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Isolation and Pathotyping of Newcastle Disease Viruses from Field Outbreaks among Chickens in the Southern Part of Egypt 2011-2012

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Abstract: Egypt is endemic for Newcastle disease virus (NDV) with continuous long-lasting outbreaks causing significant losses in the poultry industry since 1948. This study was designed to identify various NDVs associated with outbreaks occurred in different localities in Upper Egypt (Sohag, Qena and Luxor) from 2011 – 2012 among chicken flocks and estimate its virulence in chickens by the intra-cerebral pathogenicity index (ICPI). One hundred twenty six samples were collected from chickens either alive or dead showing characteristic clinical findings and post-mortem lesions of NDV. Virus propagation in embryonated chicken eggs was confirmed by hemagglutination (HA) test and identified by hemagglutination inhibition (HI) test using NDV specific antiserum. The results indicated that 45 (35.7 %) out of 126 samples were NDV positive. The ICPI revealed that isolates have a range from 1.4 - 2 index which congruent to mesogenic (2/45) and velogenic (43/45) type. These results confirmed that the circulating NDV strains are virulent for chickens and vaccination failure occur.

Key words: NDV outbreaks · Veleogenic · ICPI · Egypt · HI · Virus isolation

INTRODUCTION

Newcastle disease (ND) has been regarded as one of the most important devastating diseases of poultry because of its worldwide distribution and the severe economic losses in domestic poultry, especially in chickens [1]. ND is classified as a list A disease by the World Animal Health Organization (Office International des Epizooties, OIE) because it is highly contagious, with high morbidity and mortality in susceptible birds (up to 100%) [2, 3].The major clinical signs are respiratory distress, diarrhea, circulatory disturbances and impairment of the central nervous system [4]. Gross lesions are petechial hemorrhages and ulcers with raised borders on the mucosa of proventriculus, pneumonic lungs, hemorrhages in trachea, air sacs, brain, cecal tonsils and spleen [5].

ND is caused by avian paramyxovirus -1 (APMV-1), one of the antigenically distinct avian paramyxoviruses 1 – 11, genus *Avulavirus*, family *Paramyxoviridae* and order *mononegavirales* [6 – 8]. Newcastle disease virus (NDV) is an enveloped virus containing linear, non-segmented, negative sense, single-stranded RNA [9]. Although NDV has only one serotype, strains can differ considerably. All NDV isolates are categorized into five pathotypes based on severity of the disease in chickens;

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viscerotropic velogenic, neurotropic velogenic, mesogenic, lentogenic and asymptomatic enteric type [4]. There are several methods for pathotyping and characterization of NDV, such as intra-cerebral pathogenicity index (ICPI) in 1-day-old chicks, intravenous pathogenicity index (IVPI) in 6-week-old chickens and mean death time (MDT) in embryonated chicken eggs (ECEs) [10]. ICPI is the accepted, most sensitive *in vivo* test for determining pathogenicity of NDV according to OIE standards [3, 5].

The first confirmed outbreaks of ND occurred in 1926, in Java, Indonesia and in Newcastle-UponTyne, UK [11]. In Egypt, ND was identified for the first time in 1948 [12]. Since then, Egypt has been regarded as an endemic country by ND. In the succeeding years, an intensive vaccination program against NDV has been practiced in both large-scale poultry operations and small poultry farming. However, this virus showed the ugly face against poultry industry in Egypt causing severe outbreaks in poultry farms across Egypt resulting in heavy economic losses, in addition to increase the price of another source of animal protein. Little is known about the strains that cause these outbreaks especially in the southern part of Egypt. This study was carried out to isolate and characterize the circulating NDV strains among chicken flocks in Upper Egypt and determine its virulence nature.

