Isolation, identification and pathotyping of Newcastle disease virus from chickens in Egypt

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A B S T R A C T

Egypt is endemic for Newcastle disease virus (NDV) with continuous long-lasting outbreaks causing significant losses in the poultry industry. This study was designed to isolate and identify NDV from different localities in Egypt (Giza, Gharbiya, Kalyobiya, Sharkia, Menofia, Fayoum and Minia) among chicken flocks and estimate its virulence using Mean death time (MDT) and intra-cerebral pathogenicity index (ICPI). Forty samples were collected from chickens either alive or dead showing clinical findings and post-mortem lesions characteristic for 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 23 (57.5 %) out of 40 samples were NDV positive. The isolate from Giza was velogenic with MDT of 48 hours and ICPI of 1.625. While the isolate from Qualubiya was lentogenic with MDT of 96 hours and ICPI of 0.4375.These findings provide data on biological pathotyping of NDV in chickens in Egypt and emphasize importance of NDV surveillance for improving of strategies for the control of the disease.

KEYWORDS: NDV, MDT, ICPI, HI, VIRUS ISOLATION.

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1. INTRODUCTION

Newcastle Disease Virus (NDV) belonged to the genus Avulavirus, subfamily Paramyxovirinae, family Paramyxoviridae, causes a notifiable disease affecting many species of birds and causing severe economic losses in the poultry sector (Alexander, 1997 and OIE, 2012). NDV has a single stranded negative-sense RNA genome of approximately 15.2 kb that encodes for six structural proteins. Three of them, the hemagglutinin-neuraminidase (HN), the fusion (F), and the matrix (M) proteins, are related to the viral envelope. The remaining three proteins, nucleoprotein (NP), the phosphoprotein (P), and the RNA polymerase (L), are related to the genomic RNA (Chambers et al., 1986). Although the molecular basis of NDV virulence relies on multiple genes, the amino acid sequence motif at the cleavage site of the precursor F-glycoprotein is the critical site for major changes in virulence (Glickman et al., 1988; Peeters et al., 1999; Romer-Oberdorfer et al., 2003). Although chickens are the most susceptible host present the clinical Newcastle disease (ND), a wide host range (27 of the 50 orders) of birds are susceptible to infection (Jorgensen et al., 1998; Kuiken et al., 1998; Alexander et al., 2000 and Aldous et al 2007). The virus is transmitted by ingestion and inhalation and produces a disease of variable clinical severity and transmissibility depending on its pathogenesis. Based on the severity of the disease, NDV can be grouped into three pathotypes, lentogenic strains cause mild or unapparent respiratory disease, mesogenic strains produce respiratory and nervous signs with moderate mortality and the velogenic strains cause severe intestinal lesions or neurological disease, resulting in

high mortality (Alexander, 1989, 1997). ND remains a potential threat to poultry production, although of the effective virus control using vaccination and mass slaughtering (Westbury, 2001). In Egypt, NDV outbreaks still frequently occur in vaccinated poultry flocks, despite intensive vaccination programs are being implemented against this infection and the sources of the virulent NDV in these outbreaks are not known (Mohamed et al., 2009 and 2011, and Nabila et al., 2014). Isolation of NDV in embryonated chicken eggs and its identification by haemagglutination (HA) and haemagglutination inhibition (HI) tests with specific NDV antiserum (Alexander, 2009) is considered to be the gold standard. In recent years reverse transcription-polymerase chain reaction (RT-PCR) has been applied to identify NDV (Jestin and Jestin, 1991; Zhang et al., 2010).

The main objective of the present work is isolation, identification and pathotyping of NDV from recent field suspected outbreaks among chickens.

