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Molecular characterization of Newcastle disease virus (AOAV-1) obtained from Western region of Libya

Abdulwahab Kammon[1,2*], Elhadi Rammah[3], Abdulhamid Giweli[1,4] and Isabella Monne[5]

[1]*National Research Center for Tropical and Transboundary Diseases (NRCTTD), Alzintan, Libya*
[2]*Department of Poultry and Fish Diseases, Faculty of Veterinary Medicine, University of Tripoli, Tripoli, Libya*
[3]*Libyan Academy for Postgraduate Studies, Tripoli, Libya*
[4]*Faculty of Science, Alzintan University, Alzintan, Libya*
[5]*Istituto Zooprofilattico Sperimentale delle Venezie (IZSVe), Padova, Italy*

ABSTRACT

Background: Since its discovery in 1926, Newcastle disease (ND) is still emerging in many avian species worldwide causing severe economic losses due to high mortality.

Aim: This article aims to discuss the challenge of virulent ND in poultry in Libya, focusing on recent outbreaks investigated in Alzintan, Alrayaina, Nalut, and Surman, cities located in the western region of Libya.

Methods: Clinical signs and lesions were recorded. Tissues, as well as tracheal and cloacal swabs, were collected. RNA extraction was performed for confirmation using PCR and sequencing.

Results: Mortality, in general, reached 50%–100% in vaccinated flocks with respiratory distress, diarrhea, swelling of the face, and nasal discharges. Necropsy revealed severe hemorrhages in the proventriculus, necrosis, and hemorrhages in the intestine and cecal tonsils. All tested samples were positive for avian orthoavulavirus 1 (AOAV-1) using rRT-PCR and genetic analysis. The sequences obtained are referable to AOAV-1, which is the same strain in all tested samples. The amino acid sequences deduced from the cleavage site of the *F* protein are referable to a velogenic strain of AOAV-1 belonging to genotype VII.2. The detected strains in the current study revealed 86%–91% identity with European isolates identified between 2020 and 2022 and isolates from Asia and Africa and 97% identity to the previous isolated Libyan strains in 2013 and 2016. It is slightly different by the presence of amino acid lycine at position 111 on the cleavage site of the F0 gene as compared to previous Libyan strains in which arginine was found in the same position. The nucleotide sequence at this position changed from (aga) in AOAV-1 strains of years 2013 and 2016 to (aag) of the year 2023.

Conclusion: ND remains a significant threat to the poultry industry in Libya. Therefore, there is an urgent need to conduct an epidemiological study with a representative number of samples from all regions of the country, alongside the implementation of an inactivated vaccine targeting genotype VII.

Keywords: Newcastle disease, Broilers, Backyards, Libya, Genotype VII.

Introduction

Newcastle disease (ND) is a highly contagious viral respiratory disease affecting a vast of avian species worldwide. ND's first outbreak occurred in poultry in 1926 in Java, Indonesia, and in Newcastle-upon-Tyne, England (Hanson and Sinha, 1952). However, it seems that the disease outbreaks appeared earlier in Central Europe. Macpherson (1956) reported that the death of all chickens in the Western Isles of Scotland in 1896 was due to ND. Therefore, ND might have occurred in poultry before 1926, but its recognition as a specifically defined disease of viral etiology dates from the outbreaks during 1927 in Newcastle-upon-Tyne from which it takes its name.

Among the disease challenges, ND is a highly deadly viral infection and has been considered a significant problem for poultry production in many countries since its first discovery until now. Outbreaks of ND have a tremendous impact on both backyard and intensive commercial poultry farming and are considered an enduring hardship for poultry farmers, even with the use of vaccination (Samrawit and Mulat, 2018).