MATERIALS AND METHODS

Samples Collection: Within two years of this study (January 2011 to December 2012), One hundred twenty six ND- suspected field samples (proventriculus, lung, trachea, kidneys, intestine (including contents), cecal tonsils, spleen, brain and liver tissues)were obtained from chickens flocks suspected to have ND in different areas of Upper Egypt, Qena, Sohag and Luxor Provinces (Table 1). Most sacrificed chickens had diarrhea, also showed nervous symptoms and respiratory difficulties. These samples were labeled and transported immediately on ice to the laboratory to be either processed immediately or stored at -80°C until processed and inoculated into embryonted chicken egg (ECE).

Virus Propagation in ECE: Isolation of virus was carried out using the method described by Terregino and Capua (5). Under a laminar flow cabinet class II (Biohazard safety cabinet class II, USA)by sterile forceps and scissors, small pieces of all tissues corresponding to 2 gm were collected and homogenized as a pool (while brain and intestinal samples were processed separately from other samples) in a mortar then collected in a 15ml Falcon tube. Nine ml phosphate buffer saline (PBS), pH 7.4, containing 10000 IU Penicillin/ml, 10 mg Streptomycin/ml and 250 µg Gentamycin sulphate/ml was added to the tissue homogenate. The suspension was left at 4°C overnight and clarified by centrifuging at 2000 rpm for 10 min at room temperature (RT). Since SPF eggs were not available locally and there were no facilities available for maintaining, a disease-free flock, eggs were purchased from apparently healthy unvaccinated local flocks (13).0.2 ml of the supernatant was inoculated in duplicate into 9-11 day-old ECE via allantoic cavity. The inoculated eggs were incubated at 37°C for 5–7 days with daily observing the embryo viability. Deaths occurred during the first 24 hr of incubation was considered non-specific death. All the embryos that died after 24hr or survived till the end of incubation period were chilled in refrigerator (4ºC) overnight. The allantoic fluid (AF) was harvested, divided into aliquots and stored in sterile screw-capped vials at -80°C till further use.

Serological Detection and Identification of NDV: The presence of NDV in AF was determined by slide HA test, micro-titer plate HA and HI tests following the standard procedure [14]. Briefly, the HA test was performed using chicken red blood cells (RBCs) in 96-well V-bottom micro-titer plates. Twofold dilutions of AF in PBS were mixed with an equal volume of a 1 % (v/v) RBCs in a V bottomed 96-well micro-titer plate. The plate was then incubated for 30 min at RT. The titers were expressed as reciprocals of the highest dilution of virus that demonstrated RBCs agglutination. The HA negative AF was passaged twice in ECE before recorded as NDV negative sample. For HI test, serial twofold anti-NDV serum dilutions were made in PBS; 4 HA units of tested AF was added to each dilution and incubated at RT for 30 min. after that an equal volume of 1% chicken RBCS in PBS was added. The HI endpoint was determined as the last dilution with inhibition of HA activity.

ICPI for the NDV Isolates: The ICPI was performed to evaluate the pathogenicity of NDV isolates. The test was applied on 460 one-day old chicks according to international OIE standards [3] obtained from unvaccinated flocks, also random serum samples from these chicks were confirmed by HI as NDV negative.

Fresh AF obtained after passaging the NDV isolates in ECE with a HA titer > 2^4 (>1/16) was diluted 1/10 in sterile PBS with no additives, such as antibiotics. Then 0.05 ml of the diluted virus was injected intra-cerebral (I/C) in the chick (10 chicks / sample), as well as one group (10 chicks) was injected with 0.05 ml PBS as control. The birds were observed for the clinical symptoms every 24h/ 8 days. At each observation, the birds were scored: normal (0), sick (1) and dead (2). The quotient derived from the sum of scores and the numbers of observations represent the ICPI. An ICPI above 1.5 characterizes as a velogenic strain, 0.5-1.5 as a mesogenic strain; while an ICPI below 0.5 indicates a lentogenic strain.

RESULTS

Epidemiological Features of ND Outbreaks in the Southern of Egypt: ND struck the poultry industry in Egypt causing severe economic losses. However, many governmental and private poultry farms were established intensively in the southern part of Egypt in the last two decades. These farms suffered from severe outbreaks, also for our knowledge no reports about the NDVs circulating in the southern part in compare to some studies about NDV in the northern one. So, this study was carried out to investigate the prevalence of the virus among chickens and characterize the isolates according to its virulence in chickens in this area.

