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# Phylogenetic analysis of virulent strains of the Newcastle disease virus isolated from deceased chickens in Tanzania's Morogoro and Iringa regions

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## Abstract

**Background** Newcastle disease (ND) is a viral disease affecting a wide range of bird species and has a considerable financial impact on the world's poultry market. The ND virus (NDV) strains currently circulating in poultry throughout Africa, and especially in East Africa, exhibit significant genetic variation.

**Objectives** The primary objective of the present investigation was to investigate the NDV genotypes in chickens raised in backyards in Tanzania's Morogoro and Iringa districts, which were associated with ND outbreaks.

**Methods** Two tissue samples from chickens taken during a suspected ND outbreak in Tanzania's Morogoro (Eastern zone) and Iringa (Southern highlands zone) were subjected to reverse transcription polymerase chain reaction targeting the fusion (F) and hemagglutinin-neuraminidase (HN) genes, followed by sequencing.

**Results** Based on comprehensive analysis of the entire F and HN gene sequences, the viruses were categorized as genotype VII and displayed significant genetic similarity with NDV strains previously identified in Mozambique, South Africa, Zambia, Zimbabwe, Botswana, Southeast Asia, and China. The uniformity in the amino acid cleavage site motif of the F protein across the examined NDV isolates, characterized by 112R–R–Q/K–K–R–F117, indicates their classification as virulent strains.

**Conclusion** Regularly characterizing circulating strains and expanding the study to other parts of Tanzania may help to enhance disease control by giving a more precise picture of the situation regarding ND, especially in light of the issues posed by NDV genotype VII elsewhere.

**Keywords** Newcastle disease · Newcastle disease virus · Genotypes · Virulence · Phylogeography · Africa

## Abbreviations

| | |
|---|---|
| AOaV-1 | *Avian Orthoavulavirus 1* |
| ND | Newcastle disease |
| NDV | Newcastle disease virus |
| OIE | Office International des Epizooties |

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| | |
|---|---|
| WOAH | World Organisation for Animal Health |
| ACDPa | Advisory Committee on Dangerous Pathogens |
| BPFs | Backyard poultry farms |
| SPF | Specific-pathogen-free |
| ECEs | Embryonated chicken eggs |
| PBS | Phosphate-buffered saline |
| HA | Hemagglutination assay |
| RNA | Ribonucleic acid |
| cDNA | Complementary deoxyribonucleic acid |
| RT-PCR | Reverse transcription polymerase chain reaction |

## 1 Background

Newcastle disease (ND) impacts a diverse array of avian species and has significant global influence over the commercial poultry sector. Given ND's economic implications on the chicken industry [1], it warrants notification to the World Organisation for Animal Health (WOAH). The causative agent of ND is *Orthoavulavirus javaense*, previously named Avian orthoavulavirus 1 and commonly known as ND virus (NDV; used herein) within the family *Paramyxoviridae* and genus *Orthoavulavirus* [2]. NDV's single-stranded, negative-sense RNA genome encodes for six proteins: nucleoprotein (N), phospho-protein (P), matrix protein (M), fusion protein (F), hemagglutinin-neuraminidase (HN), and large polymerase (L). RNA editing of P messenger RNA (mRNA) facilitates the expression of supplementary proteins, V and W [3]. The interplay between the envelope surface glycoproteins F and HN mediates infection of host cells by NDV [4]. The HN protein activates the F protein, facilitating the attachment of virus particles to sialic acid-containing receptors on cell surfaces [5]. This process facilitates the fusion between the viral envelope and the host cell membrane [4]. Distinctions among lentogenic, mesogenic, and velogenic strains of Newcastle Disease Virus (NDV) are based on the cleavage site of the F protein: lentogenic strains are characterized by an amino acid sequence of 112 G/E-K/R-Q-G/E-R-L117, while all mesogenic and velogenic NDV strains exhibit the sequence 112R/K-R-Q-R/K-R-F117 within the F protein [6].

All bird species are thought to be susceptible to NDV, which infects at least 250 different species [7]. Non-avian hosts, including humans, cattle, sheep, camels, pigs, rabbits, mink, mice, and hamsters, have been shown to exhibit signs of NDV infections [8]. Although human-to-human transmission has not been reported, bird-to-human transmission has been demonstrated [9]. In humans, NDV has been identified in washings of the conjunctiva sac, nasal discharge, saliva, blood samples, and urine. Thus, ND could be of greater importance to humans than is currently appreciated [10].

Similar to other African nations, ND results in considerable losses to poultry in Tanzania [11]. In Tanzania, 86% of livestock-keeping families keep chickens, making them the most frequent type of livestock [12]. Although the adoption of vaccination against ND with LaSota and I-2 vaccines has become increasingly common in recent years on traditional farms in Tanzania [13], frequent outbreaks still occur in backyard poultry farms (BPFs) [14].