Newcastle disease virus (NDV), also known as avian paramyxovirus 1 and recently avian orthoavulavirus 1 (AOAV-1)}, is an enveloped single-stranded negative-sense RNA virus belongs to Paramyxoviridae family, which currently has seven genera that include important human and veterinary pathogens (Suarez, 2020). All different genotypes of APMV-1 circulating worldwide,

***Corresponding Author:** Abdulwahab Kammon. National Research Center for Tropical and Transboundary Diseases (NRCTTD), Alzintan, Libya. Email: *a.kammon@nrcttd.ly*

belong to a unique APMV-1 serotype. NDV genotypes are classified under two classes based on nucleotide sequences. Class I viruses have only one genotype, while class II viruses have 20 distinct genotypes (Dimitrov *et al.*, 2019). Genotypes V, VI, and VII of virulent viruses are the predominant genotypes circulating worldwide (Miller *et al.*, 2010; Neto *et al.*, 2024). Genotype VII is particularly important, given that it has been associated with many of the most recent outbreaks in Asia, Africa, and the Middle East (Zanaty *et al.*, 2019; Mngumi *et al.*, 2022; Syamsiah Aini *et al.*, 2022). Isolates of this genotype are known for their widespread genetic diversity and continuous evolution (Bello *et al.*, 2018). Therefore, molecular characterization of ND viruses is a crucial tool for epidemiological studies necessary for developing and implementing control plans. In Libya, ND outbreaks occurred in the poultry industry in March 2013, and NDV was isolated and molecularly characterized (Kammon *et al.*, 2015). The sequencing of the APMV-1/chicken/Libya/13VIR/7225-1/2013 isolate discovered for the first time the presence of a velogenic APMV-1 that belongs to genotype VIIi in class II. Kammon *et al.* (2018) reported another ND outbreak in Alzintan City that emerged in backyard chickens and pigeons in 2015. The results of Kammon *et al.* (2018) showed two viruses that genetically resembled those isolated from cloacal swabs of backyard chickens in 2013, with a velogenic type of APMV-1 belonging to genotype VIb in a pigeon. Based on these results, it may indicate that NDV is still circulating in backyard birds that are usually not vaccinated. In this work, we detected a very virulent strain of AOAV-1 from broiler chickens affected during the 2023 outbreak in some areas of the western region of Libya. A partial sequence of the *F* gene of the AOAV-1 strain was obtained, and a phylogenetic tree was constructed to compare the newly identified strain with previous ones.

Materials and Methods

Collection of samples

Dead and moribund chickens were submitted to the National Research Center for Tropical and Transboundary Diseases for diagnosis. Chickens received were from broiler flocks located in the cities of Alzintan, Alrayaina, and Nalut, in addition to a backyard flock located in Surman city. Clinical signs were recorded, and necropsies were conducted. The samples included in this study comprised of tissues, tracheal swabs, and cloacal swabs (Table 1).

RNA extraction, RT-PCR, and sequencing

The swabs and tissue samples were gently streaked on FTA® cards (Whatman, USA), air-dried, and sent to Istituto Zooprofilattico Sperimentale delle Venezie (IZSVe) in Italy. Viral RNA was extracted from FTA® cards using the Nucleospin RNA II Kit (Machery-Nagel, Duren, Germany), and the detection of AOAV-1 RNA was carried out using real time RT-PCR protocol targeting the *M* gene (Sutton *et al.*, 2019). For molecular characterization, a 260-bp hypervariable region of the *F* gene encompassing the cleavage site was first amplified with RT-PCR (Kant *et al.*, 1997). Second, amplicons were sequenced using the Brilliant Dye Terminator Cycle Sequencing kit (v3.1) (Nimagen). The products were cleaned up using the PERFORMA DTR Ultra 96-Well kit (Edge Biosystems, Gaithersburg, MD) and sequenced in a 16-capillary ABI PRISM 3130xl Genetic Analyzer (Applied Biosystem, Foster City, CA). Nucleotide sequences were aligned, and phylogenetic analysis was performed using the neighbor-joining method in the Molecular Evolutionary Genetics Analysis (Mega) 11 program. The lineages-based nomenclature illustrated by Dimitrov *et al.* (2019) was adopted. Comparison between Libyan strains of AOAV-1 and distance table to determine the percentage of identical bases were done using the Geneious software (Version 2023.2 created by Biomatters. https://www.geneious.com).

Ethical approval

Not needed for this study.

Results

Clinical signs and post-mortem lesions

Mortality reached 100% in broiler flocks located in Alzintan and Alrayaina and 80% in the flocks located in Nalut. Clinical signs included respiratory distress, diarrhea, swelling of the face, and nasal discharge. Gross lesions comprised tracheitis, severe hemorrhages in the proventriculus, necrosis, and hemorrhages in the intestine and cecal tonsils. Mortality of backyard chickens reached 50%. Gross lesions comprised necrosis and hemorrhages of combs and hemorrhages in the proventriculus. Samples were collected as per animal welfare and biosafety international standard collection methods.

