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Research Article

Genetic characterization of genotype VII.1.1 Newcastle Disease viruses from commercial and backyard broiler chickens in Egypt

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Abstract

The small-scale and backyard-raised poultry are extensively growing in Egypt. However, low biosecurity and/or vaccination practices are adopted. The current study was conducted to investigate and molecularly characterize Newcastle Disease (ND) strains circulating in small-scale and backyard poultry sectors in Giza governorate in Egypt from July 2018 to April 2020. Twelve broiler flocks (6 commercial and 6 backyard flocks) suffering from respiratory and/or nervous signs were included. Virus isolation and molecular characterization were conducted. In the present study, three NDV isolates were isolated from commercial (n=2) and backyards (n=1) flocks. The isolated viruses maintained the multi-basic motif ^{112}RRQKRF^{117} in the fusion protein cleavage site indicative of their virulent nature. Phylogenetically, the viruses are closely related to genotype VII.1.1. ND viruses are genetically different from NDV genotype II vaccine strains. Deduced amino acid sequences of the fusion (F) protein showed an amino acid change of S278P in the heptad repeat (HRb) in the two NDV isolates from commercial farms. Other amino acid substitutions were observed including the change at positions 442 (A/T) in the two commercial flocks isolates and position 317 (A/S) in the backyard ND isolate. Few silent nucleotide mutations were also observed in the F protein of the NDV isolate from the backyard flocks. In conclusion, the current study reports the genetic identification of virulent NDV viruses from commercial or backyard flocks related to genotype VII.1.1. ND circulating in Egypt. The amino acid substitutions and nucleotide changes warrant the need for continuous surveillance of NDV in these growing poultry sectors considering their importance as a spot for extensive NDV circulation and as the main source for live bird markets in Egypt.

Keywords: Newcastle disease virus, Genotype VII.1.1., Fusion protein, Backyard flocks, Commercial flocks, Egypt

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Introduction

Poultry, the largest livestock group, accounts for more than 30% of all animal protein worldwide. The industry is mainly based on commercial poultry farms which account for only 20% of the total poultry population (FAO, 2012). Backyard flocks represents a vast majority that mainly raising native breeds in developing countries. In Egypt, villagers raise poultry to meet household food demands and an additional sources of income (Nagar and Ibrahim, 2007). Due to different economic crises in the last decade, backyard production extended to raise commercial broiler chickens with low numbers (100-1500 birds). However, this sector implies low biosecurity measures with high risk of infectious diseases, such as Newcastle Disease (ND) and highly pathogenic Avian Influenza (Gomaa et al., 2020).

ND is a highly contagious and devastating viral disease of the poultry of worldwide distribution with an enormous economic impact (Alexander et al., 2012). ND is caused by virulent strains avian *Orthoavulavirus* 1 species belonging to the genus *Orthoavulavirus* of the subfamily *Avulavirinae* of family *Paramyxoviridae* (Amarasinghe et al., 2019). Class I consists of single genotype 1 with 3 sub-genotypes named 1.1.1, 1.1.2, and 1.2. Class II NDV contains at least 20 distinct genotypes (I to XXI) of non-virulent and virulent viruses. The use of the updated classification criteria revealed that genotype VII NDV is one of the most diverse NDV genotypes with three distinct subgenotypes VII.1.1, VII.1.2, and VII.2 (Dimitrov et al., 2019a).

Table 1: Type, clinical history, vaccination programs, and mortalities of examined chicken flocks. Accession numbers of characterized viruses in this study are indicated (*)

