Phylogenetic analysis of some Newcastle disease virus isolates from the Sudan

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Abstract

A reverse transcription-polymerase chain reaction (RT-PCR) was used to amplify 1412 bp of the fusion protein gene (F gene) of four Newcastle disease virus (NDV) isolates; two velogenic (TY-1/90 and DIK-90) and two lentogenic isolates (Dongla 88/1 and GD.S.1). Following sequencing, nucleotide sequences were annotated and 894 bp were compared phylogenetically with those from strains previously reported in the Sudan and the virus strains published on the GenBank. It could be demonstrated that TY-1/90 and DIK-90 strains belong to the genotype VI of NDV and are in close genetic relationship to sub- genotype VIb. TY-1/90 and DIK-90 strains were observed to be genetically unrelated to the earlier Sudanese isolates of 1970/80s and the late of 2000s suggesting a different origin. The close genetic relationship to the European and African pigeon paramyxovirus type 1 (PPMV-1) suggests a common ancestor. Dongola, GD.S.1 strains were classified into genotype II that comprises non-pathogenic lentogenic NDV strains. The present genetic classification of NDV isolates of the Sudan provides valuable information on genotypes of NDV. Further molecular epidemiological investigations of the recent outbreaks of Newcastle disease in the Sudan are needed in order to improve the efficiency of control strategies and vaccine development.

Keywords: Newcastle disease virus, Phylogenetic analysis, Sudan isolates.

Introduction

Newcastle disease (ND) or avian paramyxovirus 1 infection is a viral disease of domestic poultry and wild birds (Alexander, 1997). Gastrointestinal, respiratory and neurological symptoms of ND extent from subclinical to rapidly fatal based on the pathotype of the virus involved (Alexander, 2003). The disease is exceedingly contagious and over 250 species of birds of all age groups can be affected with the disease (Alexander *et al*., 1997). The causative virus, Newcastle disease virus (NDV), also named avian paramyxovirus type 1 (APMV-l), which has a single-stranded negative-sense RNA genome, is classified in the genus *Avulavirus* of the sub-family *Paramyxovirinae,* the family *Paramyxoviridae* and the order *Mononegavirales* (Lamb *et al*., 2000).

Diverse genotypes of APMV-1 circulate in various parts of the world and are genetically divided into two classes (class I and class II) and further classified into genotypes based on phylogenetic differences. Class I viruses have the longest of the APMV-1 genomes of 15,198 nucleotides, are genetically less diverse, generally present in wild waterfowl, and are of low virulence (Dimitrov *et al*., 2016). Viruses in this class are frequently isolated in live bird market samples and incorporate at least nine genotypes. Class II contains one genotype and its viruses are genetically more diverse, exhibit a wider range of virulence and include

at least 16 genotypes (Diel *et al*., 2012). NDV strains of low virulence commonly used as vaccines worldwide such as LaSota and B1 are incorporated in class II, genotype II. Viruses of genotypes III, which were predominantly isolated decades ago in Japan, Taiwan and Zimbabwe are now isolated sporadically (Aldous *et al*., 2003; Miller *et al*., 2010). Viruses of genotypes V, VI, and VII, are highly versatile and have been isolated from different countries and continents. Alternately, virulent viruses of genotypes XI, XIII, XVI, XIV, XVII and XVIII appear to have a more restricted geographic distribution and have been isolated predominantly from poultry (Dimitrov *et al*., 2016). Another system suggested by Aldous classifies NDV into six lineages and 13 sub lineages and later, three extra sub lineages were added (Aldous *et al*., 2003; Snoeck *et al*., 2009).

In the Sudan, NDV was first reported in Khartoum in 1951 (ARSVS, 1951). Since then the disease has been regularly mentioned in all reports of the Sudan veterinary services. Diagnosis was based on the picture of disease, but the virus was isolated and identified for the first time in 1962 (Karrar and Mustafa, 1964; Eisa, 1979). According to Ballouh *et al*. (1983), twelve NDV isolates obtained during 1963-1979 in the Sudan were mesogenic (n=4) and velogenic (n=8). During the year 1984-1985, four virus isolates were found to be velogenic (Haroun *et al*., 1992). In another study, six isolates were obtained from outbreaks in the country

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between 1988 and 1991 and found to possess the characteristics of the viscerotropic velogenic strains of NDV [VVNDV], and concluded that the VVNDV is the most prevalent pathotype in the Sudan (Khalafalla *et al*., 1992).