Due to the continuous genetic evolution and wide circulation of NDV in poultry populations, and multiple transmissions in poultry, the emergence of novel genetic NDV variants is ongoing. For these reasons, this study aimed to investigate the phylogenetic and genetic characteristics of NDV isolates from Iringa (Southern highlands zone) and Morogoro (Eastern zone) in January 2022 in Tanzania. These data facilitate tracking NDV evolution and genetic diversity and improving disease surveillance in the country. Furthermore, genetic sequences of NDV can be used in the design and future development of genotype-matched vaccines to enhance the control of NDV in Tanzania and neighboring countries.

## 2 Materials and methods

### 2.1 Sampling

In 2022, two municipalities in different regions of Tanzania, namely Iringa (Southern highlands zone) and Morogoro (Eastern zone) (Fig. 1) in Tanzania reported suspected ND outbreaks. Proventriculus tissues collected from two dead vaccinated chickens showed lesions consistent with NDV infection during postmortem examination.

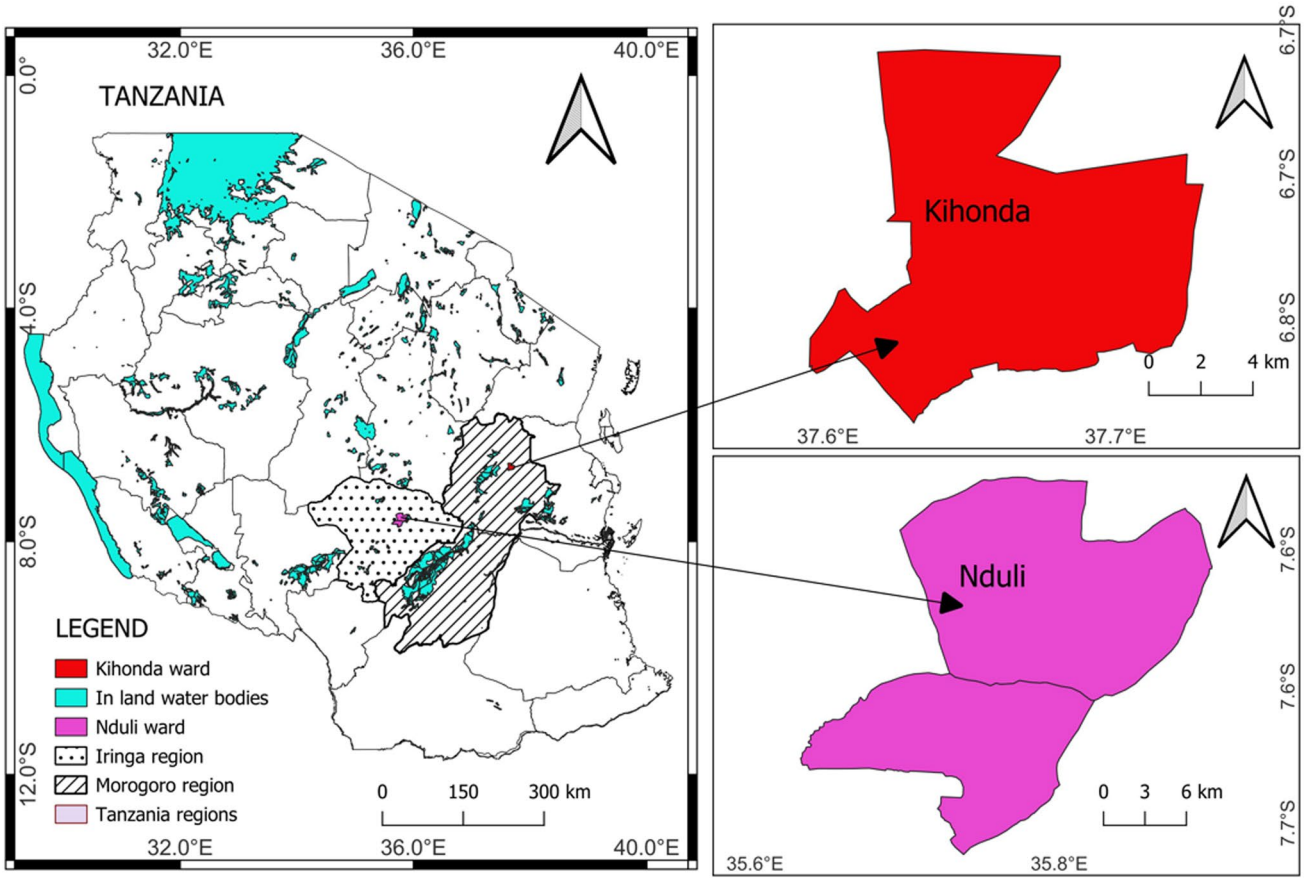


**Fig. 1** A cartographic representation of Tanzania, indicating the provenance of the samples tested in this research. The samples were procured from Nduli in the Iringa district, as well as Kihonda in the Morogoro district. The image was generated using QGIS software iteration 3.24.3, leveraging data from the DIVA-GIS repository accessible at https://www.diva-gis.org/Data

