

RESEARCH ARTICLE

Newcastle disease virus Genotype VII in Chicken Flocks in Dakahlia Governorate and the Effectiveness of some Vaccines

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Abstract

Out of fifteen chicken flocks, ten were tested Newcastle Disease Virus (NDV) positive by RT-PCR. Two selected NDV isolates from previously vaccinated flocks were selected for further investigation. The isolates were proofed negative for the presence of either infectious bronchitis virus (IBV) or avian influenza virus (AIV) genome. The mean death time and intracerebral pathogenicity indices as well as the presence of 112RRQKRF117 motif at the F gene cleavage site were indicative for the virulence of the investigated strains. The phylogenetic analysis showed that the velogenic genotype isolate clustered with published class II genotype VII and closely related to Middle East isolates. In vivo protection assay revealed that vaccination with live and/or inactivated ND vaccines induced sufficient protection ranged between 86.7 % and 100 % against morbidity and mortality as judged by HI titers and challenge test with NDV genotype VII.

Keywords: Newcastle Disease Virus, Newcastle Disease Vaccine Efficacy, Newcastle Disease Virus Genotype VII.

Introduction

Newcastle disease (ND) is an acute highly contagious viral infection affecting most avian species. The disease is endemic worldwide and causes highly economic losses due to high mortalities and reduced production [1-5]. The main route of NDV transmission is airborne route and also it can spread through direct contact with infected birds, contaminated poultry products, people with contaminated clothes or shoes, equipment, vaccines [6], contaminated water [7] and insects in a poultry house [8]. NDV varies in virulence and pathogenicity to embryonated chicken eggs, day-old chicks and six weeks-old chickens into: highly virulent (velogenic), intermediate (mesogenic) and avirulent or low virulent strains (lentogenic) [4]. NDV is composed of six structural proteins: fusion (F), hemagglutinin-neuraminidase (HN), nucleoprotein (NP), phosphoprotein (P),

matrix (M), and the RNA polymerase (L), these proteins are encoded by six genes [5].

Virulence and pathogenicity of NDV is dependent on the presence of multiple basic amino acids at the cleavage site of F0 [3]. The class II genotypes I and II NDV include mainly avirulent and lentogenic strains [9], while virulent NDV strains belong to class II genotypes III to XVI except X genotype NDV [10]. The main cause of recent outbreaks in domesticated and wild birds worldwide is NDV genotype V, VI and VII [11]. Based on virus neutralization and hemagglutination inhibition (HI) assays with monoclonal antibodies, there were antigenic differences between strains of NDV and that were confirmed by evaluating sequences of neutralizing epitopes [12-14].

Class II genotype II and VI were the most frequently isolated in Egypt [15]. However, NDV class II genotype VII is considered the major reason of the current outbreaks of ND in

Egypt [16, 17]. The current study aimed to evaluate commercial ND vaccines against challenge with NDV genotype VII.

Materials and Methods

Sample collection

Fifteen chicken samples were aseptically obtained from 15 poultry farms in between 2014 to 2015 from Dakhliya Governorate. The sampled chickens suffered from either respiratory signs and/or nervous signs and diarrhea with high morbidities that reached 60% and high mortalities that reached 50%, thus suspected to be Newcastle disease. Under sterile condition, specimens from liver, lung and spleen were collected, macerated in mortar with sterile saline (1:10) for both virus isolation and identification. Samples were subjected to quick identification by RT-PCR followed by isolation. Re-identification was performed for two positive samples by RT-PCR for H9, H5, H7 avian influenza and infectious bronchitis for exclusion of these viruses and confirmation that the allantoic fluid contained Newcastle virus only then sequencing were performed for the two samples.

Experimental Birds

Fifty one-day-old SPF (specific pathogen free) chicks were obtained from Koom Oshiem Elfayoum, Egypt and were kept in two isolators. They were fed on a balanced clean and autoclaved ration and water. These birds were used for intracerebral pathogenicity indices (ICPI) test [18].

One hundred and thirty-five one day-old HY-line chicks were used for the protection study. These chicks were fed *ad-lib* for 13 days.