Search of the literature revealed 3 publications containing phylogenetic analysis of NDV isolates from Sudan (Aldous *et al*., 2003; Ujvári *et al*., 2003; Hassan *et al*., 2010) that covered the period 1975-2006, but without a link to classical pathotyping or the circulation and origin of this devastating virus in the country. According to Ujvári *et al*. (2003) analysis of 68 strains of avian paramyxovirus type 1 of pigeons (PPMV-1) showed a close genetic relationship to Sudanese viruses from the mid-1970 suggesting that PPMV-1 could be of African origin.

Here, we genetically classified additional Sudanese NDV isolates obtained between 1988 and 1999 and discussed the origin and circulation of various virus genotypes in the Sudan.

Materials and Methods

Viruses

NDV strains were obtained from the repository of Department of Microbiology, Faculty of Veterinary Medicine, University of Khartoum. TY-1/90 and Dik-90 are virulent strains isolated from ND outbreaks that occurred in south of Khartoum during July - September 1990 (Khalafalla *et al*., 1992). Dongola 88/1 strain is a local isolate of lentogenic NDV, which was isolated in 1988 from apparently healthy chickens in Dongola city of Northern Sudan (Khalafalla, 1994). According to Khalafalla (1994) the chickens from which Dongla 88/1 isolate was obtained were vaccinated with Komarov strain of NDV. LaSota VC is a live vaccine strain (INTERVET, The Netherlands) used to vaccinate chickens in the Sudan. I2 strain is a live lentogenic thermostable NDV vaccine strain originally isolated in the Australian Center for International Agriculture Research (ACIAR) (Bensink and Spradbrow, 1999). GD.S.1 is a field virus isolated from non-vaccinated local chickens in Gedarif city in June 1993 (Abdelaziz *et al*., 2004). The viruses were propagated in embryonated chicken eggs and identified as NDV by hemagglutination inhibition (HI) test according to standard techniques (OIE, 2008).

RT-PCR

Primer design

Primers were designed from conserved nucleotide regions of the NDV fusion protein gene (F-gene). First, sequences of the NDV F-gene of various isolates of NDV were retrieved from the GenBank® (http://www.ncbi.nlm.nih.gov/Genbank/index.html) and were used for multiple sequence alignment to determine conserved regions in the F-gene. Second, conserved regions in the sequences were selected and primers were manually designed. The primers were NCD_F (5` CAG GCC TCT TGC AGC TGC 3`) and NCD_R (5` TAT AGG TAA TGA GAG CAG ACG 3`).

RNA extraction

NDV RNA was extracted from harvested virus in chicken embryonic allantoic fluids using Roti®-Quick-Kit (Roth, Germany) according to the manufactures' instructions. RNA pellet was dissolved in 15-20 µl DEPC-treated autoclaved distilled water and was immediately used in RT-PCR.

OneStep RT-PCR

RT-PCR was performed using QIAGEN® OneStep RT-PCR Kit (Qiagen, Germany). Primer stock solutions of 100 µM were first prepared and then working dilutions of 10 µM were used in the RT-PCR reactions. Briefly, NDV RNA samples, primers, dNTP mix, 5x OneStep RT-PCR Buffer, Q-solution and RNase-free water were thawed and placed on ice. All components required for RT-PCR were contained in the typical master mix except the template RNA. Five microliters of RNA were added to each 20 µl of mastermix in a PCR tube. A negative control (without template RNA) was included in every experiment. The tubes were incubated in a thermal cycler (Primus96, Peqlab Biotech, Germany) and the RT-PCR program was carried out with the following steps: reaction mixtures were subjected to a 30-min reverse transcription step at 50° C, then heating for 15 min at 95° C to activate the Hot Start *Taq* DNA polymerase, followed by 35 cycles of 95° C for 30 s, 60° C for 32 s and 72° C for 32 s and a final extension at 72° C for 2 min.

Analysis of PCR product

The PCR products were separated electrophoretically in 2% agarose gel (Sigma, UK) in Tris/Boric acid/EDTA running buffer (TBE buffer). Agarose gel was prepared by dissolving 1 g of agarose in 50 ml of TBE buffer. Ethidium bromide (1µl/40 ml agrose) was added and the gel was cast into the tray, combs were placed and the gel inside the tray was allowed to solidify for 30 min. Five µl of the PCR products were mixed with one µl of 6X loading dye and transferred into the wells. Two microliters of one Kb DNA ladder (GeneRuler™ 1kb DNA Ladder Plus #SM1331, Fermentas, Germany) was loaded into the first well of the gel. The gel was allowed to electrophorese for 45 min (120V and 30 mA), then DNA was visualized under UV light and the picture was documented using a gel documentation system (Bio-Rad, England).