## 2.2 Virus isolation

The virus was isolated according to procedures detailed in prior investigations [15] consistent with the guidelines provided by WOAH [16]. Ten-day-old, specific-pathogen-free (SPF) embryonated chicken eggs (ECEs) were treated with 100 µL of clarified tissue homogenate in their allantoic cavities. Control eggs were treated with sterile saline. Eggs were incubated at 37 °C for 4–5 days. Eggs were candled twice daily, and the embryos were visually examined to determine their viability. Any embryos that perished within 24 h post-infection (PI) were excluded from the study, as their deaths were attributed to non-specific causes. Those embryos that survived longer than 24 h or the entire incubation period were cooled at 4 °C overnight before harvesting the allantoic fluid (AF).

## 2.3 Hemagglutination assay

A hemagglutination (HA) assay using 1% chicken red blood cells was used to test the extracted AF from each infected egg using established protocols [16]. HA-negative samples were passaged for an additional two passages in ECEs, followed by an HA assay to confirm the absence of hemagglutination patterns. HA-positive samples were further tested for NDV by reverse transcription polymerase chain reaction (RT-PCR).

## 2.4 Reverse transcription polymerase chain reaction (RT-PCR)

RNA was extracted using the QIAamp Viral RNA Extraction Kit (Qiagen, California, USA) per the manufacturer's instructions. We generated cDNA from purified RNA using the SuperScript III First-Strand Synthesis System (InvitrogenTM, CA, USA). Briefly, 8 µL of RNA was mixed with 1 µL of random hexamer primers (50 ng/µL) and 1 µL of dNTPs mix (10 mM).

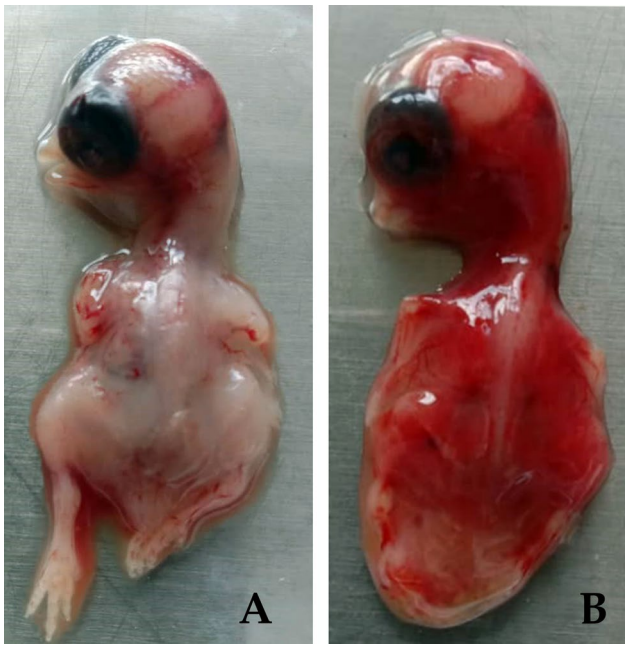


**Fig. 2** Findings from the isolation of NDV in the allantoic cavities of chicken embryos that were 10 days old. **A** The embryo that was injected with the negative control mixture. **B** An infected embryo with a positive sample that exhibits dwarfism, aberrant feathering, and subcutaneous hemorrhages throughout the body

The mixture was then incubated at 65 °C for 5 min to denature RNA secondary structures and cooled on ice for five minutes. We then added 2 µL of 10 × RT buffer, 4 µL of 25 mM MgCl2, 2 µL of 0.1 M Dithiothreitol (DTT), 1 µL of RNAseOUT (40 U/µL), and 1 µL of SuperScript III RT enzyme (200 U/µL). The mixture was incubated at 25 °C for 5 min, followed by incubation at 50 °C for 50 min and at 85ºC for 5 min to terminate the reaction. After cooling on ice, 1 µL of RNase H was added, and the mixture was incubated at 37 °C for 20 min. The final cDNA products were stored at -80 °C until further analysis. RT-PCR was conducted on the two HA-positive isolates. The initial step involved cDNA amplifications using a SuperScript III RT kit (Invitrogen, Valencia, CA, USA) as per the manufacturer's guidelines with specific primers designed for the F and HN genes [17]. The reactions underwent an initial denaturation at 95 ºC for 10 min, followed by 35 cycles consisting of 30 s at 95 ºC for denaturation, 1 min for annealing, and 2 min at 68 ºC for extension. The final extension step was carried out at 68 ºC for 10 min. The primer annealing temperatures were set at 58 ºC for the F gene and 56 ºC for the HN gene. Subsequently, electrophoresis was used to separate the PCR products on a 1.5% agarose gel stained with GelRed (Phenix, Hong Kong, China).