Virus isolation

Tissue homogenates were centrifuged at 3000 rpm for 15 min and the supernatant was collected in sterile tube containing penicillin (2000 units/mL), streptomycin (2 mg/mL), gentamycin (50 µg/mL), and mycostatin (1000 units/mL). Five ECE 10 day-old were inoculated into the allantoic cavity with 0.2 mL/egg of the supernatant. Eggs were incubated for 5 days with daily candling and any dead embryos by the end of the 5th days

were chilled for 24 hours. Eggs were then opened, and each egg was tested with slide hemagglutination test and positive eggs allantoic fluid was inspired and preserved in -20C for further identification and processing.

Haemagglutination and Haemagglutination inhibition (HI) tests

HA and HI test for Newcastle disease was done according to OIE [18].

ND Virus titration (genotype VII isolates and live vaccines)

Serial tenfold dilutions of the virus were prepared in sterile PBS containing 1000 IU penicillin and 100 mg streptomycin per ml and 0.1 ml of each dilution from 10⁻¹ to 10⁻¹⁰ was inoculated via the allantoic cavity using 5 SPF eggs per each dilution. Eggs were incubated at 37°C and candled daily for 5 days. Deaths within the first 24 hours post inoculation was considered as non-specific deaths. Died embryos were collected, chilled and examined for HA activity. Embryonic Infective Dose (EID₅₀) was calculated according to Reed and Muench [19].

Genome purification, RT-PCR and phylogenetic analysis

RNA from the tissue samples (liver, lungs, and spleen) was extracted with Thermo Scientific Gene Jet RNA purification Kit #K073 (Total RNA purification protocol of Mammalian Tissue and insect). RNA extraction was carried out according to the manufacturer guidelines.

The primers were used as following: F gene of NDV F. primer: 5'-GCA GCT GCA GGG ATT GTG GT-3', R. primer: 5'-TCT TTG AGC AGG AGG ATG TTG-3' [20],

H5 AI F. primer: 5'-GCC ATT CCA CAA CAT ACA CCC-3', R. primer: 5'-CTC CCC TGC TCA TTG CTA TG-3' [21], H9 AIV F. primer: 5'-GAA TCC AGA TCT TTC CAG AC-3', R. primer: 5'-CAA TAC CAT GGG GCA ATT AG-3' [22], H7 AIV F. primer: 5'-TCA CAG CAA ATA CAG GGA AGA G-3', R. primer: 5'-CCC GAA GCT AAA CCA GAG TAT C-3' [22], F gene NDV for sequencing F. primer: 5'-GTC AAC ATA TAC ACC TCA TC-3', R. primer: 5'-GGA GGA TGT TGG CAG CAT T-3' [23] and IBV

F. primer: 5'-AAG GAA GATAGG CAT GTAGCTT-3',

phylogenetic relationship using the neighbor-joining method [25].

R. primer: 5'GCTCTAACT CTAATAC TAG CC TAT3' [24].

Antigens and commercial ND vaccines

The obtained sequences were analyzed using MEGA 6 (Molecular Evolutionary Genetics Analysis, version 6 program for studying the

La Sota vaccine, live attenuated vaccine, MSD Company with $7\log_2$ HA units and was used as $4\log_2$ [19] (Table 1).

Table 1: List of the used NDV vaccines kindly provided by Grand Vet Company and MEVAC Company

Commercial Name	Strain	Company	Batch number
<i>Dalguban N+</i>	Genotype VII C4152R21 strain	KBNP, Korea	4513245 151230
<i>MEVAC ND</i>	NDV/ch/Egypt/ 11478AF/2011	MEVAC, Egypt	1609100101
<i>ME-Fluvac H5+ND</i>	H5N1+ND	MEVAC Egypt	1607190101
<i>Jovac NDV CLONE</i>	Live attenuated vaccine	JOVAC JORDAN	07D0416