Real-time RT-PCR and sequencing

All tested samples were positive for AOAV-1 using rRT-PCR and genetic analysis. Only one sample was not sequenced due to the very short sequence obtained. The sequences obtained are referable to AOAV-1, which is the same strain in all tested samples. The amino acid sequences deduced from the cleavage site of the *F* protein are referable to a velogenic strain of AOAV-1 (having multiple basic amino acids at the cleavage site, with phenylalanine at amino acid (aa) position 117 at the *N* terminus of the F1 protein). Based on the analysis of the partial sequence of the *F* gene, the virus belongs to genotype VII.2 according to Dimitrov *et al.* (2019), formerly grouped as five according to Aldous *et al.* (2003). AOAV-1 *F* gene sequences submitted for BLASTN analysis revealed 86%–91% identity with European isolates identified between 2020 and 2022 and isolates from Asia and Africa (Fig. 1 and Table 2). Since all detected strains were similar, one of them was compared with the previously isolated and identified strains from Libya in 2013 and 2016 and some selected

Table 1. History of clinical signs and samples collected for diagnosis of AOAV-1.

No.	City	Species	Sample code	Clinical signs	Type of sample	Age	No. of samples
1	Alzintan	Broilers	23RS-326-23VIR-1240-1 23RS-326-23VIR-1240-3	Mortality 100%, Respiratory distress, diarrhea, swelling of the face and nasal discharges.	Tracheal swabs	17 d	2 samples (Pooling of 5 samples)
2	Alrayaina	Broilers	23RS-326-23VIR-1240-2 23RS-326-23VIR-1240-15 23RS-326-23VIR-1240-16 23RS-326-23VIR-1240-17	Mortality 100%, Respiratory distress, diarrhea, swelling of the face and nasal discharges.	Tissues (trachea, lung, intestine and proventriculus)	16 d	4 samples
3	Nalut	Broilers	23RS-326-23VIR-1240-4 23RS-326-23VIR-1240-5	Mortality 80%, respiratory distress	Tracheal swab	15 d	2 samples (Pooling of 4 samples)
4	Surman	Local backyard chicken	23RS-326-23VIR-1240-6 23RS-326-23VIR-1240-7 23RS-326-23VIR-1240-11	Mortality 50%, necrosis and hemorrhages of combs	Cloacal swab	Adult	3 samples (pooling of 5 samples)
5	Surman	Local backyard chicken	23RS-326-23VIR-1240-8 23RS-326-23VIR-1240-9	Mortality 50%, necrosis and hemorrhages of combs	Tracheal swab	Adult	2 samples (pooling of 5 samples)
6	Surman	Local backyard chicken	23RS-326-23VIR-1240-10	Mortality 50%, necrosis and hemorrhages of combs	Tissue (Proventriculus)	Adult	1 sample

strains. The strain of the current study revealed 97% identity to the previously isolated Libyan strains in 2013 and 2016 (Table 2). It is slightly different by the presence of amino acid lycine at position 111 on the cleavage site of the F0 gene as compared to previous Libyan strains in which arginine was found in the same position (Figs. 2 and 3, Table 3). The nucleotide sequence at this position changed from (aga) in AOAV-1 strains of years 2013 and 2016 to (aag) in the year 2023.

Discussion

ND causes a huge deleterious impact on the poultry industry despite the use of extensive vaccination. In the current study, a velogenic strain of AOAV-1 was detected in broilers and backyard chickens in some cities of the western region of Libya. The sequences obtained are referable to AOAV-1, which is the same strain in all tested samples. The amino acid sequences deduced from the cleavage site of the *F* protein are referable to a velogenic strain of AOAV-1 having multiple basic amino acids at the cleavage site, with phenylalanine at amino acid (aa) position 117 at the *N* terminus of the F1 protein (Nagai *et al.*, 1976; OIE, 2021). Severe mortalities, reaching 100%, were recorded in vaccinated flocks. The clinical signs and gross lesions appeared to be similar to those caused by the strain isolated and characterized in 2013 (Kammon *et al.*, 2015). In the subsequent year, 2016, a study on the seroprevalence and molecular detection of NDV in backyard chickens in Tripoli was conducted by Gedara *et al.*, (2020) in which the prevalence of NDV infection in backyard chickens in different locations of Tripoli during