Type	Flock ID	Age (days)	Total No	Daily mortality (No)	Clinical signs[1]	Postmortem lesions	Isolation date	Vaccination[2]
Small-scale farms	GIZ-2/18	29	1000	10,20,60	Nervous signs and sneezing	Congested lung, trachea, and spleen	Feb-2018	Day 7 Hitchner+IB Day 17 LaSota
	GIZ-1. Comm.18-MZ683426*	27	4000	2,2,80	Diarrhea and rales	Congested lungs, air saculitis, and proventricular petechial hemorrhage	Jul-2018	Day 7 Hitchner + IB Day 17 LaSota
	GIZ-7/18-2	30	7000	8,11,15,30	Greenish diarrhea and nervous signs	Swelling of head and congested kidneys	Jul-2018	Day 7 Hitchner + IB Day 9 Inactiv. NDV Day 17 LaSota
	GIZ-7/18-3	25	8000	20,30,38	Respiratory signs and diarrhea	Enteritis, congested lung, and trachea	Jul-2018	Day 7 Hitchner + IB Day 9 Inactiv. NDV Day 17 LaSota
	GIZ-2. Comm.18-MZ683427*	25	3000	1,6,15	Sneezing and greenish diarrhea	Congested lung, liver, spleen, and trachea	Feb-2019	Day 7 Hitchner + IB Day 9 Inactiv. NDV Day 17 LaSota
	GIZ-4/19	25	1000	35,70,150,250	Greenish diarrhea	Air saculitis, enteritis, and proventricular petechial hemorrhage	Apr-2019	Day 7 Hitchner + IB Day 17 LaSota
Backyard flocks	GIZ-2/19	15	150	2,3,4	Incoordination and sneezing	Congested lung and liver	Feb-2019	None
	GIZ-2/19-2	35	700	7,9	Nasal discharge and coughing	Congested internal organs	Feb-2019	None
	GIZ-3/19	22	105	11,20	Respiratory signs	Congested lung, trachea, and liver, enteritis	Mar-2019	None
	GIZ-3/19-2	25	150	30,45	High mortalities and greenish diarrhea	Congested lung and liver	Mar-2019	None
	GIZ-2/20 Backyard.20-MZ683428*	20	100	5,8	Respiratory signs	Congested lung, liver, and trachea	Feb-2020	None
	GIZ-2/20-2	32	30	2,3,5	Sudden death and diarrhea	Congested lung and spleen	Feb-2020	None

[1]Nervous sign included torticollis, head shaking and incoordination

[2]Hitchner+ IB; is a bivalent live attenuated vaccine against NDV and infectious bronchitis virus, Inactiv. NDV; inactivated NDV vaccine.

The virulence of NDV strains can be evaluated using molecular techniques and sequencing. Also, the intracerebral pathogenicity index (ICPI) of 0.7 or more indicates virulent NDV strains. However, the in-vivo ICPI experiments need specific laboratory biosafety and biosecurity measures. Still the presence of multiple basic amino acids at the C-terminus of the F2 protein and phenylalanine at residue 117 of its F protein, is a validated method to classify the virus as virulent strain (Swayne and Brown, 2021).

In Egypt, several outbreaks of NDV by velogenic strains have been reported in different avian species such as chickens, ducks, and pigeons (Radwan et al., 2013; Ewies et al., 2017). Moreover, recent studies reported that the circulation of velogenic NDV in small-scale or backyard poultry holdings with transmission between farms is probably responsible for the rapid circulation and evolution of NDV genotype VII.1.1. in Egypt (Orabi et al., 2017; Manoharan et al., 2018; Nagy et al., 2020). The widespread of small-scale commercial and backyard poultry is of great concern due to the frequent exposure of these sectors to virulent NDV strains. The lack of biosecurity and vaccination make these sectors serve as amplification spots that may spill NDV to commercial poultry flocks or may contribute to the emergence of new strains (Conan et al., 2012).

This study investigated small-scale broiler and backyard commercial poultry flocks suffering from signs suggestive of ND infection. NDV isolation and genetic characterization were conducted in comparison to the recently published avian *Orthoavulavirus* strains in Egypt.

Materials and Methods

Field samples

Twelve broiler flocks (6 vaccinated commercial farms and 6 none vaccinated backyard flocks) of various ages from Giza governorate from July 2018 to April 2020 were included. Birds were suffering from respiratory signs and gross lesions suggestive of NDV infection (Table 1). Diseased and freshly dead chickens were subjected to clinical and postmortem examination as well as for sample collection for virus isolation. Lungs, cecal tonsils, spleen, brain, trachea, proventriculus, and liver tissue samples were collected from five diseased and freshly dead chickens/flock for virus isolation. Organs from each flock were pooled and homogenized in 10% in phosphate buffer saline (PBS) with antibiotics (streptomycin 10 mg/ml and gentamycin 250 mg/ml). The suspensions were clarified by centrifuging at 2000 rpm for 10 min at 4°C (Ewies et al., 2017).