Nucleotide sequencing and phylogenetic analysis

PCR products were sent for commercial sequencing to MWG-Biotech AG Company, Germany). Editseq program was used to edit the sequences before forward and reverse sequences of each strain were joined into one sequence using the sequence assembly software Seqman (Lasergen, DNAstar, US). Multiple sequence alignment was performed using ClustalW (http://

clustalw.ddbj.nig.ac.jp/top-e.html) and phylogenetic and molecular evolutionary analyses were conducted using MEGA 6 (Tamura *et al*., 2013). The evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei, 1987). The significance of all deduced phylogenetic trees was verified by bootstrap analysis of 1000 replicates.

The nucleotide sequences of isolates TY-1/90 and DIK-90 were compared with nucleotide sequence in the GenBank database using the online BLASTN program on the NCBI website (Altschul *et al*., 1997).

To assess the genetic lineage of the NDV strains used in this study, either phylogenetic analysis was carried out using NDV fusion protein gene sequences, which were obtained during this study, or reference sequences obtained from the GenBank. The strain name, host, region and year of isolation, genotype assignment and accession numbers, for all sequences used in the analysis are shown in Table 1.

Results

Reverse transcription polymerase chain reaction (RT-PCR)

Following RNA isolation, RNA from all the strains was reverse transcribed then amplified in a OneStep RT-PCR reaction to confirm NDV identity and to achieve molecular characterization analysis. The primer set used in this study was able to amplify fragments with the expected sizes of 1412 bp, from the NDV fusion protein gene. Examination of the amplified PCR products following electrophoresis on agarose gels revealed the expected sizes of amplicons for NDV isolates (Fig. 1).
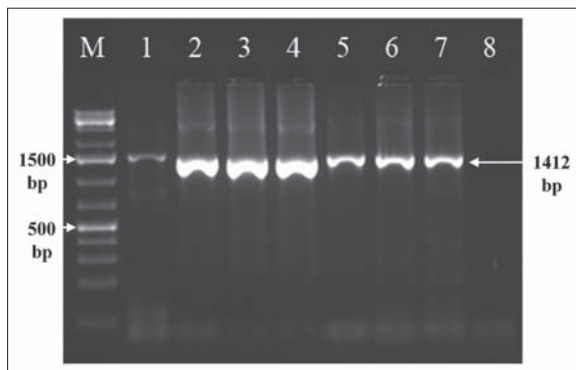
Sequencing and sequence alignment

Sequencing of the fusion protein gene of TY-1/90, DIK-90, GD.S.1, and Dongola 88/1 strains using the NCD_F forward and NCD_R reverse primers resulted

Fig. 1. RT-PCR amplification of the Newcastle disease virus fusion protein gene using F1 (forward) and R (reverse) primer combination, which gave a product size of 1412 bp. The amplicons were electrophoresed in 2% agarose gel. Lanes: M, molecular size marker; Lanes 1-7 are NDV strains I2, TY-1/90, C30, Dik-90, GD.S.1, Lasota and Dongola, respectively; Lane 8, –ve control (H$_2$O).

in readable sequences that varied in length between 906-1002 nucleotides. For the purpose of accurate alignment data, all the sequences were reduced to an equal length of 894 nucleotides. This length covered the region between nucleotide numbers 588 and 1481 of the complete coding sequence (1662 nucleotides) of the fusion protein gene.

The sequence alignment performed for TY-1/90, DIK-90, GD.S.1, and Dongola 88/1 showed that all the sequences were identical in 759 nucleotides (84.9%), while differed from each other in 135 nucleotides (15.1%). The alignment showed that the sequences were grouped into two groups according to the number of identity a group contained strains TY-1/90 and Dik-90 (Velogenic strains) and a group contained the rest of the sequences (Lentogenic strains). Strains TY-1/90 and Dik-90 showed a high degree of identity among themselves as 885 out of 894 (98.99%) nucleotides were similar.