2. MATERIAL AND METHODS

2.1. Virological Samples:

Forty samples (proventriculus, lung, kidneys, intestine, cecal tonsils, spleen and liver tissues) were obtained from ND suspected chicken flocks from Giza, Gharbya, Kalyobiya, Sharkia, Menofia, Fayoum and Minia, Egypt at November 2012 to April 2014 (Table 1). Most scarified chickens have diarrhea, nervous symptoms and respiratory difficulties. These samples were labeled and transported immediately on the ice to the laboratory and stored at –80°C until processing and isolation.

Table (1): Suspected samples collected from chickens flocks in different governorates in Egypt.

Governorate	Sample Code	Number of samples	Type of chicken	Age	Flock Capacity
Gharbiya	A1	5	15000	1 year	Layers
	A2	2	13000	26 days	Broiler
Giza	B1	2	4500	48 days	Broiler
	B 2	3	10000	45 days	Broiler
	B 3	2	14000	36 days	Broiler
	B 4	3	8000	50 days	Broiler
	B 5	4	10000	40 days	Broiler
Fayoum	C	4	8000	230 day	Layers
Sharkiya	D	1	10000	31 days	Broiler
Minia	E	3	5000	42 days	Broiler
Kalyobiya	F1	3	10000	30 day	Broiler
	F2	4	10000	28 day	Broiler
Menofiya	G	4	10000	28 day	Broiler

All the flocks were subjected for vaccination programs against NDV and other viral agents. Clinical signs include eye closure, respiration difficulties, green diarrhea, ruffled feathers; drop of production and high mortality.

2.2. Experimental hosts:

2.2.1. Specific Pathogen Free-Embryonated Chicken Eggs (SPF-ECE):

Specific pathogen free (SPF) embryonated chicken eggs (ECE) one day old were obtained from the SPF production farm, Koum Oshiem, Fayoum, Egypt. It was kept in the egg incubator at 37ºc with humidity 70% till the age of 10 day old and

was used for isolation, infectivity titration and pathotyping of NDV by calculation of the mean death time (MDT) of egg embryos.

2.2.2. Experimental one day old SPF chicks:

A total number of 30 one day old SPF chicks were purchased from SPF farm Koum Oushim El-Fayoum, reared in separated cages and kept in a strictly isolated mosquito proof room. The room was previously cleaned, thoroughly disinfected and were provided with water and feed, and used for pathotyping of NDV isolates by calculation of the intracerebral pathogenicity index (ICPI).

2.3. Newcastle Disease Virus (NDV) reference antiserum:

Reference Antiserum against NDV (Anti-NDV) was supplied by CLEVB. It is raised in chickens and has a titer of 12 $\log_2$ using HI test and used for identification of viral isolates using HI test.

2.4. Isolation of NDV on SPF - ECE:

Virus isolation was performed according to the protocol adopted by (OIE, 2012). Suspensions of organs were first centrifuged in a bench-top centrifuge at 4000 rpm for 5 min. Antibiotics (penicillin 2000 units/ml, streptomycin 0.01 µl/ml, gentamycin 50 µg/ml and mycostatin 1000 units/ml) were added to the supernatants and incubated for 1 at 4 °C. A volume of 0.2 ml of the supernatant was then inoculated into the allantoic cavity of five 10 days-old SPF-ECE. Deaths on the first 24 hours post inoculation (PI) were considered nonspecific while recorded deaths after that (from the 2nd to 4thday post inoculation) were took in consideration as specific results. Allantoic fluid (AF) was collected with a sterile syringe and centrifuged at 3000 rpm for 5 minutes to remove mixed blood and tissues, divided into aliquots and stored in sterile screw-capped vials at – 80 °C till further use. Presence of virus was confirmed by spot-HA test. Four serial virus passages were carried out in SPF –ECE.

2.5.Haemagglutination (HA) and haemagglutination inhibition (HI) tests:

HA and HI tests were carried out according to (OIE-Manual, 2012). Harvested AF from inoculated SPF-ECE was subjected for micro-plate HA test to determine the presence of haemagglutinating virus using 1% freshly prepared chicken RBCs suspension. HI test using reference NDV antiserum was employed with the HA positive samples for identification of NDV.