Experimental design

One hundred and thirty-five one-day-old HY-line chicks were fed *ad-lib* for 13 days. Fifteen blood samples were randomly collected after 13 days of feeding and the chicks were randomly equally distributed into nine groups (15 birds, each). Group (G) 9 was challenged with Genotype VII NDV, $10^{8.6}$ EID₅₀/dose/chick via intramuscular injection on 34th day. Groups G1, G3, G5 and G7 were vaccinated subcutaneous with 0.5 mL of different commercial inactivated ND vaccines at 13 day-old. While, groups G2, G4, G6 were vaccinated subcutaneous with 0.5 mL of

different commercial inactivated ND vaccines and intra/ocular with clone ND vaccine (Clone ND vaccine titer was $10^{10.4}$ EID₅₀/vial 1000 dose) at 13th days old as displayed in Table 1. Group 8 was left unvaccinated and non-challenged (control negative group). All experimental groups at the day 34th of the experiment with 0.1 mL of Genotype VII NDV, $10^{8.6}$ EID₅₀/dose/chicks via intramuscular injection. Fifteen blood samples were weekly collected from each group of chickens for 3 weeks post-vaccination and two weeks post-challenge.

Table 2: Experimental design of NDV vaccination at 13-day old

Groups	Vaccine	Route	Age of challenge I/M
G1	Dalguban N+	S/C	34 days-old
G2	Dalguban N+ + Jovac NDV Clone	S/C I/O	
G3	MEVAC ND	S/C	
G4	MEVAC ND + Jovac NDV Clone	S/C I/O	
G5	ME-Fluvac H5+ND	S/C	
G6	ME-Fluvac H5+ND + Jovac NDV Clone	S/C I/O	
G7	Jovac NDV Clone	I/O	
G8	-	-	-
G9	-	-	34 days-old

S/C: sub cutaneous I/O: intra ocular

Statistical analysis

Data were collected, summarized, and then analyzed by using SPSS, 16 (IBM Corp., Chicago, IL, USA). One-way analysis of variance (ANOVA) test was used to differentiate between the differentially treated groups, multiple comparisons test between different studied groups was done using LSD test.

Results and Discussion

Clinical signs and Post-mortem findings of the examined flocks

The examined flocks suffered from mortalities varied from 5% up to 50% and morbidity varied from 10% up to 90%. as previously reported [3-26]. Additionally, they were suffered from general signs of illness in the form of off food, depression, ruffling feathers, respiratory signs (coughing, sneezing) and specific symptoms in the form of nervous signs (torticollis, head shaking, dropping wings) and greenish watery diarrhea followed by death. The respiratory findings are characteristics for several avian viral infections such as NDV, AIV and IBV infection. However, the greenish watery diarrhea can be seen mainly in the instance of highly pathogenic AIV and NDV infections. It worth to mention here that a few of the investigated flocks were suffered from variable nervous signs such as tremors, paralyzed wings and legs, twisted necks,

circling, clonic spasms and sometimes complete paralysis.

The post-mortem (PM) lesions of both sacrificed and freshly dead birds were pinpoint hemorrhage on tips of proventriculus glands, hemorrhages on the cecal tonsils, airsacculitis, duodenal ulcer, greenish content of gizzard, tracheitis, congestion in the brain blood vessels. Taken together, the observed clinical signs and post-mortem lesions data were strongly suggestive for the presence of NDV infection.

Virus RT-PCR and taxonomic identification

Out of 15 tested flocks, 10 were NDV positive using RT-PCR assay (66.6%). The positive flocks were reproduced positive results in the HA slide test post-isolation in ECE. Two isolates were selected according to the geographical distribution and breeding purposes and were further screened by RT-PCR for the presence of either of IBV, Influenza virus subtypes H5, H9 & H7 or NDV genome. The two selected isolates were negative for IBV & Influenza virus subtypes and were positive for NDV only.

Partial sequencing of F gene of the two selected isolates were performed and submitted to the GeneBank under accession number NDV-DAK-1-15 (KX232676) and NDV-DAK-2-15 (KX232677). The sequences of the cleavage site of F gene for the two NDV strains are 112'R R Q K R F'117, specific for the virulent NDV (vNDV) (Figure 1).