Molecular detection of NDV in tissue homogenates

The RT-PCR was performed on the total RNA extracted from tissue homogenates using the Patho Gene-Spin TM DNA/RNA Extraction kit (iNtRON biotechnology, Korea) according to the manufacturer's instructions. The oligonucleotide primers specific for the F0 gene fragment (NDV-M2; TGG AGC CAA ACC CGC ACC TGC GG and NDV-F2; GGA GGA TGT TGG CAG CAT T) were used as previously described (Radwan et al., 2013; Ewies et al., 2017).

Isolation of NDV in embryonated chicken eggs

Positive NDV RT-PCR field samples used for isolation of NDV in specific pathogen-free embryonated chicken eggs (SPF-ECE). For each sample, 0.2 ml of the positive samples were inoculated into 9-day-old SPF-ECE via the allantoic cavity. The inoculated eggs were incubated at 37°C for 4-5 days with daily observing the embryo viability. Deaths within the first 24 hr were discarded. Dead (after ≥48 hr) or survived embryos were chilled overnight and the allantoic fluids were harvested and tested by the hemagglutination (HA) test titers. HA positive allantoic fluids were checked using the hemagglutination inhibition (HI) test using NDV, avian influenza H5N1, H9N2, and H5N8 specific antisera (in-house prepared) (Swayne and Brown, 2021). Additionally, a screening RT-PCR using F-gene specific oligonucleotide primers was conducted as previously described (Radwan et al., 2013; Ewies et al., 2017).

NDV full F-gene amplification, sequencing, and sequence analysis

The viral RNA was extracted from the collected allantoic fluids and the full F-genes of the NDV were amplified using specific primers for amplifying the complete F gene (NDV-F: 5'-ATGGGCTCCAAACCTTCTA-'3 and NDV-R: 5'-GGAAACCTTCGTTCCTCAT-'3) (Nagy et al., 2020) using Easy Script One-Step RT-PCR SuperMix (Transgenbiotech, China). The QIA quick PCR product extraction kit (Qiagen Inc. CA) was used to purify the amplified PCR product from the agarose gel. Samples were sent for sequencing at SolGent Co., Ltd, Korea.

BLAST analysis of obtained sequences data was performed (http://www.ncbi.nlm.nih.gov) and the sequences were analyzed with reference and recently published NDV sequences according to (Dimitrov et al., 2019b). Briefly, the phylogeny including this study isolates was constructed using a large number of sequences of the previously recorded genotypes in Egypt available at https://github.com/NDVconsortium/NDV_Sequence_Datasets/. The phylogenetic analysis involved 72 nucleotide sequences. Evolutionary analyses were conducted in MEGA7 using the Neighbor-Joining method with bootstrap test (1000 replicates) (Kumar et al., 2016). Nucleotide and amino acid identities were determined using Geneious® 7.1.3 (Copyright © 2005-2014 Biomatters Ltd.).

Results

Field survey of broiler flocks

History, clinical signs, and postmortem examinations of the investigated 12 broiler flocks from Giza governorate were carried out to isolate and characterize NDV isolates (Table 1). The diseased broiler chickens

Table 2: Nucleotide and amino acid sequence identities of the F gene of isolated ND from small-scale and backyard commercial broilers

