

Characterization of Variant Strain of Newcastle Disease Virus in Egypt

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During 2005, velogenic Newcastle disease virus (NDV) caused a major outbreak among commercial broiler chicken in Egypt. The outbreak raised concerns regarding the protective immunity of commercially available vaccines for prevention and control of this virus in poultry. The virus was isolated from broiler farm suffered from more than 95% mortalities. The isolate was confirmed not to be avian influenza virus (AIV) by rapid chromatographic strip test, and characterized as NDV using reverse transcription-polymerase chain reaction (RT-PCR) which amplified a portion of the fusion gene of NDV and haemagglutination inhibition (HI) test. This isolate confirmed to be velogenic viscerotropic NDV by mean death time (MDT) test and pathogenicity to 7-week old chickens. We tried to determine whether the existing commercial live NDV La Sota vaccine could provide protection against the isolated virus or not. Birds received a single dose of live La Sota type vaccine at 3 weeks of age and were challenged 2 weeks post-vaccination with a lethal dose of NDV. Results indicated that the live vaccine did not protect against morbidity but reduced mortality in comparison to controls. All unvaccinated control chickens challenged with NDV died within 5 days post-challenge (pc). Protection from disease did not correlate with the presence of antibody titers (determined by HI) at day of challenge. These results underscore the need to develop new NDV vaccines and vaccine strategies for use during outbreak situations to protect birds from both disease and infection and to reduce virus shedding.

Newcastle disease virus is responsible for one of the most devastating viral diseases of poultry and has substantial economic impact in the poultry industry. NDV is the only member of the genus *Avulavirus*, family *Paramyxoviridae*, subfamily *Paramyxovirinae*, in the order *Mononegavirales* and is designated avian paramyxovirus-1 (APMV-1) (Mayo, 2002). The NDV genome consists of six transcriptional units that encode the nucleocapsid protein (NP), phosphoprotein (P), matrix protein (M), fusion (F) protein, hemagglutinin protein (HN), and the polymerase protein (L). Two additional proteins, V and W, are expressed by mRNAs, which are derived from the P gene via RNA editing (Samson *et al.*, 1991; Steward *et al.*, 1993,1995). Isolates of NDV have been historically categorized into three pathotypes (Alexander, 2003). Lentogenic NDV isolates do not usually cause disease in adult birds, and utilized extensively as live-virus vaccines, mesogenic

NDV isolates that cause respiratory disease, but are not usually fatal (Hanson and Brandly, 1955), and velogenic viscerotropic or neurotropic NDV isolates with high virulence forms (Alexander, 2003). Although NDV has only one serotype, there are 10 genotypes of NDV isolates based on differences on fusion protein gene (Lomniczi *et al.*, 1998; Herczeg *et al.*, 1999; Tsaia, *et al.*, 2004). The primary molecular determinants for NDV pathogenicity are the fusion protein cleavage site amino acid sequence (Nagai *et al.*, 1976; Glickman *et al.*, 1988) and the ability of specific cellular proteases to cleave the fusion proteins of different pathotypes (Gotoh *et al.*, 1992 ; Ogasawara *et al.*, 1992). The presence of dibasic amino acids in the fusion protein sequence allows for systemic spread of velogenic NDV, whereas replication of lentogenic NDV is limited to mucosal surfaces of the host (Ogasawara *et al.*, 1992).

The main objective of the present study was to identify the causative agent of the outbreak and investigate whether the NDV isolated strain possesses such antigenic changes that enable the

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strain overcoming vaccination barrier afforded by the commonly used La Sota vaccine.

Material and Methods

Embryonated chicken eggs (ECE). Specific-pathogen-free (SPF) ECE obtained from Nile SPF (Koom Oshiem, Fayoum, Egypt) were used for isolation of the field isolate, titration of the seed stocks of NDV local isolate and vaccine strain, as well as pathogenicity test in ECE.

Chickens. Commercial 1-day-old Hyline chickens (El-Waddi Co, Egypt) were reared under strict hygienic conditions in separate rooms and used in both pathogenicity and protection studies.

Virus isolation in SPF ECE. NDV local isolate was isolated from 28-day-old broiler chickens suffering from both respiratory and renal distresses from Fayoum Governorate in 2005. Tracheal and proventricular scraping suspensions were pooled, centrifuged at 500 xg for 10 min. Antibiotic mixture (1000 µg penicillin and 1000 mg streptomycin/ml) was added to the supernatant clear fluid. The supernatant fluid was inoculated into chorioallantoic sac of 10-day-old SPF ECE. Allantoic fluid was harvested after 72 h and re-passed for another egg set.