The Samples were collected from commercial chicken flocks and village chickens exhibited ND signs either clinically or PM lesions during Jan. 2011 – Dec. 2012. All commercial chicken farms were of open system except some farms in Qena Province were of closed system. The farms records included flock size, birds' type, ages and vaccination shown in Table 1. The common vaccination schedule against ND used in the area of study was live Hitchner B1 given in drinking water (DW) at 5 -7 days of age, LaSota strain given in DW at 18-19 days of age and LaSota in DW at 28 day of age. Inactivated vaccine had not been used in these flocks. Village chickens had no vaccination history. Morbidity rate was high and elevated up to 95% in some flocks. The mortality rate ranged from 10% - 90%, with deaths occurring within 24 - 72hr after the onset of clinical signs. The peak of mortality occurred in Nov. 2012, but increased mortality was also in Mar., Jun. and Sept. of the same year.

Clinically, the most common clinical signs were greenish diarrhea surrounding the cloaca, incoordination (e.g. backward movement of the bird & falling to one side or the other), tremors, torticollis, opisthotonus and death (Fig. 1 (A - C)). Most commonly observed PM lesions were marked hemorrhages on the tips of the proventricular glands, button-like ulcers in the wall of intestine, cecal tonsils were enlarged, thickened, necrotic and hemorrhagic as shown in Fig. 1 (D - F).

Virus Isolation and Identification: Out of 126 ND-suspected field samples propagated in 9 -11 days old ECE via allantoic sac, 47 showed positive rapid HA within few seconds which indicated that the isolates were hemagglutinating viruses. All HA positive embryo died within 24 - 96 hr post-inoculation (PI). The embryos showed congestion and hemorrhage in the whole bodies, subcutaneous tissues of the heads of the embryos were filled with blood and the blood vessels over the bodies were prominent (Fig. 2).

The rapid HA positive samples were titrated using micro HA test; HA titers were ranged from 1:32 - 1:1024 (Table 2).45/47 (95.7%) of the HA positive AF, were inhibited by anti-NDV hyper-immune serum using HI test whereas, 2 were not inhibited which indicated these were other than NDV (Table 2). The HI titers of the positive viruses were from 1:32 - 1:4096 (Table 2).

Pathotyping of NDV Isolates in Chicken Using ICPI: To classify the isolates biologically and clarify whether it's avirulent (vaccine strains) or virulent strains. The pathogencity of NDV isolates obtained from the field samples to chicken was assessed by ICPI test. This assay was performed using 460 chicks' one day old divided into 46 isolated groups, 10 chicks/group. Each group inoculated I/C in the caudal part of the brain with 0.05ml of 1/10 diluted freshly prepared AF but the control group injected with PBS and kept under observation every 24hr/8days.

Clinically, chicks showed clinical signs were ranged from general nonspecific signs as birds appeared depressed, comatose, closed eyes, ruffled feathers, off food, somnolence and sternal or lateral recumbence to specific signs mainly; nervous in nature i.e., paresis and paralysis of legs (one or both) and wings (one or both), twisting of the head and neck, muscle tremors, incoordination in gait and opisthotonos (Fig. 3 A-C).PM lesions, there were petechial hemorrhages appeared on the tips of the glands of the proventriculus of some birds especially at the junction between the proventriculus and esophagus; congestion of the blood vessels of the brain (cerebellum and cerebrum) and congestion appeared on a yellow liver with enlargement of the gall bladder as in Fig. 3 (D-F). The ICPI was calculated according to OIE standard manual [3].