BLAST search and phylogenetic analysis of partial fusion gene

Results of the BLAST search for TY-1/90 partial F gene nucleotide sequences revealed 96% identity with NDV strain APMV1/chicken/Japan/Osaka/2440/1969 (AB853926.2) isolated in Japan in 1969, strain IS 744/99 (JF795620.1) isolated in Israel in 2011 and strain ETH10065 (KC205475.1) isolated in Ethiopia in 2014. Strain DIK-90 demonstrated 96% nucleotide identity with strain APMV1/chicken/Japan/Osaka/2440/1969 (AB853926.2), strain Warwick (Z12111.1) isolated in Great Britain in 1966, Pigeon/Nigeria/VRD07-173/2007 (JQ039395.1) isolated in Nigeria in 2007, strain dove/Nigeria/VRD07-163/2007 (JQ039385.1) isolated in Nigeria in 2007 and strain 2011_Ethiopia_ETH10065 (KC205475.1) isolated in Ethiopia in 2014. In light of the percent identity matrix created by Clustal 2.1 (Table 2), Sudan isolates of NDV of the 2000s (chicken/Sudan/02-06) showed 96.18-100% nucleotide sequence homology between them, while Sudan isolates of 1970s in addition to isolate Chicken/Sudan/Obeid isolated in 1987 shared 95.99- 98.41% identity. The Sudan 1970/80s shared 87.58-89.81% identity with the Sudan 2000s isolates. On the other hand, isolates of 1990s analyzed in this study (TY-1/90 and DIK-90) showed only 41.61-43.62% nucleotide homology with both the Sudan 1970/80s and 2000s isolates (Table 2).

Based on the phylogenetic analysis performed in this study, all studied NDV strains can be divided into two clusters, which represent the genotypes II (strains I-2, Clone 30, GD.S.1, LaSota VC and Dongola 88/1) and VI (TY-1/90 and DIK-90). TY-1/90 and DIK-90 strains clustered close to sub-genotypes b, f, g and e of genotype VI at one branch of the tree, while the other Sudanese strains of the 1970s, 1980s and 2000s clustered close to sub-genotypes a, d and h of genotype VI at the other branch (Fig. 2).

Table 1. Referential Newcastle disease virus (NDV) strains used in this study for phylogenetic analysis of the fusion protein gene.

Sno	Strain	Host	Year	Country	Genotype	Accession number
1	Ulster 2C/67	Fowl	1967	N. Ireland	I	D00243
2	V4 Queensland/66	Fowl	1966	Australia	I	M24693
3	Beaudette C/45	Fowl	1945	USA	II	X04719
4	La Sota/46	Fowl	1946	USA	II	M24696
5	Texas G.B 48	Fowl	1948	USA	II	M24698
6	B1	Fowl	2000	USA	II	AF309418
7	V4	Fowl	2003	China	II	AY225110
8	Clone 30	Fowl	2005	_	II	Y18898
9	LaSota	Fowl	1999	Netherlands	II	AF077761
10	Miyadera/51	Fowl	1951	Japan	III	M18456
11	Miyadera/51/1	Fowl	1951	Japan	III	M24701
12	Herts/33	Fowl	1933	UK	IV	M24702
13	Italien/45	Fowl	1945	Italy	IV	M17710
14	Chicken/Sudan/Obied/1987	Fowl	1987	Sudan	IV	GQ258674
15	Cormorant/US(CA)D9704285	Cormorant	1997	USA	V	GQ288381
16	Cormorant/US(CA)92-23071	Cormorant	1992	USA	V	GQ288388
17	VRDC/NDV/F/N9	Fowl	2012	India	V	KJ621049
18	Iraq AG68	Fowl	1998	Iraq	VI	AF001108
19	Q-GB 506/97	Fowl	1998	Denmark	VI	AF001129
20	Pigeon/Guangdong/GZ293	Pigeon	2014	China	VI	KT381592
21	TX3503/04	Pigeon	2008	USA	VI	KT381592
22	SD-2/75	Fowl	1975	Sudan	VI	AY151384
23	SD-3/75	Fowl	1975	Sudan	VI	AY151383
24	SD-4/75	Fowl	1975	Sudan	VI	AY151385
25	Kuwait 256	Fowl	1968	Kuwait	VIa	AF001109
26	Lebanon 70	Fowl	1970	Lebanon	VIa	AF001110
27	Ch/98-1	Pigeon	1998	China	VIb	AF358785
28	VRD07-163/2007	Dove	2012	Nigeria	VIb	JQ039385
29	DK-6/95	Ostrich	1995	Denmark	VId	AF001130
30	A-24/96	Fowl	1996	Austria	VId	AF001133
31	Warwick/66	Fowl	1966	Great Britain	VIe	Z12111
32	JX-1/94	Fowl	1994	China	VIf	AF458021
33	ZhJ-2/86	Fowl	1986	China	VIg	AF458016
34	Sh-1/97	Fowl	1997	China	VIg	AF458018
35	TW-C69-2-9	Fowl	1969	Taiwan	VIh	AY372129
36	TW-C81-4-3	Fowl	1981	Taiwan	VIh	AY372133
37	JS-2/98(Go)	Goose	1998	China	VII	AF456439
38	AE 232/1/96	Partridge	1996	UAE	VIIb	AF109884
39	ZW3422/95	Fowl	1997	Zimbabwe	VIIb	AF109877
40	CH-A7/96	Fowl	1996	China	VIIc	AY028995
41	JS-3/00	Fowl	2000	China	VIIc	AF458010
42	Chicken/Sudan/02/2005	Fowl	2005	Sudan	VIId	GQ258669