Virus	1	2	3	4	5	6	7	8	9	10	11	12	13
1. GZ-1.Comm/18 acc.no. MZ683426		100	99.2	99.4	99.4	98.9	99.4	99.4	99.6	98.9	88.9	88.4	88.7
2. GZ-2.Comm/19 acc.no. MZ683427	99.9		99.2	99.4	99.4	98.9	99.4	99.4	99.6	98.9	89	88.4	88.8
3. GZ-11.Comm/20 acc.no. MZ683428	98.3	98.3		99.4	99.8	99.2	99.4	99.4	99.6	98.8	89.1	88.5	88.9
4. VII 1.1. Egypt/Ch/MN51/2019	98	97.8	97.9		99.6	99	99.6	99.6	99.8	99	89.1	88.5	88.9
5. VII 1.1. Egypt/Ch/D30-F0/2018	98.3	98.1	97.9	98.3		99.4	99.6	99.6	99.8	99	89.3	88.7	89.1
6. VII 1.1. Egypt/Ch/R78-F0/2018	98.2	98.1	97.6	98.1	99		99	99	99.2	98.9	88.8	88.2	88.6
7. VII.1.1. 2016—Egy-Beh—ck—NR737	99	98.9	97.7	98.7	98.8	98.7		99.6	99.8	99	89	88.4	88.8
8. VII 1.1. Ck/Eg/Luxor/2012/16	98.5	98.3	97.6	98.7	99.3	98.6	99		99.8	99	88.9	88.3	88.7
9. VII 1.1. NDV/quail/Eg/SDU-2/2016	98.4	98.3	97.6	98.7	99.4	98.5	98.9	99.8		99.2	89.1	88.5	88.9
10. VII.1.1. NDV-FU8-EGYPT-NLQP-14	98.3	98.1	97.5	98.5	99	98.5	98.8	99.3	99.4		88.4	87.9	88.2
11. II NDV Hitchner B1	83.2	83.1	82.9	83.2	83.3	83.2	83.6	83.6	83.5	83.6		99.5	99.8
12. VG/GA F gene complete	83	82.9	82.6	83	83.1	83	83.4	83.4	83.3	83.4	99.7		99.3
13. LaSota F gene complete	83.2	83	82.8	83.1	83.3	83.1	83.6	83.5	83.5	83.5	99.2	98.9	
	Nucleotide identity (%)												

Amino acid identities (%)

Table 3: Amino acid substitutions in the F gene of NDV VII.1. of NDV strains in comparison to the selected vaccine and field strains. The amino acid sequences of vaccine strains in the reported positions are gray shaded

Virus	Cleavage site	Heptad repeat (HRb)	Other amino acid positions	
	112-117	278	317	442
Consensus residues	RRQKRF/ GRQGRL	S	A	A/V
GZ-1.Comm/18 acc.no. MZ683426	RRQKRF	P	A	T
GZ-2.Comm/19 acc.no. MZ683427	RRQKRF	P	A	T
GZ-11.Comm/20 acc.no. MZ683428	RRQKRF	S	S	A
VII 1.1. Egypt/Ch/MN51/2019	RRQKRF	S	A	A
VII 1.1. Egypt/Ch/D30-F0/2018	RRQKRF	S	A	A
VII 1.1. Egypt/Ch/R78-F0/2018	RRQKRF	S	A	A
VII.1.1. 2016—Egy-Beh—ck—NR737	RRQKRF	S	A	A
VII 1.1. Ck/Eg/Luxor/2012/16	RRQKRF	S	A	A
VII 1.1. NDV/quail/Eg/SDU-2/2016	RRQKRF	S	A	A
VII.1.1. NDV-FU8-EGYPT-NLQP-14	RRQKRF	S	A	A
II NDV Hitchner B1	GRQGRL	S	A	V
II VG/GA F gene complete	GRQGRL	S	A	V
II LaSota F gene complete	GRQGRL	S	A	V

suffered from greenish diarrhea, nervous signs, respiratory signs, torticollis, and deaths with variable morbidity and mortality rates. Postmortem lesions were suggestive for NDV infection, including congested lungs and trachea, air saculitis, proventricular gland and cecal tonsils hemorrhages.

NDV isolation and characterization

ND screening RT-PCR revealed positive ND cases in 8 out of the 12 examined flocks (data not shown). The positive PCR samples inoculated in SPF ECE showed positive HA results after embryo deaths within 48- 96hr post-inoculation. The HA titers of the isolated viruses ranged between 5-8 $\log_2$. All the HA positive allantoic fluids were inhibited by NDV specific antiserum. However other hemagglutinating viruses were excluded since the specific antisera of H5N1, H9N2 and H5N8 did not inhibit the HA of positive allantoic fluid.