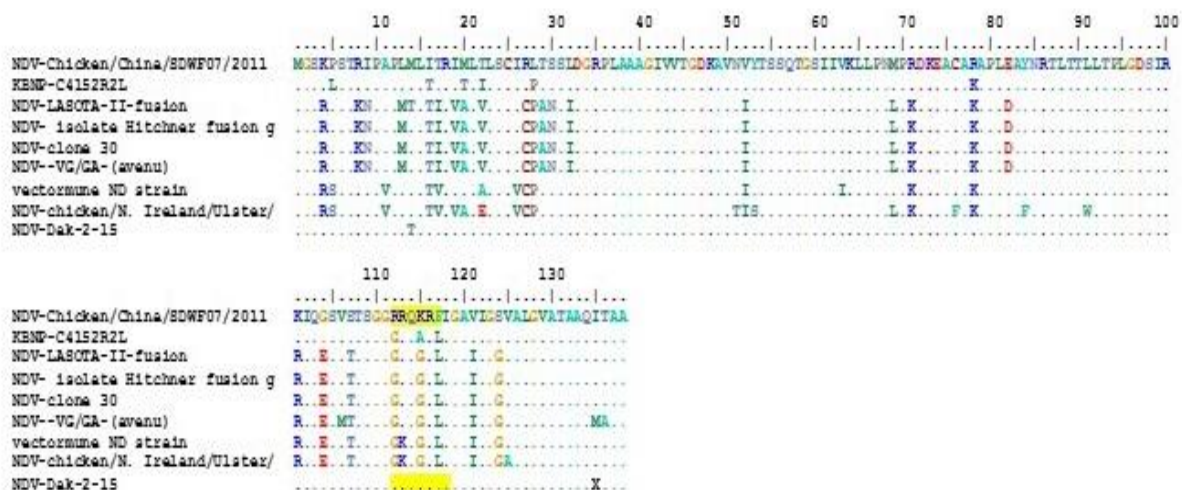


Figure 1:- Deduced amino acids and cleavage site sequence among NDviruses.

The two investigated strains (NDV-DAK-1-15 and NDV-DAK-2-15) were completely identical in the cleavage site with Egyptian strains described by Radwan and colleagues [16]. Hence, they found that the sequence of isolate NDV/Chicken/Giza/Egypt/MR0/2012 carries the motif 112'RRQKRF'117 that is consistent with viruses of velogenic strains of the subgenotypeVIIId [27]. The cleavage site of the Egyptian strain 112'R-R-Q-K-R-F'117 has four basic amino acids at positions 112–116 characteristic for vNDV strains. In

addition, the presence of the phenylalanine (F) residue at position 117 has been described as being a possible contributor to the neurological effects [3].

The phylogenetic analysis of partial sequence of F gene revealed that the two strains NDV-CH-DAK-2-15 and NDV-DAK-1-15 (Figure 2) were closely related to the isolates from China, suggesting that the virus transmission could have occurred through migratory water birds from the Far East (NDV-CH-DAK-2-15) virus belonged to class II genotype VII.

