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Molecular characterization of Newcastle disease virus (genotype VII) from broiler chickens in Egypt

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ABSTRACT

Newcastle disease (ND) outbreaks have been occurred in the Egyptian poultry causing high mortalities with severe economic losses. Samples were collected from 3 to 4 weeks-old broiler chickens suffering from high mortalities respiratory and/or nervous signs, located at Beni-Suef, Minia, Fayoum, Ismailia and Menofia governorates and Postmortem examination showed hemorrhages at both proventricular glands and cecal tonsils. The samples were subjected to virus isolation trials by inoculation of the processed samples in the allantoic sac of 9-day-old specific pathogen free eggs (SPF). Hemagglutinating activity was tested by hemagglutination test and the isolated viruses were molecularly characterized by RT-PCR targeting the partial F-gene of NDV. Partial F gene sequence analysis showed that the isolated NDV strains belong to genotype VII with the characteristic amino acid sequences of the F0 protein proteolytic cleavage site motifs (^{112}RRQKRF^{117}) for the velogenic NDV strains. No significant differences in both nucleotide and amino acid sequences were observed among different examined strains. The vNDV isolates in this study were identical to each other and they were similar to strains isolated from Egypt during 2012–2014 with minor genetic variation. This study indicated that though intensive ND vaccination programs using LaSota like strains, the vNDV strains of genotype VII are still circulating in the field causing significant economic losses. The genetic variation between the used vaccine strains and the circulating NDV viruses may explain the inability of the currently used vaccines to protect chicken against vNDV genotype VII. Further studies are needed to screen the protection of the currently used vaccines against recently isolated vNDV strains.

1. Introduction

Newcastle disease virus (NDV) is one of the main poultry pathogens severely endangering out the poultry industry causing a highly contagious septicemic, fatal and destructive disease that attacks chiefly chicken and turkeys. Since its first appearance in Indonesia in1926 (Alexander, 2000) the NDV is still widespread in most countries worldwide with the only exception; The Oceania countries that showed a relative freedom from the disease (Dimitrov et al., 2016). In Egypt, the disease had been firstly identified in 1948 (Daubney and Mansy, 1948). The disease became widely spread throughout the country and recorded as an endemic disease by the beginning of the 1960 (El-Nassary and Eskarous, 1960).

The main disease symptoms are respiratory distress, diarrhea, and circulatory disturbance and in chronic cases, impairment of the central nervous system (Alexander et al., 1992). In chickens, the pathogenicity of NDV is dependent on virus strain, the age of chicken and environmental conditions as well as the dose and the route of administration. Infection with a virulent strain causes sudden deaths without major clinical signs in young chickens, while in older birds the disease may be more protracted and with characteristic clinical signs respiratory distress including rales, sneezing, and wet eyes and/or nervous manifestations.

The NDV belongs to avian paramyxovirus serotype-1 (APMV-1), that spreads primarily through direct contact between infected and healthy birds (Kaleta et al., 1979). Virulent NDV isolates possess a high capacity to mutate, allowing the development of multiple virulent genotypes evolving simultaneously. The virulence of NDV strains is determined by multiple factors: tissue or organ tropism, the host immune system and/or the efficacy of replication (Fan et al., 2016). However, based on the genome length and sequence of the F gene, NDV strains have been classified into classes I and class II (fifteen genotypes I-XV). Class I include avirulent strains and comprise a single genotype while class II contains 15 genetic groups including 10 previously established (I–IX, and XI) and five

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new genotypes (X, XII, XIII, XIV and XV). Class II also contains both virulent and avirulent vaccine strains such as LaSota and Hitchner B1 being used worldwide (Diel et al., 2012; Huang et al., 2004; Tsai et al., 2004).

Currently, the circulating strains associated with disease outbreaks worldwide predominantly are from genotypes V, VI, and VII of class II (Aldous et al., 2003; Lin et al., 2003). Genotype VII was initially divided into two subgenotypes: VIIa, representing viruses that emerged in the 1990 s in the Far East and spread to Europe and Asia and VIIb, representing viruses that emerged in the Far East and spread to South Africa (Aldous et al., 2003). The two sub-genotypes of VII are further divided into VIIc, d, and e, which represent isolates from China, Kazakhstan and South Africa (Bogoyavlenskiy et al., 2009; Wang et al., 2006), and VIIf, g, and h, which represent African isolates (Snoeck et al., 2009). In Egypt, The sub-genotype VIId is predominant causing several ND outbreaks in poultry (Radwan et al., 2013).

In many countries legislation exists to control ND outbreaks that may occur. In Egypt, prophylactic vaccination of birds even in absence of outbreaks is being applied. Other countries have a policy of ring vaccination around outbreaks to establish a buffer zone (Alexander and Senne, 2008). Control of NDV outbreaks is not effective after the onset of the disease but different types of vaccines are used for preventing clinical pictures and mortalities induced by the virus. Although the current vaccines offer substantial protection against disease, they do not completely prevent infection or virus shedding and disease can occur in vaccinated birds (Cattoli et al., 2010).

The aim of the current study is to characterize the NDV strains responsible for multiple outbreaks in vaccinated poultry farms in Egypt during 2014 and 2016.