Virus titration. NDV local isolate and La Sota vaccine (Intervet International, Boxmeer, The Netherlands) were titrated as described by (Villegas and Purchase 1989). Virus titers were expressed as 50% embryo infectious doses (EID₅₀) according to (Reed and Meunch, 1938).

Avian influenza virus antigen detection. The allantoic fluid of the second passage was screened for AIV using rapid chromatographic strip test (Animal Genetic Inc. Korea) according to manufacturer instructions.

Haemagglutination inhibition test. NDV specific antibodies were titrated by HI assay according to (Beard, 1989). The test was conducted in round bottomed microtiter plates (Dynatech Lab., Alexandria, Virginia). Serial two fold dilutions of serum were prepared in phosphate buffered saline (PBS). Total volume of diluted serum was 25µl per well, then an equal volume of predetermined 4HA units of NDV was dispensed to each well. Incubation was conducted at 37°C for 1h in a humidified chamber. Fifty microliters of 1% RBC was dispensed to each well. The plates were left at room temperature until positive serum well exhibit tight well circumscribed button of unagglutinated sedimented cells. Result of each

sample was numerically reported as Log₂ of the highest dilution of serum at which there was complete inhibition of agglutination.

Reverse transcriptase polymerase chain reaction (RT-PCR). RNA from the local NDV isolate was extracted from infectious allantoic fluid using QIAamp viral RNA mini kit (Qiagen, Hilden, Germany) according to manufacturer instructions. The RNA elute was subjected to reverse transcription (RT) with Reverse-iT™ One-Step RT-PCR Kit (ABgene, Epsom, Surrey, United Kingdom) using NDV F-gene specific primers originally described by (Collins *et al.*, 1993) (+)5'-TACACCTCATCC-CAGACAGG-3' and (-) 5'-AGTCGGAGGATGTTGGCAGC-3'. Briefly, total RNA elute was incubated at 47°C for 30 minutes followed by an additional incubation at 94°C for 4 minutes. The reaction was subsequently run for 30 cycles at 94°C for 30 seconds, 55°C for 15 seconds, and 72°C for 20 seconds, followed by an additional elongation at 72°C for 7 minutes. Reaction product was visualized by ethidium bromide staining under UV transillumination after electrophoresis on 1% agarose gel.

Pathotyping of the NDV isolate.

Mean death time (MDT) (Alexander, 1989). Fresh infective AF was diluted in sterile saline solution in ten fold dilution from 10⁶ to 10¹⁰. For each dilution 0.1 ml of AF was inoculated into the allantoic cavity of five 10 days old embryonated chickens eggs and incubated at 37°C. Remaining virus dilutions were retained at 4°C and inoculated into another five embryonated eggs with 0.1 ml of each dilution 8 h later. Embryos were incubated at 37°C and examined twice daily for 7 days for their viability. Minimum lethal dose is the highest dilution of virus where all the embryos were dead. MDT is the mean time in hours for the minimum lethal dose to kill all the embryos.

Intravenous pathogenicity index to 7-week old chickens (Alexander, 1989). Fifteen 7-week old chickens were infected with NDV local isolate 0.1 ml of 1:10 dilution of allantoic fluid by intravenous route. Birds were examined daily for 10 days and each bird is scored 0 if normal, 1 if sick, 2 if paralyzed and 3 if dead. IVPI value is the mean score per bird per observation.

Vaccination trial. Two groups (A, B) of one-old chickens were used to evaluate the protection provided by La Sota vaccine