## 2.5 Sequencing, sequence assembly, alignment, and phylogeny

Dideoxynucleotide Sanger sequencing of PCR products was conducted at Macrogen Laboratory (Macrogen Inc., Amsterdam, The Netherlands) on an ABI 3730xl DNA analyzer (Applied Biosystems, Foster City, CA).. Sequence Scanner Software v2.0 (appliedbiosystems) was used to check the quality of the chromatograms and Bioedit Sequence Alignment Editor v7.2.5.0 [18] was used to assemble and edit the obtained nucleotide sequences. The obtained sequences and those found in the NCBI nucleotide database were compared using the NCBI BLAST tool. Open reading frames (ORF) were predicted using the GENEQUEST program of DNASTAR Lasergene 17 (version 17.5.0.48; Madison, WI). Nucleotide alignment was performed by the ClustalW method built in MEGAX software [19]. Comparisons were made between the sequences from this work and reference sequences from the NCBI database. The reference sequences selected for phylogenetic analysis were chosen according to a set of criteria designed to capture global genetic diversity. These criteria included representation from diverse geographic regions, strains from different genotypes, availability of complete or near-complete genome sequences, and sourcing from reputable databases with comprehensive metadata. The full nucleotide sequence of the F and HN genes was used as the basis for a phylogenetic analysis using MEGA X software. The Maximum Likelihood method utilized the General Time-Reversible (GTR) model incorporating a discrete gamma distribution (+ G) and permitting invariant sites (+ I). The statistical analysis was based on 1000 bootstrap re-sampling [19]. Analysis of amino acid substitutions in F and HN proteins were assessed [20]. The sequences derived from this investigation were uploaded to the NCBI GenBank with accession numbers PP195786 for the HN gene and PP216548 and PP216549 for the F genes.