(*Contd...*)

Table 1. *Continued*.

| Sno	Strain	Host	Year	Country	Genotype	Accession number
43	Chicken/Sudan/08/2004	Fowl	2004	Sudan	VIId	GQ258675
44	Chicken/Sudan/05/2004	Fowl	2004	Sudan	VIId	GQ258672
45	Chicken/Sudan/04/2003	Fowl	2003	Sudan	VIId	GQ258671
46	Chicken/Sudan/03/2003	Fowl	2003	Sudan	VIId	GQ258670
47	Chicken/Sudan/06/2006	Fowl	2006	Sudan	VIId	GQ258673
48	Taiwan 95	Fowl	1995	Taiwan	VIIe	U62620
49	Nd/chicken/TR/53	Fowl	2014	Turkey	VIIi	KT585633
50	Nd/chicken/TR/19-2	Fowl	2012	Turkey	VIIi	KT585617
51	F48E9	Fowl	1948	China	IX	AF163440
52	FJ-1/85	Fowl	1985	China	IX	AF458009
53	TW-C69-10-36	Fowl	1969	Taiwan	X	AY372163
54	TW-C81-4-6	Fowl	1981	Taiwan	X	AY372137

Table 2. Percent Identity Matrix created by Clustal 2.1 for published velogenic Sudanese isolates and representative VI genotypes of Newcastle disease virus from different regions of the world based on nucleotide sequence of partial F gene.