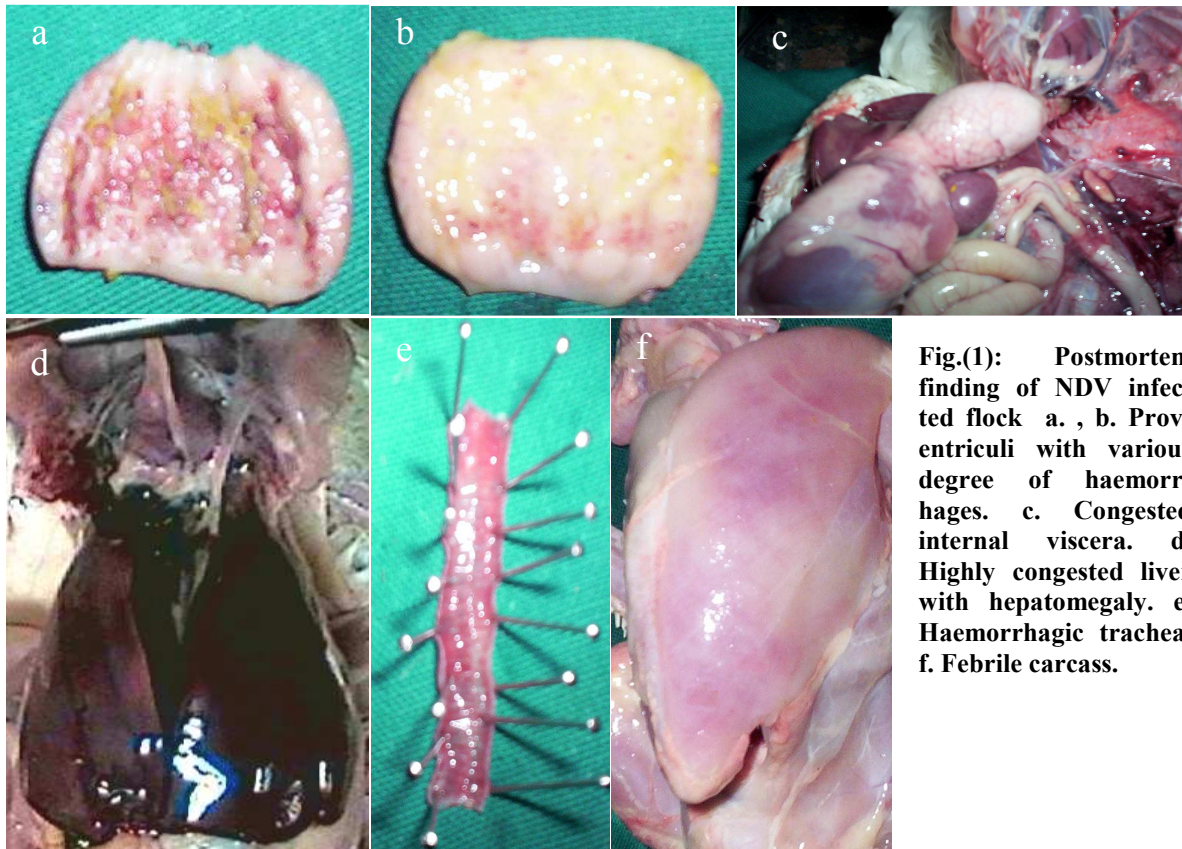


Fig.(1): Postmortem finding of NDV infected flock a. , b. Proventriculi with various degree of haemorrhages. c. Congested internal viscera. d. Highly congested liver with hepatomegaly. e. Haemorrhagic trachea. f. Febrile carcass.

against challenge with NDV local isolate. HI antibody titers were measured at one day old chickens to determine the due time of vaccination (3-week of age). Birds in group A were vaccinated by eye drop La Sota vaccine ($10^{6.0}$ EID₅₀) according to manufacture instructions while that of group B were kept as control unvaccinated. Two weeks post vaccination; chickens in group A and B were challenged by eye drop with $10^{5.9}$ EID₅₀ per bird of NDV local isolate (Kapczynski and King 2005). Clinical signs and mortalities were recorded.

Results

Clinical history. Newcastle disease was diagnosed during July 2005 in Fayoum Governorate, Egypt. The outbreak occurred in commercial broiler farm with flock density of 8000. The first signs were severe depression, green diarrhea, paresis and death two or three days after the onset of the disease. Other signs included severe conjunctivitis, facial swelling, birds standing dull with drooping wings. Within a period of 17-20 days after the appearance of the disease, the mortality increased progressively and by the end of such period 250 birds were the only survivors. Postmortem examination of dead

birds showed generalized congestion in all organs with highly injected liver and splenomegaly (Fig.1c,d), haemorrhagic tracheitis (Fig.1e) and moderate (Fig.1b) to severe haemorrhages (Fig.1a) on the proventricular gland tips were also observed. The carcasses were febrile with congested muscles (Fig.1f). Emergency vaccination using NDV La Sota failed to stop or even reduce deaths.

Virus identification. The virus was haemagglutinating virus. AIV detection was confirmed to be negative by performing AIV antigen detection in replicate manner. The isolate was serologically identified as NDV by HI test using NDV polyclonal chicken serum.

Virus characterization. RT-PCR was performed on the allantoic fluid of the field isolate. Agarose gel electrophoresis of the RT-PCR product revealed a single band within the expected size; 305 bp (Fig.2) thus confirming that the virus is Newcastle disease virus.