The F-gene genetic analysis of selected NDV isolates

The full-length F was successfully amplified 6 isolates and the full F gene sequences were obtained for 3 isolates (2 small scales and 1 backyard flock). The F gene sequences were deposited to the GenBank under accession numbers MZ683426, MZ683427, and MZ683428 for the isolates GIZ-1. Comm/2018, GIZ-2. Comm/2019, and GIZ-11. Backyard/2020, respectively. Nucleotide and deduced amino acid sequences revealed that the 2 isolates from small-scale farms have

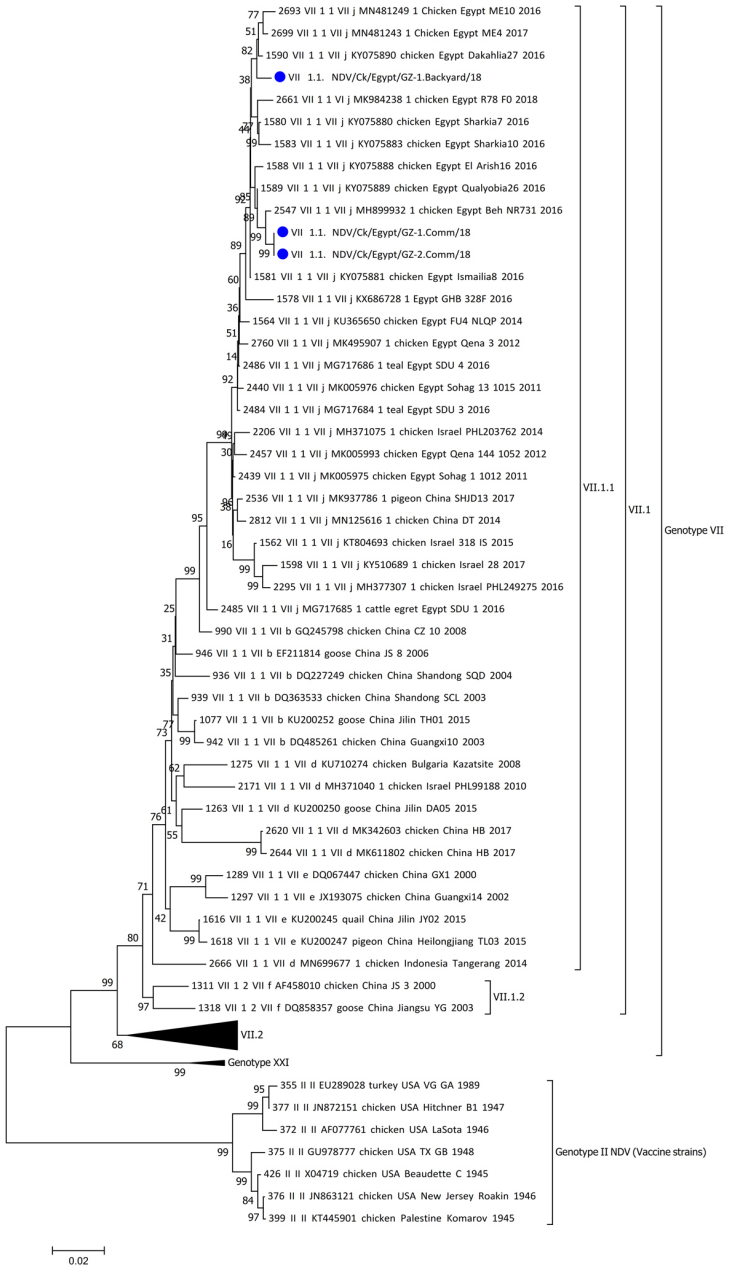

Figure 1: Phylogenetic tree is based on the full nucleotide sequences of the F gene. Isolates of the current study are indicated (blue dots). The analysis involved 72 nucleotide sequences. Evolutionary analyses were conducted in MEGA7 using the Neighbor-Joining method with bootstrap test (1000 replicates) (Kumar et al., 2016)

98-99.5 and 99-99.6% nucleotide and amino acid identities to the recent NDV isolates, respectively. However, the isolate from backyard poultry showed 97.3-98.5 and 98.8-99.6% nucleotide and amino acid identities to the same recent isolates. Though the backyard isolate was 98.3% identical to those isolated from small-scale farms, it showed 99.2% identity on the amino acid level (Table 2).