Fig. 1: Clinical and pathologic features of ND cases. A. Periorbital edema and nasal discharge. B. Nervous signs (twisted neck and paralysis). C. Whitish greenish colored diarrhea soiling the vent area. D.Pin point hemorrhages on the tips of proventricular glands. E. Enlargement and haemorrhages of cecal tonsils and with opening, showed extensive hemorrhages and necrosis in the mucosa occurs in infection. F. Hemorrhagic, necrotic and focal diphtheroid lesions (button like ulcers) affecting the mucosa of the intestines

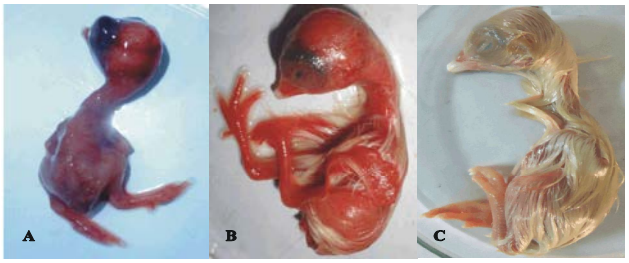

Fig. 2: Effect of NDV on inoculated ECE. A. Chicken embryo (2 days PI) is diffusely red and the subcutaneous tissue of the head is filled with blood and the blood vessels over the body were prominent. B. Chicken embryo (4 days PI), showing diffuse congestion and hemorrhage in the whole body and edema in the head region. C. Control non-inoculated chicken embryo

The results showed that 43/45 (95.56%) of the NDV isolates have an ICPI value >1.5 (Table 3), which congruent to velogenic NDV isolates, whereas 2/45 (4.44 %) have lower value 1.4 – 1.46 for mesogenic isolates (Table 3).

DISCUSSION

ND causes a serious economic loses in the poultry industry although the intensive vaccination regimes carried out in Egypt. In the last 50 years there has been a major genetic change in the strains of NDV that have been identified in poultry, although they still remain as a single serotype [15].

The data regarding the pathogenicity of NDVs circulating among chickens in Egypt is limited, especially in the southern part. So, the isolation and pathotyping of the NDVs from recent outbreaks among chickens is a critical for the control of ND and vaccination evaluation.

Although commercial chickens were vaccinated, almost all flocks were accompanied by high mortality (up to 90%), clinical and PM findings of ND (Table 1 and Fig. 1). In addition to this, the isolation of NDV from the clinical samples collected from different localities was confirmed by HA and HI tests, with a high titer of 1: 1024 and 1:4096, respectively (Fig. 2 and Table 2). The HI test detected 45 out of 47 HA positive samples, where the other 2 HA positive were confirmed as influenza viruses.

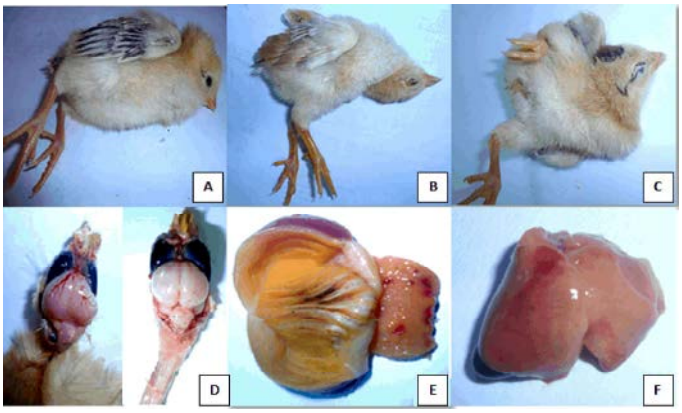

Fig. 3: Clinical and pathologic features of Newcastle disease virus (NDV) in chicks used for ICPI test. A. Paralysis of both legs with lateral recumbency. B. Twisting of the head and neck. C. Opisthotonos with whitish diarrhea. D. Congestion of the blood vessels of brains (left),control normal brain (right). E. Ulcers and hemorrhagic foci on the tips of proventricular glands especially at esophageal-proventricular junction. F. Enlarged and congested liver with distended gall bladder

Table 1: Epidemiological data of the ND outbreaks among chickens flock in Upper Egypt (Qena, Sohag, and Luxor Provinces) during 2011 - 2012