Pathogenicity indices. The mean death time into chicken embryos was 60h. while inoculation of the virus in 7-week old chicken resulted in death of all inoculated birds indicated that this virus is extremely virulent for chickens with 2-3 IVPI.

Table.1 Results of protection afforded by La Sota strain against challenge with NDV field isolate.

Group ^A	Number	Vaccination ^B	Challenge ^C	Prechallenge HI titers		Number of birds	
				Log ₂ ^D	Range ^E	Sick	Dead
A	20	+	+	9	8-11	16/20	4/20
B	20	+	-	9	8-11	0/20	0/20
C	5	-	+	0	0	5/5	5/5

^{A,B} Birds in groups A and B were vaccinated at 3 weeks of age by La Sota vaccine (eye drop route) according to manufacture instructions while that of group B were kept as control unvaccinated.

^C Chickens in group A and B were challenged by eye drop with 10^{5.9} EID₅₀ per bird of NDV local isolate, two weeks post vaccination.

^{D,E} Mean HI antibody titers calculated as Log₂ titers. Range of HI titers within the group.

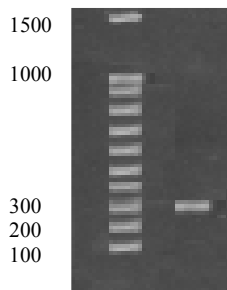


Fig.(2): Agarose gel electrophoresis a. Lane contained DNA molecular weight markers and b. PCR product of NDV isolate.

Protection test. The results obtained from the experiment to assess the ability of La Sota live vaccine to protect chickens against challenge with the NDV field isolate are shown in Table 1. Although high HI immune response was obtained prior to challenge, unsatisfactory protection level was obtained. Results showed that the vaccine strain La Sota reduced overall clinical protection to 20% when challenged with Egyptian isolate, although it protected 16 out of the 20 chicks challenged with isolate against mortality but did not protect against clinical disease in 80% of vaccinated birds. None of the control chicks were protected after challenge. Clinical signs include diarrhoea, nasal discharge and birds standing dull with drooped wings. Postmortem examination of dead birds revealed generalized congestion in all parenchymatous organs and haemorrhages on the proventricular gland tips.

Discussion

In this study, isolation of an apparently new variant of Newcastle disease virus from broiler flock which experienced drastic mortality pattern

in spite of being vaccinated by commonly used NDV vaccine. Preliminary haemagglutination inhibition and further RT-PCR tests as well as negative AIV antigen detection test revealed that the isolated strain is Newcastle disease virus. It has been demonstrated that the pathogenicity of Newcastle disease virus for chickens is dependent on the ability of the host non-trypsin-like proteases to cleave the precursor fusion protein F0 to F1 and F2; in some cases a similar mechanism is also required for haemagglutinin-neuraminidase protein (Rott and Klenk, 1988). Pathogenicity test to 7-week old chickens reproduce similar disease with 100% mortality rate. Diarrhoea, paresis and severe respiratory signs were detected in most birds.

It was known that ND viruses exist as a single serotype based on the neutralizing test and cross-protective analysis (Alexander, 1997) but 10 genotypes of NDV isolates based on differences on fusion protein gene are existed (Lomniczi *et al.*, 1998; Herczeg *et al.*, 1999; Tsaia *et al.*, 2004). In view of recent outbreaks of ND in vaccinated chicken flocks of Egypt, we conducted a laboratory investigation to determine whether there was evidence of cross-protection between vaccine strain La Sota and field strain. It is well known that NDV antibody response is essential for clinical protection against challenge with virulent NDV (Reynolds and Maraqa, 2000) but this was not the case in our study. Although very high prechallenge HI antibody titers were obtained, unsatisfactory protection was afforded. Results revealed that La Sota-vaccinated chickens were only partially protected against challenge with recent isolate.

Indeed it was an antigenic variant that escapes the protective immune response of the La Sota vaccine strain. In the last decades it has been reported many NDV outbreaks in vaccinated flocks (Yang *et al.*, 1997,1999). Although antigenic differences detected between virulent viruses and vaccine strains are insufficient to result in failure of vaccination (Alexander *et al.*, 1997), it does not seem reasonable to suspect that all cases are due to technical errors in vaccination performance or bad quality of the used vaccine. It is apt assumed that the disease caused by a pathogenic NDV strain having certain antigenic peculiarities helping to overcome vaccination barrier (Panshin *et al.*, 2002).