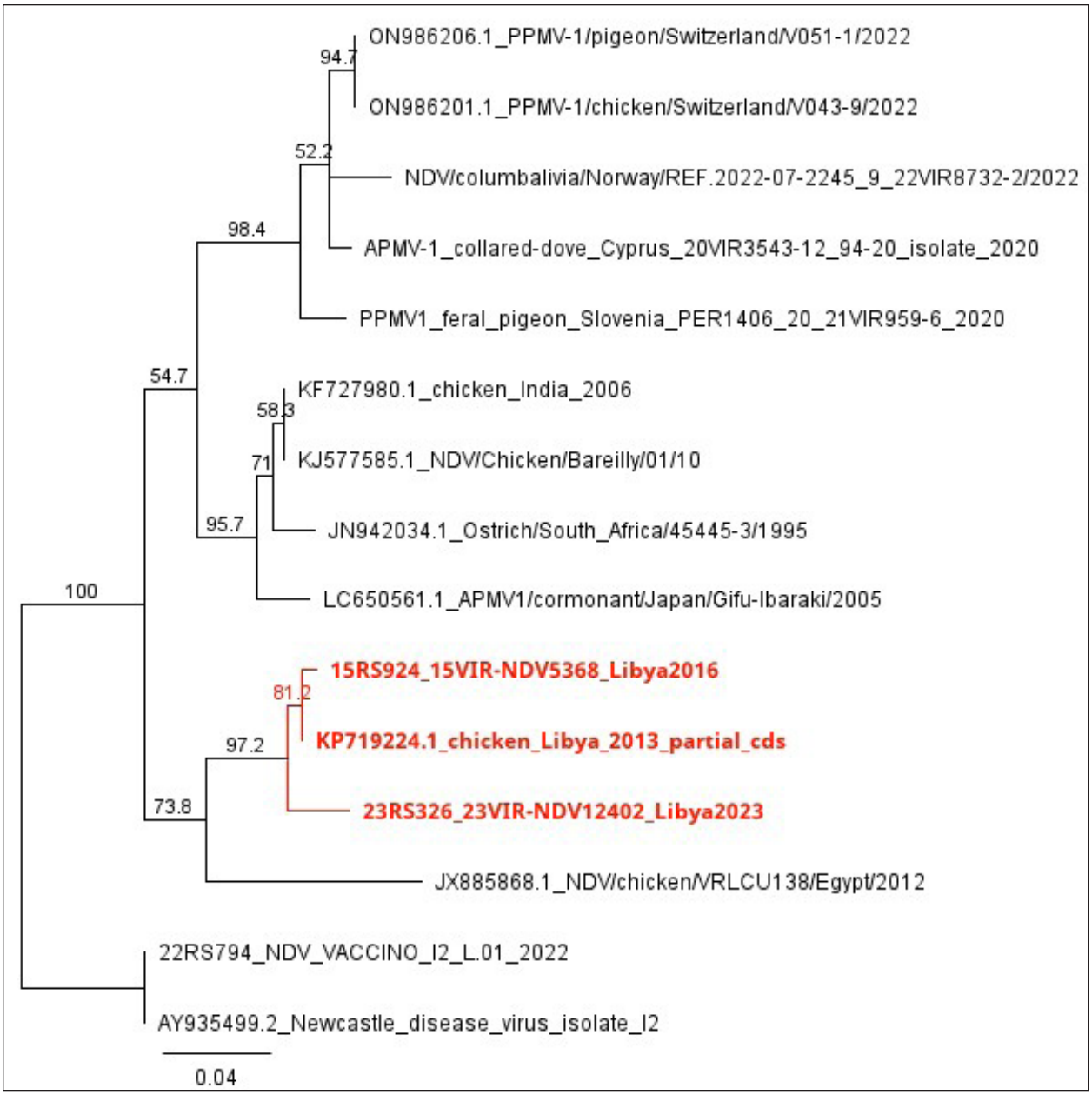

Fig. 1. Phylogenetic tree constructed by neighbor-joining method with 1,000 bootstraps using the MEGA11 program. NDV genotype VII strains from the current study and previous isolates are indicated in red.