2.6. Reverse Transcription-Polymerase Chain Reaction (RT-PCR):

2.6.1. Extraction of viral RNA

The genomic viral RNA was extracted from AF by using the QIAamp viral RNA mini kit (QIAGEN, Hilden, Germany) according to the manufacture's protocol and was stored at –86°C until use.

2.6.2. Oligonucleotide primers:

They were synthetized by Appligene. The choice of primers was made according to Jestin and Jestin (1991), with the assistance of a microcomputer: PC/gene program (Intelligenetics Inc and Genofit SA), along the F gene sequences. Primer 1 was a 19-mer oiigonucleotide, 5' CTTTGCTCACCCCCCTTGG 3' localized at position 315 to 333 of the cDNA; primer 2 was a 18-mer oligonucleotide, 5' CTTCCCAACTGCCACTGC 3' localized at position 572 to 589 of the cDNA. They were used for the amplification of 275 bp amplicons corresponding the cleavage activation site of F gene of NDV. The Amplitaq® kit (Perkin-Elmer Cetus) containing PCR buffer 5x (contain 12.5 Mm MgCl2), dNTPs Mix (10mM each) and nuclease free water was used for DNA amplification; the dNTPs of this kit were also employed for r cDNA preparation. The

test was calibrated in such a way that dNTP were 1.25 mM each, PCR primers were 100 ng each, Taq polymerase was adjusted to 1 U in a total volume of 20 ml. cDNA was amplified with a programmable thermal cycler (Techne PHC-1). The PCR program was defined as denaturation at 94°C for 1.5 min, annealing at 51 °C for 2.5min, elongation at75 °C for 1 min for 35 cycles. After the 35th cycle, the time of extension at 75 °C was 2 min.

2.6.3. Agarose gel electrophoresis

The PCR products were separated in 1.5% agarose gel in TAE buffer stained with ethidium bromide and compared with molecular mass marker (50 bp DNA markers) and visualized by ultraviolet (UV) transillumination.

2.7. Pathogenicity test:

Pathotyping of the NDV isolates were carried out using calculation of Mean Death Time (MDT) and Intracerebral Pathogenicity Index (ICPI) according to (OIE-Manual, 2012).

3. RESULTS

3.1. Virus isolation from suspected samples on SPF-ECE:

Trials for isolation gave positive results after the third passage in 7 out of 7 samples from Gharbia, 2 out of 14 samples from Giza, 7 out of 7 samples from Kalyobiya, 4 out of 4 samples from Fayoum, 1 out of 1 sample from Sharkia, 3 out of 3 samples from Minia, and in 4 out of 4 samples from Menofia (table 2).

These results were confirmed by application of HA test on the collected allantoic fluid of inoculated ECE with HA titers of 6 $\log_2$ HA units/ml for isolates from Gharbia, 10 $\log_2$ HA units/ml for isolates from Giza, 9 $\log_2$ HA units/ml for isolates from Kalyobiya, 6 $\log_2$ HA units/ml for isolates from Fayoum, 5 $\log_2$ HA units/ml for isolates from Sharkia, 8 $\log_2$ HA units/ml for isolates from Minia and 7 $\log_2$ HA units/ml for isolates from Menofia (table 2).

3.2. Serological identification of suspected NDV isolates using HI test:

Application of HI test for identification of suspected NDV isolates using the specific NDV antiserum, gave positive results with 23 out of 40 samples (7 out of 7 samples from Gharbia, 2 out of 14 samples from Giza, 7 out of 7 samples from Kalyobiya, 3 out of 3 samples from Minia, and in 4 out of 4 samples from Menofia) as shown in table (2).

Isolates from Giza and Kalyobiya showed the highest serum titers while suspected isolates from samples collected from Fayoum and Sharkia showed negative results for NDV identification using HI test.

