 06 March 2025

**References**

1. Rima B, Balkema-Buschmann A, Dundon WG, Duprex P, Easton A, Fouchier R, et al. ICTV virus taxonomy profile: Paramyxoviridae. J Gen Virol. 2019;100(12):1593–4.
2. World Organization for Animal Health (WOAH). NEWCASTLE DISEASE (INFECTION WITH NEWCASTLE DISEASE VIRUS). 2021.
3. Desingu PA, Singh SD, Dhama K, Vinodhkumar OR, Nagarajan K, Singh R, et al. Pathotyping of Newcastle disease virus: A novel single BsaHI digestion method of detection and differentiation of avirulent strains (lentogenic and mesogenic vaccine strains) from virulent virus. Microbiol Spectr. 2021;9(3):e00989–21.
4. Alexander DJ. Newcastle disease diagnosis. Newcastle disease: Springer; 1988. pp. 147–60.
5. Ganar K, Das M, Sinha S, Kumar S. Newcastle disease virus: current status and our Understanding. Virus Res. 2014;184:71–81.
6. Czeglédi A, Ujvári D, Somogyi E, Wehmann E, Werner O, Lomniczi B. Third genome size category of avian paramyxovirus serotype 1 (Newcastle disease virus) and evolutionary implications. Virus Res. 2006;120(1–2):36–48.
7. Jadhav A, Zhao L, Ledda A, Liu W, Ding C, Nair V, et al. Patterns of RNA editing in Newcastle disease virus infections. Viruses. 2020;12(11):1249.
8. Römer-Oberdörfer A, Werner O, Veits J, Mebatsion T, Mettenleiter TC. Contribution of the length of the HN protein and the sequence of the F protein cleavage site to Newcastle disease virus pathogenicity. J Gen Virol. 2003;84(11):3121–9.
9. Dimitrov KM, Abolnik C, Afonso CL, Albina E, Bahl J, Berg M, et al. Updated unified phylogenetic classification system and revised nomenclature for Newcastle disease virus. Infect Genet Evol. 2019;74:103917.
10. Lomniczi B, Wehmann E, Herczeg J, Ballagi-Pordany A, Kaleta E, Werner O, et al. Newcastle disease outbreaks in recent years in Western Europe were caused by an old (VI) and a novel genotype (VII). Arch Virol. 1998;143:49–64.
11. Liu H, Wang Z, Wu Y, Wu Y, Sun C, Zheng D, et al. Molecular characterization and phylogenetic analysis of new Newcastle disease virus isolates from the Mainland of China. Res Vet Sci. 2008;85(3):612–6.
12. Miller PJ, Haddas R, Simanov L, Lublin A, Rehmani SF, Wajid A, et al. Identification of new sub-genotypes of virulent Newcastle disease virus with potential panzootic features. Infect Genet Evol. 2015;29:216–29.
13. ETHIOPIA FDRO, CENTRAL STATISTICAL AGENCY (CSA). AGRICULTURAL SAMPLE SURVEY VOLUME II REPORT ON LIVESTOCK AND LIVESTOCK CHARACTERISTICS 2021.
14. (SELCO) SFi. Empowering Smallholder Poultry Production Through Renewable Energy: Challenges and the Way Forward. 2022.
15. Sime AG. Review on poultry production, processing, and utilization in Ethiopia. Int J Agricultural Sci Food Technol. 2022;8(2):147–52.
16. Terefe D, Belaineh R, Chaka H, Sombo M, Mekuria A, Gugsa KL, et al. Serological and molecular study of Newcastle disease virus in village chickens in selected rift-Valley areas, Ethiopia. J Vet Sci Technol. 2015;6:1–4.
17. Nasser M, Lohr J, Mebratu G, Zessin K-H, Baumann M, Ademe Z. Oral Newcastle disease vaccination trials in Ethiopia. Avian Pathol. 2000;29(1):27–34.