Virus isolate	(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)	(11)	(12)	(13)	(14)	(15)	(16)	(17)	(18)	(19)
(1) Warwick	100	93.8	86.8	85.3	89.4	92.6	92.8	44.1	42.3	42.3	42.9	44.5	44.0	44.0	43.7	42.7	43.3	43.0	43.0
(2) Sh-1/97	93.8	100.	92.0	91.4	94.0	94.1	94.4	41.9	42.3	42.3	43.0	43.4	43.6	43.4	42.6	41.6	43.2	41.9	41.9
(3) TX3503/04	86.8	92.0	100.	94.8	94.0	93.9	94.0	43.6	43.0	43.0	43.6	44.3	43.4	43.4	43.2	42.5	43.3	42.7	42.5
(4) Pigeon/Gdg/GZ293	85.3	91.4	94.8	100.	92.7	92.4	92.5	44.6	44.6	43.3	44.0	44.8	44.0	44.0	44.7	43.0	44.0	43.3	43.0
(5) 5Ch/98-1	89.4	94.0	94.0	92.7	100.	94.2	94.5	45.3	45.3	44.0	44.3	45.6	44.8	44.8	44.5	43.6	44.0	43.9	43.6
(6) TY-1/90	92.6	94.2	93.9	92.4	94.2	100.	99.0	42.4	42.6	42.3	43.0	43.4	43.6	42.6	42.6	41.3	42.0	41.6	41.6
(7) Dik_90	92.8	94.4	94.0	92.5	94.5	99.0	100.	42.8	42.6	42.3	43.0	43.4	42.6	42.6	42.4	41.3	42.0	41.6	41.6
(8) TW-C69-2-9	44.1	41.9	43.6	44.6	45.3	42.4	42.7	100.	88.5.	89.0	89.0	96.6	97.4	97.9	97.4	95.0	96.8	96.3	96.3
(9) Chicken/Sudan/06	42.3	42.3	42.9	43.3	44.0	42.6	42.6	88.5	100.	96.2	96.2	97.8	87.9	87.6	87.3	88.2	88.5	88.2	88.2
(10) Chicken/Sudan/08	42.3	42.3	43.0	43.3	43.6	42.3	42.3	89.0	96.2	100.	98.4	86.3	87.9	87.6	87.3	87.6	87.9	88.2	88.2
(11) Chicken/Sudan/03*	43.0	43.0	43.6	44.0	44.3	43.0	43.0	89.0	97.8	98.4	100.	87.9	89.5	89.2	88.9	89.2	89.5	89.8	89.8
(12) DK-6/95	44.5	43.4	44.2	44.8	45.6	43.4	43.4	96.6	86.3	87.9	86.3	100.	95.1	95.4	94.9	92.5	94.6	93.6	93.3
(13) Lebanon 70	44.0	42.6	43.4	44.0	44.8	42.6	42.6	97.4	87.9	87.9	89.5	95.1	100.	99.2	98.7	96.8	97.5	97.6	97.3
(14) Kuwait 256	44.0	42.6	43.4	44.0	44.8	42.6	42.6	97.9	87.6	87.6	89.2	95.4	99.2	100.	99.5	96.5	97.1	97.3	97.1
(15) Iraq AG68	43.7	42.4	43.1	43.7	44.5	42.4	42.4	97.4	87.3	87.3	88.9	94.9	98.7	99.5	100.	96.0	96.8	96.8	96.5
(16) SD-4/75	42.7	41.6	42.5	43.0	43.6	41.3	41.3	95.0	88.2	87.6	89.2	92.5	96.8	96.5	96.0	100.	98.4	97.6	97.3
(17) Chicken/SD/Obied	43.3	42.0	43.3	44.0	44.0	42.0	42.0	96.8	88.5	87.9	89.5	94.6	97.5	97.1	96.8	98.4	100.	98.4	98.4
(18) SD-2/75	43.0	42.0	42.7	43.3	43.9	41.6	41.6	96.3	88.2	88.2	89.8	93.6	97.6	97.3	96.8	97.6	98.4	100.	99.7
(19) SD-3/75	43.0	41.9	42.5	43.0	43.6	41.6	41.6	96.3	88.2	88.2	89.8	93.3	93.6	97.0	96.5	97.3	98.4	99.7	100.

Discussion

Four strains of NDV, 2 velogenic (TY-1/90 and DIK-90) and two lentogenic (Dongola and GD.S.1), were selected for this study. A number of 894 nucleotides of the fusion protein gene of each of the strains were sequenced and analyzed before a phylogenetic tree was constructed.