Phylogenetic analysis showed that the 3 isolates are closely related to the recently isolated NDV strains from Egypt and belonged to genotype VII.1.1 (Figure 1). Compared to recently isolated NDV genotype VII 1.1 strains, the isolated viruses had the characteristic velogenic multi-basic motif ^{112}RRQKRF^{117} cleavage site. Detailed analysis of different domains of the F gene revealed conservation in most of the domains of the gene in the genotype VII 1.1. except for the Heptad repeat (HRb), in which the 2 small-scale farm isolates showed an amino acid change of S278P. Additional changes were an amino acid change in position 442 (A/T) and position 317 (A/S) in the 2 small-scale and backyard isolates, respectively (Table 3).

Discussion

Poor biosecurity measures in the small-scale and backyard-raised poultry predispose them to more frequent exposure to infectious diseases including the ND virus (Chaka et al., 2013; Ogali et al., 2018). Therefore,

monitoring of the potential contribution of these growing sectors to the ND virus evolution and emergence is required (Conan et al., 2012). The current study was conducted to investigate and molecularly characterize NDV strains circulating in small-scale and backyard poultry sectors in Giza governorate of Egypt.

The investigated flocks suffered from greenish diarrhea, nervous and respiratory signs with variable mortalities which are characteristic of ND infection (Swayne and Brown, 2021). The isolated viruses were characterized as velogenic NDV based on their fusion protein cleavage site sequences and were closely related to other genotype VII.1.1 NDV strains isolated from Egypt (Shehata et al., 2019; Abdel-Latif et al., 2020; Nagy et al., 2020). One of the OIE criteria of the virulent ND viruses is the presence of multi-basic motifs at the F gene cleavage site (Swayne and Brown, 2021). The current study characterized viruses maintained the same multi-basic motif ^{112}RRQKRF^{117} cleavage site.

To understand the genetic properties of NDV affecting small-scale and backyard flocks, the deduced amino acid sequences of F protein were evaluated. Interestingly, the 2 small-scale farm isolates showed an amino acid change of S278P in the HRb. Previous reports indicated that substitutions in the fusion peptide and HR regions of F protein may affect the fusion activity of ND viruses (Sergel-Germano et al., 1994; McGinnes et al., 2001). Other amino acid substitutions were observed, including the change at positions 442 (A/T) in the 2 small-scale flocks isolates and position 317 (A/S) in the backyard ND isolate.

These mutations are not located in the previously reported signal peptide, fusion peptide, transmembrane domain, cytoplasmic tail, or antigenic sites of the F protein (McGinnes et al., 2001). Similarly, the backyard NDV isolates shared lower nucleotide identity with those isolated from small-scale and previously published NDV sequences (98.3%). However, the amino acid identities were 99.2% indicating few silent nucleotide mutations. These changes on both the amino acid and nucleotide levels alarm for the need for continuous surveillance of ND in the growing sectors of small-scale and backyard poultry flocks.

Consistent with previous studies indicating that virulent NDV strains are genetically different from vaccine strains. The isolated NDV strains in this study shared low genetic identities with the vaccine strains LaSota, VG/GA, and Hitchner (Nabila et al., 2014; Manoharan et al., 2018; Nagy et al., 2020; Tran et al., 2020). Though commercial genotype II vaccines with strict biosecurity can efficiently prevent ND outbreaks, the attenuated genotype VII.1.1. matched vaccines were suggested to provide better especially in endemic countries (Ji et al., 2018; Ali et al., 2019; Sultan et al., 2020).

In conclusion, the current study identified 3 virulent NDV isolates from commercial broiler chickens raised in small-scale or backyard facilities. Phylogenetically the isolates belong to genotype VII.1.1. NDV circulating in Egypt. The observed mino acid and nucleotide changes indicate the necessity for ongoing surveillance of ND in both small-scale and backyard poultry flocks considering their relevance as a potential source for the widespread and transmission of poultry viruses.

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