18. World Animal Health Information. System (WAHIS). Six-monthly report. 2024.
19. Chaka H, Goutard F, Gil P, Abolnik C, Servan de Almeida R, Bisschop S, et al. Serological and molecular investigation of Newcastle disease in household chicken flocks and associated markets in Eastern Shewa zone, Ethiopia. Trop Anim Health Prod. 2013;45:705–14.
20. Mamo T, Yimer L. Serological investigation of Newcastle disease in selected districts of Buno Bedelle zone, Ethiopia. Veterinary Medicine: Res Rep. 2021:253–9.
21. Alemu EE, Senbata B, Sombo M, Guyassa C, Alemayehu DH, Kidane E, et al. Molecular detection and characterization of Newcastle disease virus from chickens in Mid-Rift Valley and central part of Ethiopia. Veterinary Medicine: Res Rep. 2024;149:57.
22. Mulisa DD, W/Kiros MK, Alemu RB, Keno MS, Furaso A, Heidari A, et al. Characterization of Newcastle disease virus and poultry-handling practices in live poultry markets. Ethiopia SpringerPlus. 2014;3:1–6.
23. Chaka H, Goutard F, Bisschop SP, Thompson PN. Seroprevalence of Newcastle disease and other infectious diseases in backyard chickens at markets in Eastern Shewa zone, Ethiopia. Poult Sci. 2012;91(4):862–9.
24. Haile B, Fentie T, Kassa T. The role of live chicken markets as a source of replication and dissemination of Newcastle disease virus in chickens, Northwest Ethiopia. Poult Sci. 2020;99(11):5415–21.
25. Awais M, Wajid A, Goraichuk IV, Batool A, Rahim A, Anif A, et al. Surveillance and assessment of risk factors for Newcastle disease virus from live bird retail stalls in Lahore district of Pakistan. Avian Dis. 2022;66(3):278–85.
26. Tsaxra JB, Gallardo RA, Abolnik C, Chengula AA, Msoffe PL, Muhairwa AP, et al. Spatiotemporal patterns and risk factors for Newcastle disease virus among chickens in a Tanzania live bird market. Transbound Emerg Dis. 2024;2024(1):5597050.
27. Zelalem GA, Kidist T, Belachew B, Tadios H, Addisalem H. Evaluation of the Newcastle disease antibody level after vaccination regimes in chickens in Debrezeit agricultural research center, Ethiopia. J Veterinary Med Anim Health. 2014;6(1):7–12.
28. Damena D, Fusaro A, Sombo M, Belaineh R, Heidari A, Kebede A, et al. Characterization of Newcastle disease virus isolates obtained from outbreak cases in commercial chickens and wild pigeons in Ethiopia. Springerplus. 2016;5:1–8.
29. Bari FD, Gelaye E, Tekola BG, Harder T, Beer M, Grund C. Antigenic and molecular characterization of virulent Newcastle disease viruses Circulating in Ethiopia between 1976 and 2008. Research and reports. Veterinary Medicine; 2021. pp. 129–40.
30. Worku T, Dandecha M, Shegu D, Aliy A, Negessu D. Isolation and molecular detection of Newcastle disease virus from field outbreaks in chickens in central Ethiopia. Veterinary Medicine: Res Rep. 2022:65–73.
31. Yadeta W, Amosun E, Mohammed H, Woldemedhin W, Sherefa K, Legesse A, et al. Isolation and genetic characterization of genotype VII velogenic pathotype Newcastle disease virus from commercial chicken farms in central Ethiopia, distinct from the local vaccine strains. Viruses. 2024;16(8):1249.
32. Miller PJ, King DJ, Afonso CL, Suarez DL. Antigenic differences among Newcastle disease virus strains of different genotypes used in vaccine formulation affect viral shedding after a virulent challenge. Vaccine. 2007;25(41):7238–46.
33. Wajid A, Basharat A, Bibi T, Rehmani SF. Comparison of protection and viral shedding following vaccination with Newcastle disease virus strains of different genotypes used in vaccine formulation. Trop Anim Health Prod. 2018;50(7):1645–51.
34. Sultan HA, Elfeil WK, Nour AA, Tantawy L, Kamel EG, Eed EM, et al. Efficacy of the Newcastle disease virus genotype VII. 1.1-matched vaccines in commercial broilers. Vaccines. 2021;10(1):29.