3.3. *Molecular identification of the NDV isolates using RT-PCR:*

Identification of the NDV viral genome of the local isolates from Giza and Qualubiya using RT-PCR for amplification of the Fusion protein encoding gene using Taq polymerase enzyme with the upstream and downstream specific primers. Electrophoresis of the amplified products revealed the presence of specific PCR product at the correct expected size of the fusion protein encoding gene (275 bp), with both isolates of NDV as shown in figure No. (1).

3.4. Pathotyping of NDV isolates by calculation of the mean death time (MDT) and intracerebral pathogenicity index (ICPI):

Both isolates from Giza and Qualubiya governorates were subjected to pathotyping to classify them biologically if they virulent were strains or avirulent (vaccine) strains using MDT and ICPI tests. The results showed that isolates from Giza was velogenic with MDT of 48 hours and ICPI of 1.625, while the isolates from Qualubiya was lentogenic with MDT of 96 hours and ICPI of 0.4375, as shown in tables (3).

Table (2): Isolation and identification of suspected NDV isolated on SPF-ECE:

Governorate	Number of samples	* Number of positive samples on isolation	HA titer (log_2 HA units/ml)	Samples positive for HI test	
				Number	log_2 serum titer
Gharbia	7	7	6	7	4
Giza	14	2	10	2	6
Kalyobiya	7	7	9	7	6
Fayoum	4	4	6	0	0
Sharkia	1	1	5	0	0
Minia	3	3	8	3	2
Menofia	4	4	7	4	4
Total	40	28	-	23	-

* Positive result represented by death of the embryo at /more than 24 hours with +ve HA on allantioc fluid.

Table (3): Pathotyping of NDV isolates:

NDV isolate	MDT	ICPI	Pathotype
Giza governorate	48	1.625	Velogenic
Kalubiya governorate	96	0.4375	Lentogenic

MDT values: <50 hours denotes for velogenic strains, 50–90 hrs denotes for mesogenic strains and >90 hrs denotes for lentogenic strains, while ICPI values <0.5 denotes for lentogenic strains, 0.5–1.5 denotes for mesogenic strains and >1.5 denotes for velogenic strains, according to OIE (2009).

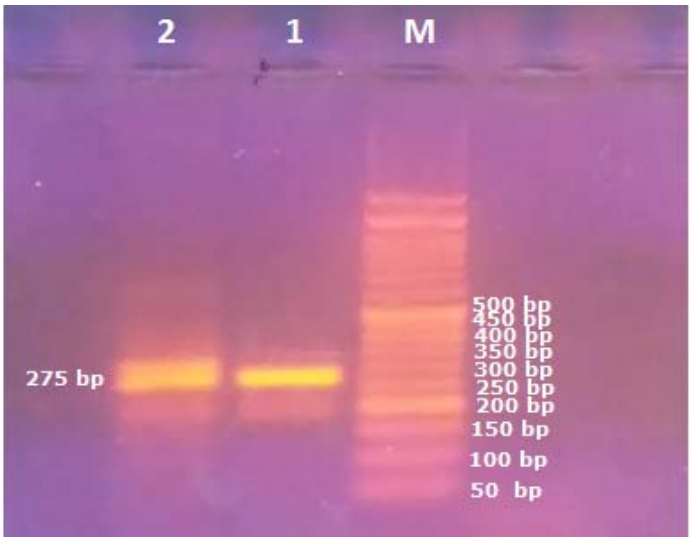

Figure No. (1) Electrophoresis of the amplified PCR products of NDV fusion protein gene for local isolates detected at the size of 275 bp.
Lane M: 50 bp nucleic acid marker.
Lane 1: NDV isolate from Giza governorate.
Lane 2: NDV isolate from Kalubiya governorate.