Evolution of NDV, may be related to the accumulation of point mutations that induce amino acid substitution in the neutralizing epitopes, the cysteine residues, and N-linked glycosylation sites of the F protein (Chambers, *et al.*, 1986; Toyoda *et al.*, 1989; Yusoff *et al.*, 1989). Immune pressure of the host may contribute to the difference in evolutionary rates as selective immune pressure of the host enhanced the evolutionary process of NDV (Yu *et al.*, 2001).

In Egypt a strict vaccination program against ND has been performed for decades. These results suggested that selective immune pressure of the host enhanced the evolutionary process of NDV. Although, genetic analysis proves valuable tool in tracking the spread and origin of NDV in countries. However, neither the highly virulent and moderately virulent NDV strains nor the low-virulent and avirulent NDV strains can be distinguished by the cleavage site motifs (Yu *et al.*, 2001). In addition, the phylogenetic relationship among NDV strains does not correlate well with cleavage site sequences and virulence (Yu *et al.*, 2001). This indicated that the virulence of NDV strains can be qualified rather than quantified by the analysis of cleavage site motifs and that pathogenicity tests such as MDT, ICPI and IVPI cannot be replaced by analysis of the cleavage site to precisely characterize virulence and pathogenicity of NDV strains.

This experiment demonstrated that La Sota vaccine was not full efficacious. This is supported by the evidence from the field in which very full vaccination program used was apparently insufficient to protect against challenge with this particular strain of the virus. The results presented here emphasize the

importance of maintaining continued surveillance of vaccination procedures.

The continued outbreaks of velogenic NDV in domestic poultry worldwide emphasize the importance for continued research on vaccine efficacy against newly isolated strains. Studies on comparison of antigenic structure of the pathogenic NDV viruses isolated from different outbreaks with that of the viruses used for the vaccination are of crucial importance to determine the key question concerning the estimation of the possible risk to overcome the vaccination barrier.

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توصيف عترة مغايرة من فيروس مرض النيوكاسل بمصر

أثناء عام 2005 تسبب فيروس مرض النيوكاسل في وياء كبير بين قطعان دجاج التسمين في مصر وقد لفت هذا الوباء الأنظار لمقدار الحماية التي توفرها اللقاحات المتداولة تجارياً في السيطرة على المرض في الدواجن وتم عزل الفيروس من قطع تسمين بلغت نسبة النافق به 95% ولقد تم استبعاد أن يكون الفيروس المسبب للمرض هو فيروس إنفلونزا الطيور عن طريق إجراء اختبار الكروماتوجرافي السريع للكشف عن هذا الفيروس وتم توصيف الفيروس كفيروس النيوكاسل باستخدام اختبار النسخ العكسي لسلسلة تفاعلات إنزيم البلمرة لجزء من الجين (F) كما تم عمل اختبار تثبيط تالازن كرات الدم الحمراء. وتم التأكد بأن الفيروس المعزول حشوي وضاري وذلك عن طريق حساب متوسط الوقت اللازم لقتل أجنة البيض المخضب وتقييم ضراوة الفيروس للدجاج عمر ثمانية أسابيع، كما تم تقييم مدى الحماية التي يوفرها اللقاح الحي المتداول تجارياً (لاسوتا) ضد العدوى بالفيروس المعزول حديثاً فتم عمل تجربة تم فيها تحصين كناكيت تبلغ من العمر ثلاثة أسابيع وتم عمل عدوي تجريبية لها بجرعة قاتلة من الفيروس المعزول وأشارت النتائج إلى أن اللقاح لم يوفر حماية تامة ضد ظهور الأعراض المرضية ولكنه نجح في تقليص عدد الطيور النافقة مقارنة بالمجموعة الضابطة والتي حدث بها نفوق لكل الطيور في غضون خمسة أيام من إجراء العدوي التجريبية، ومن المشاهدات الهامة هو عدم وجود ارتباط قوي بين مقدار الحماية من المرض وكمية الأجسام المضادة الموجودة بالطيور قبل إجراء العدوي مباشرة. ولقد بينت هذه النتائج أن هناك احتياج ضروري لتطوير إستراتيجيات التحصين وإنتاج لقاحات جديدة ضد فيروس مرض النيوكاسل من العترة المعزولة محلياً قادرة على وقاية الطيور من ظهور الأعراض المرضية والنفوق على حد سواء لكي يقلل من إفراز الفيروس الضاري من الطيور المصابة.