35. Roohani K, Tan SW, Yeap SK, Ideris A, Bejo MH, Omar AR. Characterisation 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.
36. Wise MG, Suarez DL, Seal BS, Pedersen JC, Senne DA, King DJ, et al. Development of a real-time reverse-transcription PCR for detection of Newcastle disease virus RNA in clinical samples. J Clin Microbiol. 2004;42(1):329–38.

37. Organization WH. WHO manual on animal influenza diagnosis and surveillance. World Health Organization; 2002.
38. Grund C, Werner O, Gelderblom H, Grimm F, Kösters J. Avian paramyxovirus serotype 1 isolates from the spinal cord of parrots display a very low virulence. J Veterinary Med Ser B. 2002;49(9):445–51.
39. Wen G, Hu X, Zhao K, Wang H, Zhang Z, Zhang T, et al. Molecular basis for the thermostability of Newcastle disease virus. Sci Rep. 2016;6(1):22492.
40. Liu H, Zhao Y, Zheng D, Lv Y, Zhang W, Xu T, et al. Multiplex RT-PCR for rapid detection and differentiation of class I and class II Newcastle disease viruses. J Virol Methods. 2011;171(1):149–55.
41. Reed LJ, Muench H. A simple method of estimating fifty per cent endpoints. 1938.
42. Maqbool R, Gul I, Wani S, Kashoo Z, Gul N, Islam SU et al. Molecular characterisation and dynamics of the fusion protein of an emerging genotype VIIi of Newcastle disease virus. Agricultural Res. 2024:1–12.
43. Ramzy N, Elsamadony H, Mekky H, Fedawy H, Saad A. Isolation, genotyping, and sub-genotyping of Newcastle disease virus from commercial broiler chickens in Northern Egypt. 2024.
44. Kumar S, Stecher G, Li M, Knyaz C, Tamura K. MEGA X: molecular evolutionary genetics analysis across computing platforms. Mol Biol Evol. 2018;35(6):1547–9.
45. Kinde H, Utterback W, Takeshita K, McFarland M. Survival of exotic Newcastle disease virus in commercial poultry environment following removal of infected chickens. Avian Dis. 2004;48(3):669–74.
46. Fentie T, Heidari A, Aiello R, Kassa T, Capua I, Cattoli G, et al. Molecular characterization of Newcastle disease viruses isolated from rural chicken in Northwest Ethiopia reveals the circulation of three distinct genotypes in the country. Trop Anim Health Prod. 2014;46:299–304.
47. 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.
48. Adam AHA. The geographical nature of the red sea area and its impact on the material culture: case study: Aqiq port. Human interaction with the environment in the red sea. Brill; 2017. pp. 228–53.
49. Molouki A, Sotani M, Fallah Mehrabadi MH, Shoushtari A, Abtin A, Mahmoudzadeh Akhijahani M, et al. Predominance of fourth panzootic Newcastle disease virus subgenotype VII. 1.1 in Iran and its relation to the genotypes Circulating in the region. Curr Microbiol. 2021;78(8):3068–78.
50. Mihreteab B, Kgotlele T, Neguse F, Petros Y, Habtemariam H, Berhane Y, et al. Phylogenetic analysis of Newcastle disease virus detected in Eritrea between 2017 and 2021. Avian Pathol. 2023;52(6):426–31.
51. Abd Elfatah KS, Elabasy MA, El-Khyate F, Elmahallawy EK, Mosad SM, El-Gohary FA, et al. Molecular characterization of velogenic Newcastle disease virus (Sub-genotype Vii. 1.1) from wild birds, with assessment of its pathogenicity in susceptible chickens. Animals. 2021;11(2):505.
52. Amer S, Kutkat M, Abdel-Baki M, Maatouq A, Kutkat O, Ahmed H, et al. Epidemiological surveillance for the newly classified Newcastle disease virus genotype VII. 1.1 in chicken flocks in Egypt. Adv Anim Vet Sci. 2022;10(3):451–8.

## Publisher's note