4. DISCUSSION:

In Egypt, outbreaks of NDV are still frequently occurring in vaccinated poultry flocks, despite the intensive vaccination programs (Mohamed et al., 2011 and Nabila et al., 2014). The main objective of the present work is the isolation, identification, pathotyping and genotyping of NDV isolates from infected chicken flocks from different governorates (Giza, Qualubiya, Sharkiya, Fayoum, Minia and Menofiya) in Egypt between the years 2013 and 2014. Concerning isolation of NDV from field samples from infected chicken farms in different localities from Giza, Kalyobiya, Sharkia, Fayoum, Minia and Menofia governorates, suspected NDV from Suspensions of organs (proventriculus, spleen and liver tissues). The present study reported trials for virus isolation on SPF-ECE then the detection of HA activity and then identified by HI test. This was in agreement with that described by OIE (2012) and Abdelrahim and Elhag (2014). Regarding isolation of ND virus on Specific Pathogen Free-Embryonated Chicken Eggs (SPF-ECE), isolation of the virus was performed through the inoculation into allantoic cavity of SPF-ECE for three passages. The Samples inoculated in SPF-ECE induced signs in 7 out of 7 samples from Gharbia governorate, 2 out of 14 samples from Giza governorate, 4 out of 4 samples from Fayoum governorate, 1 out of 1 sample from Sharkia governorate, 3 out of 3 samples from Minia governorate, 7 out of 7 samples from Kalyobiya governorate and in 4 out of 4 samples from Menofia governorate. Similar results were reported by Hussein et al. (2013); Ahmadi et al. (2014) and Salehinezhad et al. (2014). Field samples from suspected chick flocks positive for isolation on SPF-ECE were subjected for titration after each passage starting from the second passage. HA titers of the isolates ranged from 5 $\log_2$ HA units/ml to 10 $\log_2$ HA units/ml after the 3rd passage. This result agreed with that obtained by Bilal et al. (2014), Mantip et al. (2011) and Pansota et al. (2013). Serological identification of the suspected viral isolates showed that 23 out of 40 samples gave positive results by HI using the specific NDV anti serum. NDV isolates from Giza and Kalyobiya governorates showed the highest serum titers during NDV identification using HI test. These results were similar to that of Ghaniei and Mohammad et al. (2012) and Uddin et al. (2014). Molecular identification of NDV using RT-PCR for amplification of the Fusion protein encoding gene using Taq polymerase enzyme with the upstream and downstream specific primers, revealed the presence of the amplified products of both reference strain and local isolates at the correct expected size (275 bp) on electrophoresis. Results of RT-PCR as a sensitive test for NDV detection confirmed the results of HI and agreed with those of Fazel et al. (2012) and Munir et al. (2012). Concerning the pathotyping of the NDV isolates. The present study revealed that Pathotyping of NDV isolates by calculation of the mean death time (MDT) and intracerebral pathogenicity index (ICPI). This was in agreement with that described by OIE (2012) and Munir et al. (2012). Regarding MDT, the result showed that isolate from Giza was velogenic with MDT of 48 hours and isolate from Qualubiya governorates was Lentogenic with MDT of 96 hours. This result agreed with that obtained by Mantip et al. (2011), Munir et al. (2012), OIE (2012), Abdelrahim and Elhag (2014) and Mehrabanpour et al. (2014). Regarding ICPI, the result showed that isolate from Giza was velogenic with ICPI of 1.625 and isolate from Qualubiya governorates was Lentogenic with ICPI of 0.4375. This result agreed with that obtained by Nabila et al. (2014) and Kianizadeh et al. (1999). While Abdelrahim and Elhag (2014) reported that the intracerebral pathogenicity index found to be 0.9 and stated that its value for all the isolates ranged from 1.7 to 1.96.

Finally, it is concluded that rapid detection, identification and Pathotyping of NDV is crucial for adaptation of an effective control

of the disease. Further studies on the genetic characters and antigenic and characters and the efficacy of commonly used NDV vaccines for protection against the NDV isolate are required.

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