Location		Samples no. / farm	Type of chicken	Vaccine	Age (day)	Flock capacity	% Morbidity	% Mortality	Samples / province	Total
Sohag	Tema	11	Broiler	Done	30	2500	> 90	> 90	52	126
	Akhmem	3	Broiler	Done	26	2000	75-80	40-55		
	Tahta	20	Broiler	Done	36	4500	>95	> 90		
	Gerga	6	Broiler	Done	32	2800	70-80	40-55		
	El-Monshaa	5	Broiler	Done	39	1500	80	35/80		
	El-Balyana	7	Broiler	Done	24	3200	70-80	40-55		
Qena	Nag Hammadi	10	Broiler	Done	18	6000	80	40	66	
	Qena City	31	Broiler	Done	36	12000	>95	> 90		
		5	VCa	Unknown	60	100-120	30	10		
	Abu Tisht	3	VC	Unknown	230	30-50	50	10		
	Qus	6	VC	Unknown	70	100-150	35	10-20		
	El Wakf	7	Broiler	Done	53	1000	70-80	40-60		
	Qeft	4	VC	Unknown	90	20-30	60	20-30		
	Akhmem	3	Broiler	Done	26	2000	75-80	40-55		
	Tahta	20	Broiler	Done	36	4500	>95	> 90		
	Gerga	6	Broiler	Done	32	2800	70-80	40-55		
	El-Monshaa	5	Broiler	Done	39	1500	80	35/80		
	El-Balyana	7	Broiler	Done	24	3200	70-80	40-55		
Luxor	El Bayadeya	3	Broiler	Done	55	900	60	20-30	8	
	Esna	5	Broiler	Done	35	200	70-80	40-55		

[a]village chickens

Table 2: The distribution of HA and HI titers of NDV isolates obtained from the collected samples in the southern part of Egypt

	HA titer							HI titer							
Location	1/32	1/64	1/128	1/256	1/512	1/1024	Total	1/32	1/128	1/256	1/512	1/1024	1/2048	1/4096	Total
Sohag	1	3	11	6	6	3	30	1	9	12	4	2	-	-	28
Qena	-	3	8	2	1	1	15	-	-	1	-	4	8	2	15
Luxor	-	-	-	-	2	-	2	-	2	-	-	-	-	-	2
Total (%)	1(2.13)	6(12.8)	19(40.42)	8(17)	9(19.14)	4(8.51)	47(100)	1(2.22)	11(24.44)	13(28.9)	4(8.9)	6(13.3)	8(17.8)	2(4.44)	45(100)

Table 3: The ICPI values and pathotypes of NDV strains used in this study

	Location					
ICPI values	Sohag	Qena	Luxor	NDV pathotype	Virulence to chicken	Percent (%)
<0.5	-	-	-	-	-	0
0.5-1.5	-	2	-	Mesogenic	Moderate	4.44
>1.5	28	13	2	Velogenic	High	95.56

The ICPI of the identified isolates revealed its virulence to chickens producing characteristic clinical and PM features of NDV in infected chicks (Fig. 3) with a high incidence of the velogenic NDV isolates (95.56%) in all provinces (Table 3). The NDV infection even in a well-vaccinated flock can occur because some of the birds will have had a poor vaccine response and will be susceptible to infection. This attributed to ND vaccines do not protect vaccinates from infection and viral shedding. Also, parental immunity contributed to vaccination inefficiency in young chicks [16].

In conclusion, the previous results indicated that, 1) NDV isolates circulating among chickens are virulent and associated with outbreaks in commercial poultry farms and backyard reared chickens in southern Egypt. 2) Although these farms follow strict vaccination regimes, the NDV causes severe economic losses in almost of these forms up to 90%. 3) Vaccination failure due to inefficient vaccination or virus genetic drift should be considered to draft the efficiency of the commercial available vaccines against these isolates or to prepare a new one. 4) Continuous monitoring of the flocks' immunological status should be carried out to evaluate the antibody response to administrated vaccines.

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