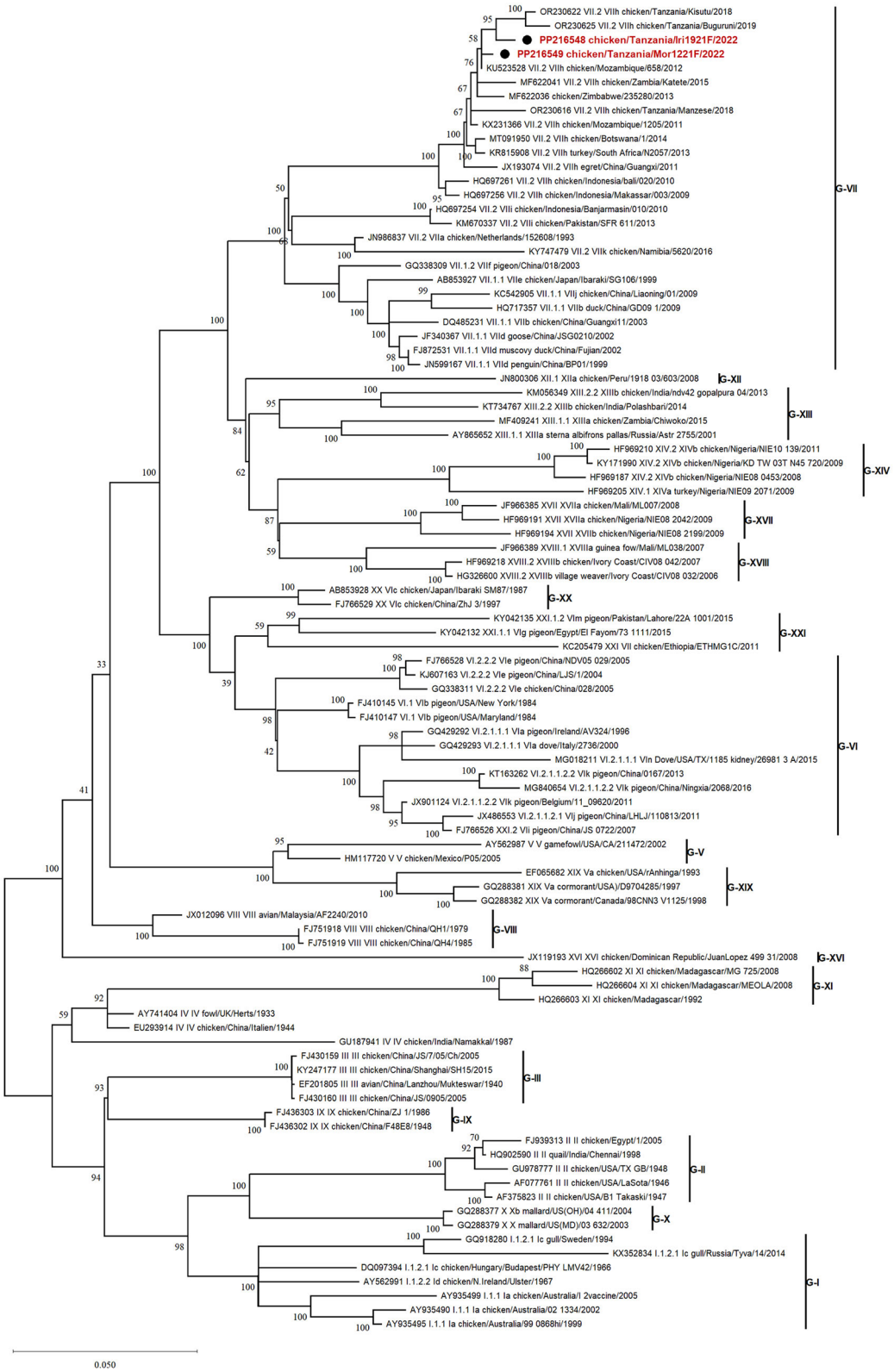


**Fig. 3** Phylogenetic analysis using the whole NDV strain *F* gene sequences. The two nucleotide sequences that are the subject of this investigation are designated in red (subgenotypes VII.2) and indicated with ●. We included 94 class II sub-genotype sequences. The sequences were aligned using ClustalW in MEGA X. We reconstructed a phylogenetic tree using the maximum likelihood approach with 1000 bootstraps. To estimate the evolutionary history while taking into consideration invariant sites (+I), the general time-reversible (GTR) model with a discrete Gamma distribution (+G). The first, second, third, and noncoding codon locations are all included in the inquiry. The names given to each sequence in the phylogenetic tree correspond to the GenBank accession number, followed by the sub-genotype, isolation country, strain identifier, and isolation year

**Table 1** Comparison of F genes nucleotide sequences described in this study with previously published NDV strains available at GenBank

| Accession number | Strain origin | Year of isolation | % of similarity to Mor1221 | % similarity to Iri1921 | References |
|---|---|---|---|---|---|
| KU523528 | Mozambique | 2012 | 99.70 | 99.04 | [21] |
| MF622045 | South Africa | 2013 | 99.04 | 98.38 | [22] |
| MF622036 | Zimbabwe | 2013 | 98.86 | 98.20 | [22] |
| MT091950 | Botswana | 2014 | 98.80 | 98.14 | [23] |
| MF622041 | Zambia | 2015 | 98.68 | 97.90 | [22] |
| ON210973 | Malaysia | 2011 | 98.92 | 98.26 | [24] |
| KU175233 | China | 2013 | 98.68 | 98.02 | [25] |

## 3 Results

### 3.1 Virus isolation and confirmation

The inoculated embryos showed extensive hemorrhage and congestion and died within 48–96 h after the first passage (Fig. 2). AF from the inoculated ECE was examined for hemagglutinating activity by HA followed by confirmation using RT-PCR. Both samples tested positive for hemagglutination activity and were positive for the presence of F and HN genes of NDV by RT-PCR, confirming the presence of NDV in the two isolates.

### 3.2 Sequence and phylogenetic analyses

We obtained two full F gene sequences from Iringa (Iri1921; accession number PP216548) and Morogoro (Mor1221; accession number PP216549). Both sequences were 1662 bp in length.

The nucleotide assembly sequences generated for the HN genes of our two isolates yielded a single good-quality result, namely one from the Morogoro isolate (Mor1221). We were unable to obtain the HN gene for the second isolate from Iringa (Iri1921). The full HN gene of the Morogoro strain (Mor1221) has the accession number PP195786 (1755 bp in length).

#### 3.2.1 F gene

A phylogenetic analysis of the F genes of strains Iri1921 and Mor1221 showed that both viruses clustered within sub-genotype VII.2 (Fig. 3). Based on sequence similarity, NDV strains previously isolated from Africa and Asia were closely related to the two strains presented here (Table 1). The sequence at the cleavage site suggested that both viruses are virulent due to the polybasic amino acid motif 112RRQKRF117, typical of virulent viruses [6].