The phylogenetic analysis showed that TY-1/90 and DIK-90 strains belonged to genotype VI within a
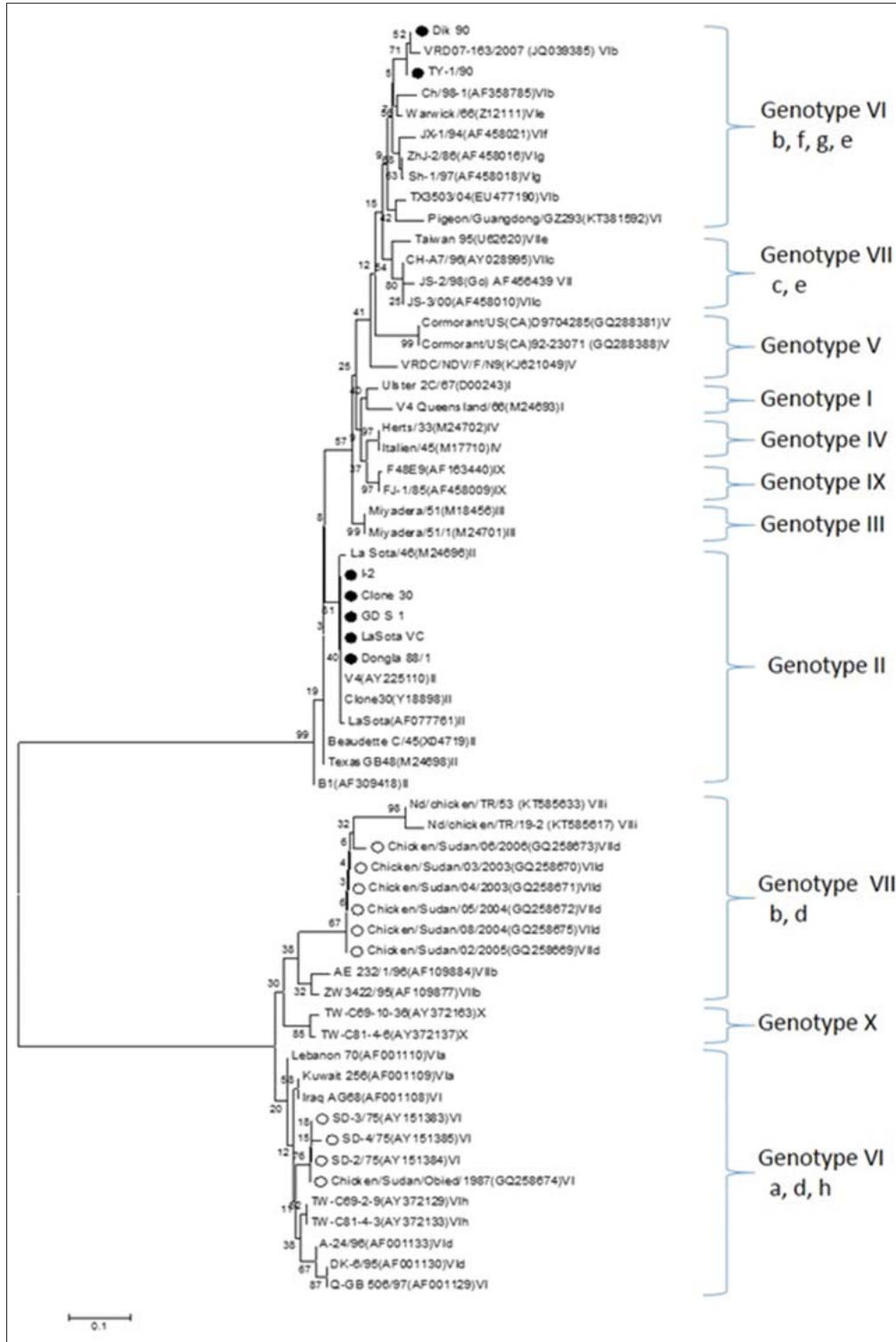
Fig. 2. Rooted phylogenetic tree of the nucleotide sequences of the fusion protein gene of Newcastle disease virus strains. The evolutionary history was inferred using the Neighbor-Joining method. The optimal tree with the sum of branch length = 2.20497769 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site. The analysis involved 61 nucleotide sequences. Evolutionary analyses were conducted in MEGA7. Isolates analyzed in this study are marked with filled circle, whereas those reported earlier from the Sudan are marked with empty circles.

sub-group containing sub-genotypes b, f, g and e. The BLAST search revealed a 96% nucleotide homology with VI genotype NDVs mostly from Africa and Asia that were characterized as belonging to sub genotypes a, b, f and e (Van Borm *et al.*, 2012; de Almeida *et al.*, 2013; Umali *et al.*, 2013). As the partial sequence of the F gene does not allow precise delineation of NDV viruses at the sub-genotype level (de Almeida *et al.*, 2013), it is not possible to designate a sub-genotype for isolates TY-1/90 and DIK-90. These two strains originated from natural severe outbreaks of ND that occurred in Tayba, 30 Km south of Khartoum, in July 1990 (TY-1/90) and Dikheinat, 15 Km south of Khartoum, in September 1990 (DIK-90) (Khalafalla *et al.*, 1992), respectively. Affected chickens exhibited symptoms characterized by profuse diarrhea and paralysis of wings and legs demonstrating the involvement of digestive and nervous systems. According to the classical pathotyping, TY-1/90 and DIK-90 strains were described as viscerotropic velogenic NDV in light of the fact that they kill chicken embryos quickly and produce haemorrhagic lesions in the intestinal tract in infected chickens (Khalafalla *et al.*, 1992). The mean death time (MDT) and intracerebral pathogenicity index (ICPI) values used in the pathotyping were similar to those described by Hanson *et al.* (1973) for the viscerotropic pathotypes of NDV.