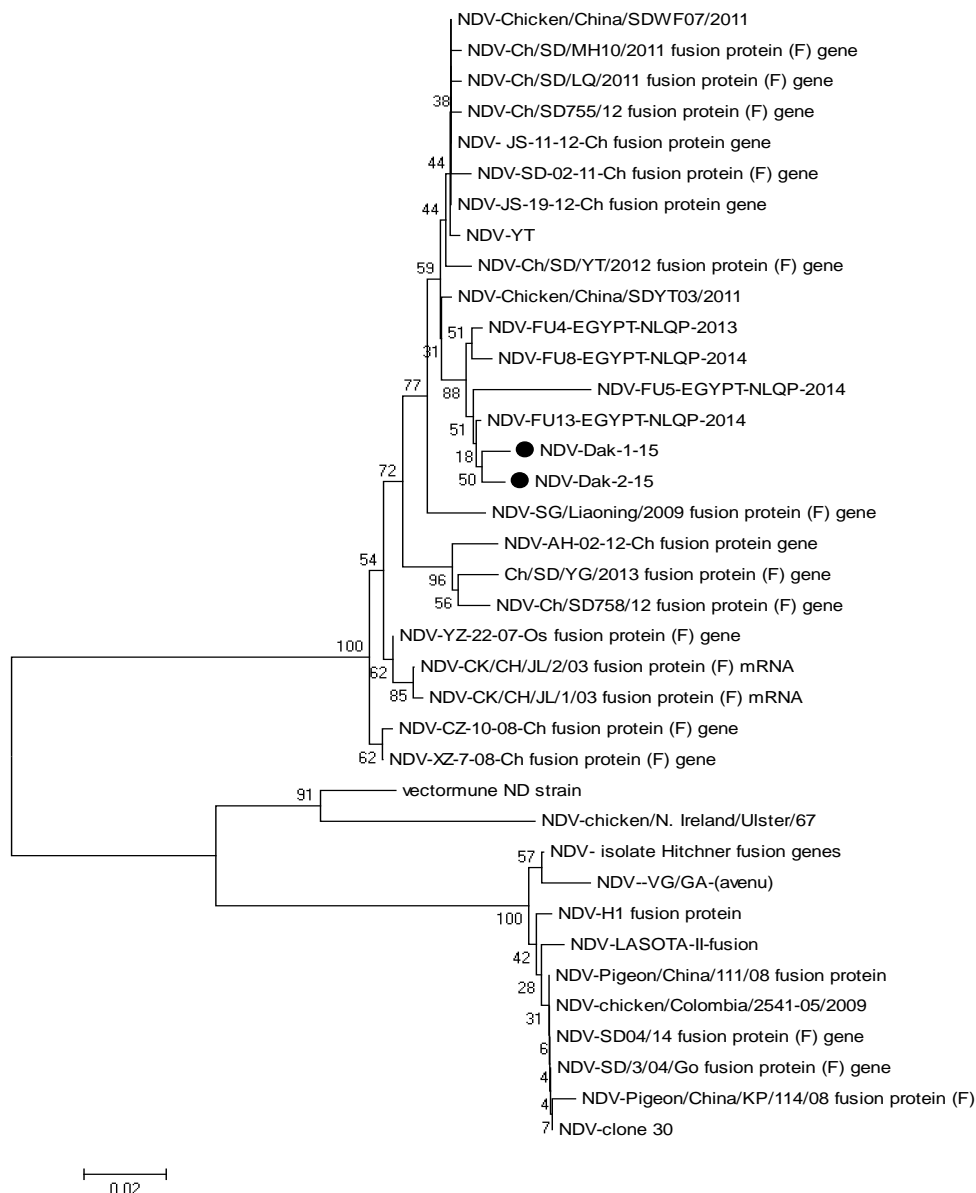


Figure 2: Phylogenetic analysis of partial F protein of NDV genotype VII (NDV-CH-DAK-1-15) and NDV genotype VII (NDV-CH-DAK-2-15) with other NDV strains and vaccinal strains. The two sequences are labeled with black circle. Phylogenetic relationship was estimated through a bootstraps trail of 1000 with the MEGA Version 6 using Cluster W alignment algorism and neighbour-joining method for tree construction.

NDVs with an ICPI $\geq 0,7$, the cleavage site of the fusion protein contain multiple basic amino acids with at least three lysine (K) or arginine (R) [19] and mean death time (MDT) must be less than 60 hours [28] are classified as velogenic viruses. Both of NDV genotype VII (NDV-CH-DAK-1-15) and NDV genotype VII (NDV-CH-DAK-2-15) were velogenic of MDT 48 hours and 50 hours with ICPI 1.8 and 1.7, respectively. In addition, the partial sequencing of the F gene (cleavage site) showed the presence of polybasic amino acid motifs 112RRQKRF117.

Protection study

Average maternal NDV-derived-antibody (Table 3) titer was $5 \log_2$ on the 13th days of age (vaccination day), which decreased weekly in control-non-vaccinated chicks (G 8 and 9) to reach non-protective titers on the 27th and nil on the 34th days age and onwards.