#### 3.2.2 HN genes

A phylogenetic analysis of the HN gene of virus strain Mor1221 revealed that it clustered within sub-genotype VII.2 (Fig. 4). The MR1221 HN was closely related to NDV strains from Southeast Asia and Africa (Table 2).

### 3.3 Analyze of the amino acid substitution of F and HN proteins

Amino acid substitution analysis of the F (Fig. 5A) and HN (Fig. 5B) proteins of Tanzanian subgenotype VII.2 in the current study was conducted in comparison with some African strains (Uganda, Zambia, Mozambique, South Africa, Botswana, and Zimbabwe) of NDV under genotype VII. This analysis revealed strong conservation in the F and HN protein sequence regions of the Tanzanian NDVs identified in the current study (shown in italics) with other strains.

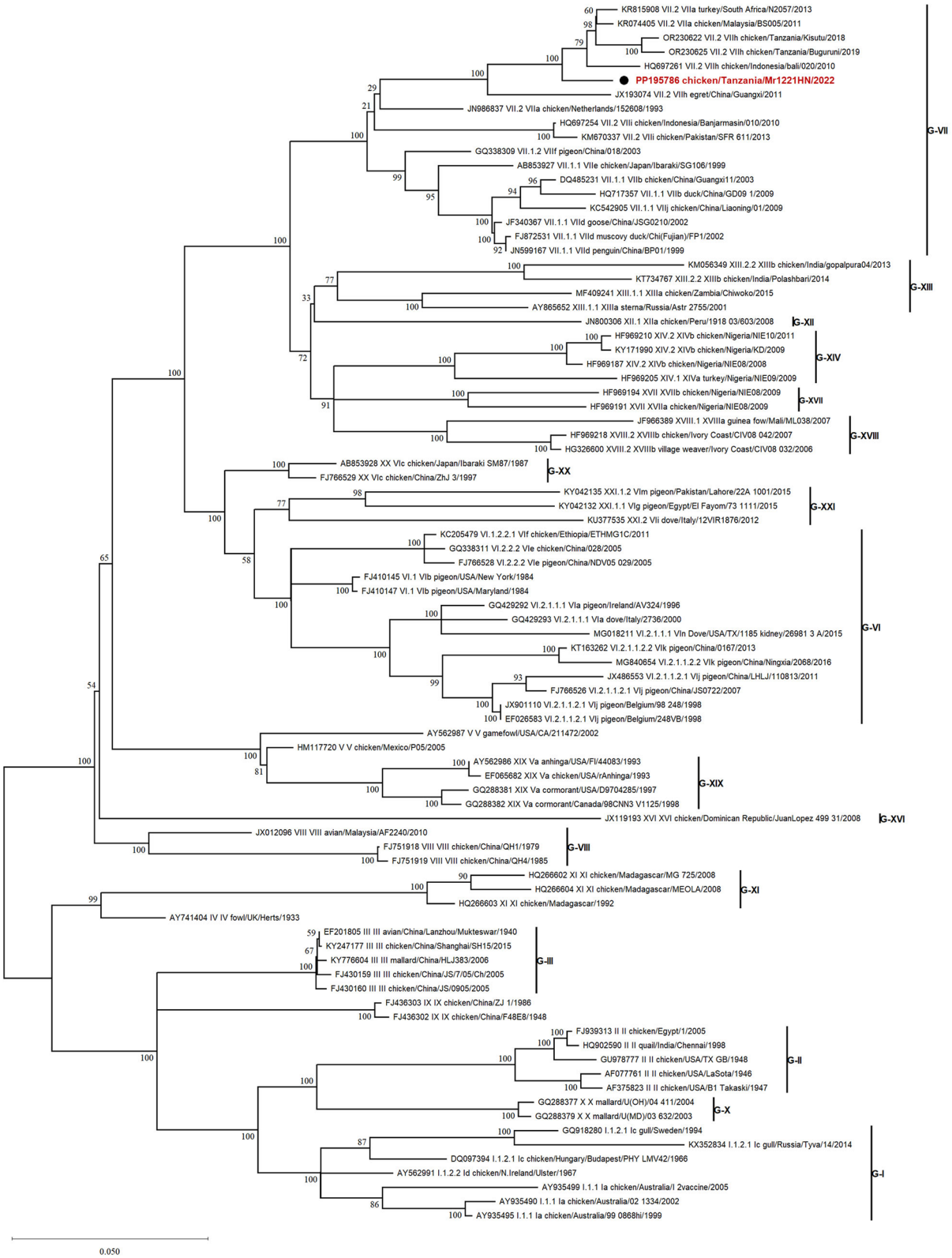


**Fig. 4** Phylogenetic analysis using the whole NDV strain *HN* gene sequence. MR1221 HN is designated in red (subgenotypes VII.2) and indicated with ●. We used 87 class II sub-genotype sequences. The sequences were aligned using ClustalW in MEGA X. We reconstructed a phylogenetic tree using the maximum likelihood approach with 1000 bootstraps. To estimate the evolutionary history while taking into consideration invariant sites (+I), the general time-reversible (GTR) model with a discrete Gamma distribution (+G). The first, second, third, and noncoding codon locations are all included in the inquiry. The names given to each sequence in the phylogenetic tree correspond to the GenBank accession number, followed by the sub-genotype, isolation country, strain identifier, and isolation year