Genotype VI has been shown to be characterized by frequent branching and repeated epizootics caused by molecular variants separated by only short evolutionary intervals (Lomniczi *et al.*, 1998). The genotype was divided into eight subtypes (a-h) (Liu *et al.*, 2003; Tsai *et al.*, 2004; Lien *et al.*, 2007). Genotype VIb is comprised mostly of viruses associated with panzootic infection in pigeons (*Columba livia*) (Aldous *et al.*, 2003) and isolates studied so far usually belonged to mAb group P (Alexander *et al.*, 1997). Pigeon isolates were further divided into two genetic lineages (Ujvári *et al.*, 2003). The majority of isolates clustered into a single genetic lineage, termed VIb/1, within the genotype VI of NDV strains of chickens, whereas a small number of isolates that originated in Croatia after 1995, are grouped in a highly diverged lineage, termed VIb/2. Our phylogenetic analysis showed that the Sudanese strains TY-1/90 and DIK-90 studied in this study cluster close to genotype VIb that included four strains isolated from pigeons and doves. This finding supports the assumption that the pigeon PMV-1 (PPMV-1) could be of African origin (Ujvári *et al.*, 2003).

The mid-1970s recorded outbreaks of ND occurred in chicken in Sudan were caused by Genotype VI including SD-2/75, SD-3/75 and SD-4/75 strains (Ujvári *et al.*, 2003). The aforementioned Sudanese isolates clustered together, in this study within the VI genotype group (containing sub-genotypes a, d and h)

viruses in a separate branch that likewise contains the Middle Eastern isolates of subtype VIa. The previously described Sudanese isolates of 1970/80s and 2000s isolates shared a relatively high (87.58-89.81%) sequence homology, while isolates of 1990s analyzed in this study (TY-1/90 and DIK-90) shared only 41.61-43.62% nucleotide homology with them. Furthermore, the results of the phylogenetic analysis affirm that TY-1/90 and DIK-90 are distinct from previously reported NDV isolates of the Sudan as each group cluster in different branches of the tree. Accordingly, the origin of these two groups of NDVs seems to be different.

Our results demonstrated that the Sudanese strains Dongola 88/1 and GD.S.1 fit in with genotype II. Genotype II consists primarily of viruses that were isolated from 1945 to 2000 worldwide, varying from virulent to mild isolates (Aldous *et al.*, 2003). Generally, genotype II has two subclusters a velogenic and a lentogenic (Qin *et al.*, 2008). The Beaudette C/45 and Texas48 isolates, which have a polybasic-F0 cleavage site motif typical for velogenic NDV strain fall into one subcluster, while the reference vaccine strains (LaSota, B1, Clone 30, La Sota/46 and V4), which have a motif typical of lentogenic NDV strain fall into the other subcluster. All the above-mentioned isolates analyzed in this study fell into the lentogenic cluster of genotype II.

For Dongola 88/1 strain, this result is in accordance with our previous findings of Khalafalla (1994). Khalafalla described Dongola strain, which was isolated in 1988 from apparently healthy chickens in Dongola town of northern Sudan, as a lentogenic pathotype of NDV, causing a mild or inapparent infection of the respiratory tract. The study of the mAb binding pattern placed Dongola strain in group E of paramyxoviruses (Khalafalla, 1994). The results obtained for GD.S.1, which was isolated from Gedarif in June 1999, are in contrast with the previous description of the strain as velogenic (Abdelaziz *et al.*, 2004). These two isolates are probably originated lentogenic strains of NDV used as vaccines. Further whole genome sequencing is expected to unveil whether these isolates were vaccine or field lentogenic strains.

A limitation of this study is that we used partial F gene sequences to characterize genetically our NDV isolates instead of the full F gene as proposed by Diel *et al.* (2012).

It can be concluded that both lentogenic and velogenic NDV exist in Sudan. Genotype VI of NDV has been maintained since 1970s and until at least the 1990s. This genotype has probably given origin to PPMV-1 strains of the 1980s. Amid the same period, other genotypes of NDV existed in Sudan. Genotype IV was isolated from domestic fowls on 2 occasions; in 1987 (Hassan *et al.*, 2010) and in 1991 (Aldous *et al.*, 2003). More

recently, genotype VIId was isolated from vaccinated immature and adult layer flocks in Khartoum State and eastern Sudan (Gadarif) between 2003 and 2006 (Hassan *et al*., 2010). Along these lines, further studies using phylogenetic analysis are necessary to enable a full view on the evolving status of ND in Sudan, to trace the sources of infection, and to assist in taking decisions to improve the disease control efforts.

Conflict of interest
The authors of this article declare that there is no conflict of interest.

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