summer and winter was 45% and 53%, respectively. However, sequencing was not attempted. Based on the analysis of the partial sequence of the *F* gene, the virus strain of the current study belongs to genotype VII.2 according to Dimitrov *et al.* (2019), formerly grouped as five according to Aldous *et al.* (2003). The recently detected strain is slightly genetically different from the strains isolated in 2013 and 2016. Nonetheless, both strains belong to AOAV-1 genotype VII.2, which is of utmost importance since it has caused many of the most recent outbreaks worldwide (Miller *et al.*, 2015; Makki *et al.*, 2021; Eid *et al.*, 2022; Fernández-Díaz *et al.*, 2023; Henriques *et al.*, 2023).

AOAV-1 *F* gene sequences submitted for BLASTN analysis revealed 86%–91% identity with European isolates identified between 2020 and 2022 and isolates from Asia and Africa. Migratory birds may play a very important role in transmitting AOAV-1 from Europe to Libya. Turan *et al.* (2020) reported the presence of subgenotype VII.2 of NDVs in Common Moorhen and Mallard in north west of Turkey and revealed some degree of molecular evolution when compared to the

Table 2. Percentage of identical bases/residues.

Virus strain	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
1. 23RS326_23VIR-NDV12402_ Libya2023		97	97	88	89	91	91	90	86	86	86	87	87	85	85
2. 15RS924_15VIR-NDV5368_ Libya2016	97*		99	90	90	92	92	91	86	86	86	87	87	87	87
3. KP719224.1_chicken_ Libya_2013_partial_cds	97	99		90	91	93	93	92	87	87	87	88	88	87	87
4. JX885868.1_NDV/chicken/ VRLCU138/Egypt/2012	88	90	90		87	88	88	87	85	86	86	86	86	85	85
5. LC650561.1_APMV1/ cormonant/Japan/Gifu-Ibaraki/2005	89	90	91	87		97	97	96	91	91	91	90	90	87	87
6. KF727980.1_chicken_ India_2006	91	92	93	88	97		100	98	91	92	92	92	92	88	88
7. KJ577585.1_NDV/Chicken/ Bareilly/01/10	91	92	93	88	97	100		98	91	92	92	92	92	88	88
8. JN942034.1_Ostrich/South_ Africa/45445-3/1995	90	91	92	87	96	98	98		89	90	90	90	90	87	87
9. NDV/columbalivia/ Norway/REF.2022-07-2245_9_22VIR8732-2/2022	86	86	87	85	91	91	91	89		96	96	95	96	85	85
10. ON986206.1_PPMV-1/pigeon/ Switzerland/V051-1/2022	86	86	87	86	91	92	92	90	96		100	97	98	86	86
11. ON986201.1_PPMV-1/chicken/ Switzerland/V043-9/2022	86	86	87	86	91	92	92	90	96	100		97	98	86	86
12. PPMV1_feral_ pigeon_Slovenia_ PER1406_20_21VIR959-6_2020	87	87	88	86	90	92	92	90	95	97	97		97	88	88
13. APMV-1_collared-dove_ Cyprus_20VIR3543-12_94-20_ isolate_2020	87	87	88	86	90	92	92	90	96	98	98	97		86	86
14. 22RS794_NDV_VACCINO_ I2_L.01_2022	85	87	87	85	87	88	88	87	85	86	86	88	86		100
15. AY935499.2_Newcastle_ disease_virus_isolate_I2	85	87	87	85	87	88	88	87	85	86	86	88	86	100	

*Values represent percentages (%) of identity inferred by the analysis of 224 nucleotide positions of the *F* gene of AOVA-1 using the Geneious version 2023.2 created by Biomatters (https://www.geneious.com).

earlier NDV-VII.2 isolate in Turkey. Thus, surveillance studies of NDV evolution and prevalence among migratory and free-living birds simultaneously with domestic avian species would significantly provide important information on its role in transmission in Libya. Such efforts are also important for the use of appropriate protective vaccines as well as biosecurity measures to hinder the virus transmission cycle and protect domestic poultry.