**Table 2** Comparison of HN gene nucleotide sequences described in this study with previously published NDV strains available on GenBank

| Accession number | Strain origin | Year of isolation | % of Similarity to Mor1221 HN gene | References |
|---|---|---|---|---|
| HQ697255 | Indonesia | 2015 | 98.86 | [26] |
| KT760569 | China | 2013 | 97.44 | [25] |
| KR074405 | Malaysia | 2013 | 96.75 | [27] |
| OP921644 | Cambodia | 2012 | 97.90 | [28] |
| OP921685 | Vietnam | 2012 | 97.78 | [28] |
| OR230616 | Tanzania | 2018 | 97.05 | [29] |
| KR815908 | South Africa | 2013 | 97.83 | [30] |

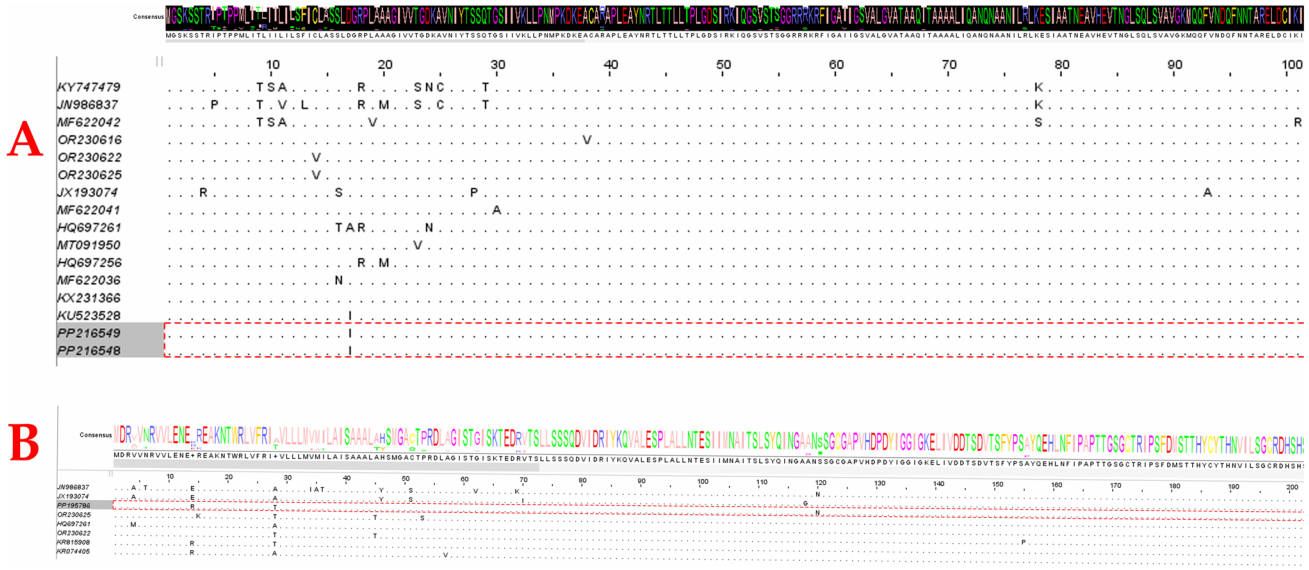


**Fig. 5** Comparative analysis of the amino acid (aa) residues of the fusion protein (**A**) and the hemagglutinin-neuraminidase protein (**B**) of the Tanzanian NDVs identified in the current study (shaded in grey color) with other strains

## 4 Discussion

We obtained HN and F gene sequences of NDV isolates from BPFs in Tanzania in 2022. To improve knowledge of the distribution and genetic variability of NDV in Tanzania, we characterized the sequences phylogenetically. The viruses sequenced here were categorized as subgenotype VII.2 according to the classification scheme suggested by Dimitrov et al. [31]. We added one complete HN gene and two complete F gene sequences to the GenBank database. Both viruses identified are closely related to previously reported isolates from Mozambique (KU523528) based on the F gene. Sequencing of the HN gene showed similarities with other sequences previously isolated in Asian countries such as Malaysia, China, and Indonesia.