2. Materials and methods

2.1. Field samples

Sixty-eight pooled tissue samples were collected from vaccinated broiler flocks from Beni-Suef (21 flocks) Fayoum (11 flocks), Ismailia (14 flocks), Menofia (14 flocks), and Minia (8 flocks) governorates. Investigated flocks suffered from respiratory distress (rales, sneezing and wet eyes) and/or nervous manifestations (head deviation and torticollis) (Table 1). Died and moribund birds were subjected to necropsy and tissue samples were collected including lung, proventriculus, liver, duodenum, spleen, and cecal tonsils.

Tissue samples from each flock were pooled and ground in sterile phosphate buffer saline pH 7.0–7.4 containing gentamycin (50 μg/ml) and mycostatin (1000 units/ml) in a 1:5 (w/v) dilution, centrifuged and tissue supernatant was collected and stored at −20 °C till being used in virus detection and isolation.

2.2. Virus isolation

Processed samples were inoculated into the allantoic sac of 9-day-old specific pathogen free embryonated chicken eggs (SPF-ECE) (OIE, 2012). Inoculated eggs were incubated at 37 °C for 96 h and candled daily for embryo viability. All inoculated eggs allantoic fluids from dead and surviving embryos were harvested after overnight chilling at 4 °C and tested for hemagglutination using 1% washed chicken red blood cells (OIE, 2012). Initial virus identification was conducted using hemagglutination inhibition test with chicken hyperimmune sera against NDV produced in SPF chickens. Briefly, formalin inactivated vNDV strain (ck/Eg/BSU-BS-KN1/13 acc. No. KR010945) was mixed with the oil adjuvant Montanide ISA70 (SEPPIC, Puteaux, France) according to the manufacturer's instructions. Two-week-old SPF chickens were inoculated subcutaneously (0.5 ml/bird) with adjuvanted inactivated antigen containing approximately 128 HA unit of the virus. Birds received a booster immunization 2 weeks later and sera were collected 2 weeks after the booster dose. Sera were heat inactivated at 56 °C for 30 min (Allan and Gough, 1974). The HI test was conducted using 4 HA units of each antigen and twofold dilution of the sera and titers were determined using 1% chicken red blood cells (OIE, 2012).

Table 1
Distribution of collected field samples indicating the year and location of collection.

Year of collection	Sample No Beni-Suef	Fayoum	Minia	Ismailia	Menofia
2014	9	8	8	14	14
2016	12	3	–	–	–
Total	21	11	8	14	14

2.3. Conventional RT-PCR and gene sequencing

The viral RNA was extracted from harvested allantoic fluids by Bioflux® viral RNA Mini Spin column kit (Bioflux, China) according to the manufacturer's instructions. RT-PCR was used for the detection of partial F-gene of vNDV using the following primers Forward: 5′-ATGGGCTCCAGACCTTCTACCA-3′ and Reverse: 5′-CTG CCACTGCTAGTTGTGATAATCC-3′ that flanks a 535 bp of the F gene of NDV as previously described (Radwan et al., 2013). A single step RRT-PCR assays using Quant one step RT-PCR Tiangen Kit (Tiangen Inc., China) was used according to the manufacturer's instructions. The final reaction volume was 25 μL; including 7 μL RNA template, 5 μL 5X RT-PCR enhancer, 2.5 μL 10X RT-PCR buffer, 1.5 μL of each forward and reverse primers (20 pmol), 1.25 μL hot master Taq-polymerase, 1 μL super pure dNTPS, 0.25 μL RNasin, 0.25 μL Quant RTase, and 4.75 μL RNase-free water. Thermal cycling RT-PCR conditions included a reverse transcription at 50 °C for 15 min, then an inactivation of reverse transcription enzyme and initial denaturation at 95 °C for 15 min, followed by 35 cycles at 95 °C for 15 s., 30 s at 54 °C, and 45 s at 72 °C. The addition final extension was performed at 65 °C for 10 min.

Amplified RT-PCR products The RT-PCR products were subjected to electrophoresis in 1–1.5% agarose gel using TBE (pH 8). DNA fragments were examined under UV light. For gene sequencing, the target bands of specific size were excised from the gel and purified with the QIAquick gel extraction kit (Qiagen, Valencia, CA) according to the manufacturer instructions and the DNA was quantified and sequenced directly using the ABI Prism 3100 automated sequencing machine (Applied Biosystems, Foster City, CA). A BLAST search was conducted for each sequence (http://www.ncbi.nlm.nih.gov/BLAST). Sequence comparisons and phylogenetic relationships through a bootstrap of 1000 trials were determined with the MEGA version 6 program using the Clustal W alignment algorithm (Tamura et al., 2013).

Table 2
Virus isolation and initial HI characterization.

Governorate/Year	No	Embryonated SPF egg passage			NDV HI confirmed samples
		P1	P 2	P3	
Fayoum					
2014	8	1	2	2	4
2016	3	3	–	–	3
Beni-Suef					
2014	9	9	6	–	9
2016	12	8	–	–	8
Minia 2014	8	2	2	3	5
Menofia 2014	14	–	1	1	2
Ismailia 2014	14	–	–	–	–
Total					31

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