The strain of the current study revealed 97% identity to the previous isolated Libyan strains in 2013 (accession no. KP719224.1) and 2016. It is slightly different by the presence of amino acid lycine at position 111 on the cleavage site of the F0 gene as compared to previous Libyan strains in which arginine was found in the same position. The nucleotide sequence at this position changed from (aga) in AOAV-1 strains of years 2013 (accession no. KP719224.1) and 2016 to (aag) in the year 2023. This change is out of the region known for velogenic strains of AOAV-1 having multiple basic amino acids at the cleavage site starting from position 112, with phenylalanine at amino acid (aa) position 117 at the *N* terminus of the F1 protein according to OIE definition of velogenic NDV (OIE, 2021). However, in contrast to this definition, Heiden *et al.* (2014) found that regions of the *F* protein other than the polybasic cleavage site, have a role in virulence of AOAV-1. They found that the substitution of different regions

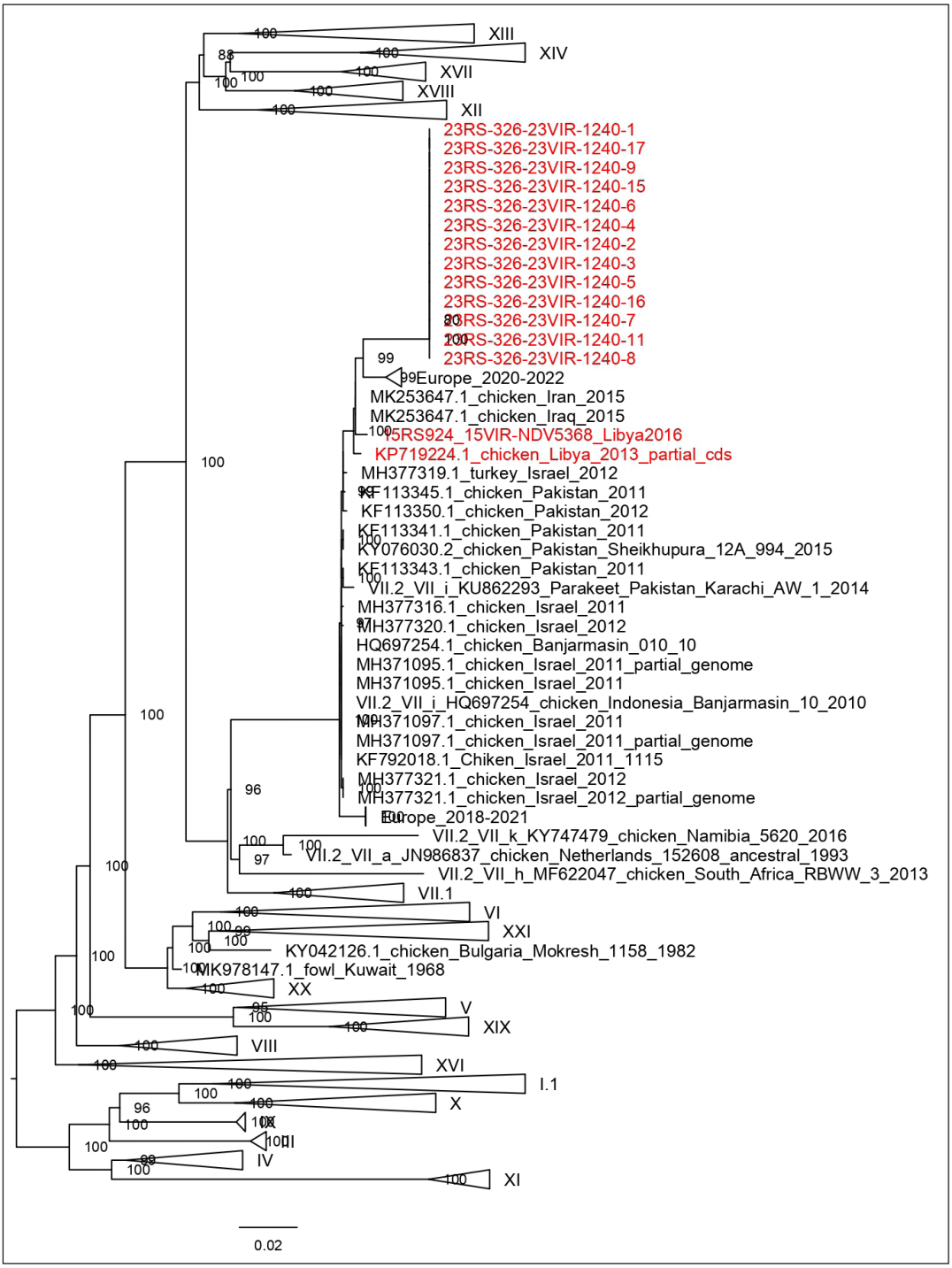