Previous studies have suggested that subgenotype VII strains isolated in southern Africa originated in Asia, which could explain the similarity shared by the Tanzania HN gene with strains identified in Southeast Asian countries [32, 33]. This provides more evidence that genotype VII viruses in southern Africa originated in Southeast Asia.

Since its first detection in Mozambique in 2011 [30], subgenotype VII has spread to Zimbabwe, Malawi, Zambia, South Africa, and Botswana. Before its appearance in South Africa and Mozambique [21], sub-genotype VII.2 viruses, which have the potential to cause ND panzootics, were endemic in some Southeast Asian countries, such as Indonesia [26], Malaysia [34], Cambodia [35], and Vietnam [36]. In recent years, this sub-genotype has spread to many parts of Tanzania [15, 37]. Sub-genotype VII.2 was recently identified in a live bird market in Zambia and was related to strains previously isolated in other southern African nations [32]. Notably, several sub-lineages of genotype VII have appeared in the Far East and

spread to other geographical areas in South America [38, 39], Europe [40, 41], Asia [24, 25, 42, 43] and Africa [15, 30, 37, 44]you. This suggests global circulation of NDV sub-genotype VII.2.

ND outbreaks in vaccinated commercial flocks caused by genotype VII have resulted in up to 60–80% of deaths in Indonesia [45], and Japan [46, 47], indicating that more extensive NDV surveillance is needed. Since 2020, a new, rigorous program for monitoring vaccinations to combat several animal diseases, including NDV, has been in place in Tanzania. This program ensures compliance with a vaccination schedule for NDV and manages the quality and pricing of vaccines on the market [48, 49]. However, despite these measures, breeders still suffer outbreaks of ND, causing huge losses in their flocks.

The numerous NDV outbreaks in vaccinated chickens necessitate ongoing observation of the evolution and transmission of NDV. In addition, ongoing assessment of ND vaccination effectiveness is required to prevent future outbreaks. Several factors may be responsible for vaccine failure in poultry [50]: problems associated with the vaccine (inactivation of the vaccine due to expiration, antigenic differences between existing vaccine and field strains, inadequate level of protection; factors associated with the host/birds (stress factors, interference with maternal antibodies/immunity, immunosuppressive and coexisting diseases, genetic factor); factors associated with administration of vaccines (inappropriate route of administration, insufficient dosage, improper formulation of vaccine and diluent used). Notably, both strains identified here were from vaccinated chickens, highlighting the ineffectiveness of current vaccination strategies. The results of the alignment of amino acid substitutions in the F and HN proteins of subgenotype VII.2 present a compelling rationale for considering this subgenotype as a potential vaccine target. This is particularly true given that conserved regions could be targeted by vaccines [51].

## 5 Conclusions

Our study's results imply that BPFs in the Morogoro and Iringa regions of Tanzania are home to virulent genotype VII NDV. As the chickens tested in this study had been vaccinated, these results suggest that current vaccines are ineffective at preventing severe ND. Regular surveillance will lead to improved disease control and a better understanding of the ND status, characterization of circulating strains.

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**Author contributions** Conceptualization, C.F.A., A.A.C, J.W-L. and G.M.; methodology, C.F.A., J.N.H., A.A.C., M.R.M., J.W-L. and G.M.; software, C.F.A., J.N.H; validation: A.A.C., J.W-L. and G.M; formal analysis: C.F.A., J.N.H. and M.R.M.; data curation: C.F.A.; writing—original draft preparation: C.F.A.; writing—review and editing: C.F.A., J.N.H., A.A.C., M.R.M., J. W-L. and G.M.; Supervision, A.A.C., J.W-L. and G.M. All authors have read and agreed to the published version of the manuscript.

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**Data availability** The nucleotide sequences generated in this study are available at NCBI GenBank under Accession Numbers PP216548 and PP216549 for the F genes and PP195786 for the HN gene.

## Declarations

**Ethics approval and consent to participate** Ethical clearance was obtained from the Office of the Vice Chancellor, Sokoine University of Agriculture, Tanzania (Ref. No. SUA/ADM/R.1/8A/737) and Permission to undertake animal health research in Tanzania from the United Republic of Tanzania Ministry of Livestock and Fisheries (Ref. No. DB.16/324/01/137).

**Consent for publication** Not applicable.

**Competing interests** The authors declare no conflict of interest. The funder had no role in the design of the study; in the analyses, and interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

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