Fig. 2. Phylogenetic tree constructed by neighbor-joining method with 1,000 bootstraps. The analysis was performed using the Geneious version 2023.2 created by Biomatters (https://www.geneious.com) to clearly compare between the Libyan isolates and selected other isolates. The Libyan AOAV-1 isolates from the current and previous studies are indicated in red.

of the *F* protein of Clone 30 by those of PPMV-1, while maintaining the polybasic amino acid sequence at the *F* cleavage site, resulted in recombinant viruses with ICPIs ranging from 0.59 to 1.36. It is not clear if the point mutation of the current Libyan AOAV-1 strain has a role in its virulence in vaccinated flocks.

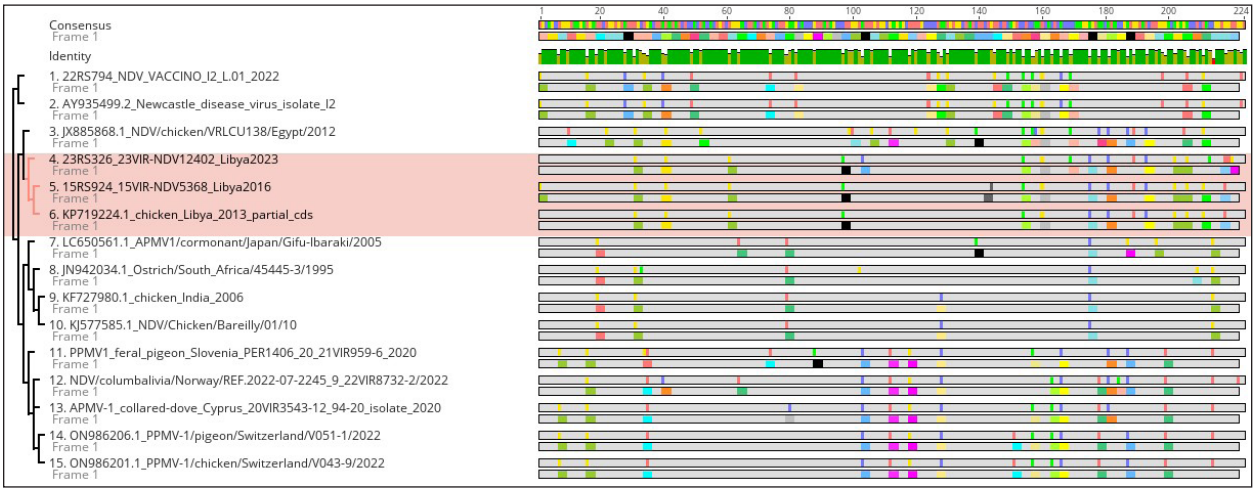

Fig. 3. Alignment of Libyan isolates and selected other isolates. The Libyan AOAV-1 isolate from the current study and previously are indicated in red. Analysis performed using Geneious version 2023.2 created by Biomatters (https://www.geneious.com).

Table 3. Amino acid sequence at the F0 cleavage site of Libyan AOAV-1 and some other strains.

GenBank access no.	Virus strain	Virulence	Cleavage-site (110-118)
None	23RS326_23VIR-NDV12402_Libya2023	High	G**K**RRQKR*FI
None	15RS924_15VIR-NDV5368_Libya2016	High	GRRRQKR*FI
KP719224.1	Chicken_Libya_2013_partial_cds	High	GRRRQKR*FI
LC650561.1	APMV1/cormonant/Japan/Gifu-Ibaraki/2005	High	GGRRQRR*FI
KF727980.1	Chicken_India_2006	High	GGRRQKR*FI
KJ577585.1	NDV/Chicken/Bareilly/01/10	High	GGRRQKR*FI
JN942034.1	Ostrich/South_Africa/45445-3/1995	High	GGRRQKR*FI
None	NDV/columbalivia/Norway/REF.2022-07-2245_9_22VIR8732-2/2022	High	GGKRQKR*FI
ON986206.1	PPMV-1/pigeon/Switzerland/V051-1/2022	High	GGRRQKR*FI
ON986201.1	PPMV-1/chicken/Switzerland/V043-9/2022	High	GGRRQKR*FI
None	PPMV1_feral_pigeon_Slovenia_PER1406_20_21VIR959-6_2020	High	GGRRQKR*FI
None	APMV-1_collared-dove_Cyprus_20VIR3543-12_94-20_isolate_2020	High	GGRRQKR*FI
JX885868.1	NDV/chicken/VRLCU138/Egypt/2012 fusion glycoprotein (F) gene, partial cds	High	GGRRQKR*FI
None	22RS794_NDV_VACCINO_I2_L.01_2022	Low	GGRKQGR*LI
None	AY935499.2_Newcastle_disease_virus_isolate_I-2	Low	GGRKQGR*LI

*Cleavage point. All virulent viruses have phenylalanine (*F*) at position 117.

However, a mutation in the A2 antigenic epitope of NDV has been reported to induce escape mutation to monoclonal antibodies generated using the NDV LaSota strain (Funsho-Sanni *et al.*, 2022). Moreover, point mutation identified in the field isolates from Japan was attributed to the potential escape of the virus from vaccine pressure. The identification of such mutation may be useful for future site-directed mutagenesis to understand the dynamics of NDV infection in vaccinated chickens (Umali *et al.*, 2013).

In response to the ND outbreaks in Libya in 2013, live as well as killed vaccines were recommended and successfully protected poultry. However, in the current outbreak, vaccinated flocks with live vaccines were severely affected. In Egypt, Lebdah *et al.* (2024) used a live attenuated vaccine with an inactivated genetically matched strain vaccine and then boosted

it with one of the available live vaccines. They found that this program is the most effective against current field vNDV namely genotype VII infection. Despite the point mutation of the new strain of AOAV-1, the reason for current ND outbreak severity is not exactly known. There are many factors that may contribute to the vaccination failure such as the quality of drinking water, handling of the vaccines, weakness of biosecurity measures, and so on. Although AOAV-1 worldwide isolates differ genetically, they all belong to the same serotype; thus, if given correctly, the vaccines prepared with any AOAV-1 should protect poultry from clinical disease and mortality in the face of a virulent challenge (Miller *et al.*, 2007; Perozo *et al.*, 2008). In fact, there are excessive genetic and antigenic variations between conventional live vaccines and field viruses. Some studies have found that the amino acid sequence homologies of the *F* protein between La Sota strain and genotype VII strain range from 87% to 89% (Xiao *et al.*, 2012). Thus, genotype matching especially inactivated and recombinant vaccines might be the solution in this case taking into consideration efficacy, as well as the safety of such vaccines. In Korea, a recombinant (live & inactivated) genotype VII vaccine strain (KBNP-C4152R2L) was produced (Cho *et al.*, 2008). This vaccine was found to provide better protection than La Sota in terms of reducing virus shedding (Sultan *et al.*, 2020; Dewidar *et al.*, 2022). In China, Hu *et al.* (2009), developed an attenuated genotype VII NDV vaccine that induced a faster, stronger, and more protective antibody response against the challenge of genotype VII virus and significantly decreased virus shedding. This vaccine was included for the first time in the Chinese Veterinary Pharmacopoeia.

In conclusion, ND continues to pose a significant threat to the poultry industry in Libya. Therefore, there is an urgent need to conduct an epidemiological study encompassing representative samples from all regions of the country. Concurrently, implementing an inactivated vaccine targeting genotype VII is essential. Moreover, it is crucial to elucidate whether the point mutation of the AOAV-1 F gene, particularly at positions outside the cleavage site, plays a role in virulence and potential vaccination escape.

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Conflict of interest

The authors certify that there are no conflicts of interest.

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Authors' contributions

This study is a joint work and contribution from all authors. Abdulwahab Kammon was responsible for the final revision and proofing.

Data availability

All data are provided in the manuscript.

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