Table 3: Weekly NDV-HI Ab titers (\log_2) post-Newcastle vaccination at 13 days old of experimental chicks

Groups	HI titer on the day 13 of the experiment (the day of vaccination)	Weeks post-vaccination			2 weeks post challenge
		1	2	3	
G1	5	2.8 ± 0.40^c	1.8 ± 0.90^d	4.2 ± 0.62^b	8.9 ± 0.08^a
G2.		3.5 ± 0.34^{bc}	6.2 ± 0.65^{ab}	7.4 ± 0.27^a	9.4 ± 0.19^a
G3		2.7 ± 0.33^c	2.5 ± 1.23^{cd}	4.3 ± 0.67^b	9.8 ± 0.11^a
G4		3.5 ± 0.43^{bc}	7.2 ± 0.40^a	7.8 ± 0.23^a	9.4 ± 0.20^a
G5		5.5 ± 0.22^a	2.7 ± 0.67^{cd}	5.1 ± 0.45^b	9.8 ± 0.09^a
G6		3.8 ± 0.17^b	6.0 ± 0.45^{ab}	7.3 ± 0.25^a	9.8 ± 0.18^a
G7		5.3 ± 0.21^a	4.5 ± 0.34^{bc}	4.0 ± 0.38^b	9.7 ± 0.14^a
G8		4.8 ± 0.17^a	1.8 ± 0.60^d	0.0 ± 0.0^c	0.0 ± 0.0^b

Administration of either combination from both live and inactivated NDV vaccines or live vaccine alone led to significant elevation of NDV-GMTs 2 weeks post-vaccination (Table 3). On the other hand, neither of the applied inactivated NDV vaccines alone could induce statistical significant elevation in the NDV-GMTs at two weeks post-vaccination compared with the non-vaccinated control group. Although all vaccinated groups had statistical significant increases in the GMTs (Table 3), birds received combination of live and inactivated vaccines (G2, 4 & 6) had

It seems from our results (Table 3) at 1 week post-vaccination of birds that there was significant declining in antibodies in birds either vaccinated with Mevac ND, Mevac ND+colone, Mevac ND-H5, Mevac ND-H5+colone, or Dalgupan+colone. The previous could be attributed to the neutralization of vaccinal virus by maternal antibodies or due to the normal declining of maternal immunity by time post-hatching. In the same time point (one week post-vaccination), groups vaccinated with either Dalgupan ND or Jovac NDV Clone as a single vaccine have slight increase in NDV GMT compared with the control non-vaccinated ones that may be attributed to non-interference of the vaccine with maternally derived antibodies or may due to high immunogenicity of the vaccine.

superior and statistical significant GMTs compared to groups single NDV vaccinated groups. Two factors affect on the response of bird immunity to vaccination the first one having enough time between vaccination and challenge to develop sufficient immunity and the second one the flock (herd) immunity [29].

Morbidity post-challenge as judged by weakness, off food, unbalanced gait, torticollis, and/or greenish diarrhea were 100% in non-vaccinated-challenged group and mortality were 66.7% versus nil in non-vaccinated non-challenged group.

Respectively, inactivated ND oil adjuvant Mevac ND, Delguban N+ and *ME-Fluvac H5+ND* vaccines afforded 86.7%, 100%, and 100% protection from morbidity and 86.7%, 100%, and 100% protection from mortality against challenge with $10^{8.6}$ –EID₅₀ of ND genotype IIV NDV/CH/DAK/2/2015. Vaccination with live NDV Clone alone afforded 93.3% protection against the challenge as judged by morbidity and mortality.

Post vaccination of inactivated recipient birds with clone increase protection in groups receiving Mevac ND oil adjuvant vaccines from 86.7% to 100% but not for group receiving Mevac ND-H5 AI vaccines with clones. Mortality post-challenge with ME-Fluvac ND-H5 AI group was 0.0% that increase to 7.1% in such group by boosting with clone.

Antibodies produced against the hemagglutinin (HN) and fusion (F) trans-membrane surface glycoproteins are able to neutralize NDV upon subsequent infection [30, 31]. Because all NDV are the same serotype so that there are cross protection between different genotypes so clinical signs and deaths from ND should be prevented by all vaccines. However, some studies have been demonstrated that vaccines formulated with homologous strains can decrease the amount of challenge virus shed than heterologous vaccine [32-34]. Our results agreed with Saad *et al.* [35] who approved that adequate heterologous antibody levels, induced by the proposed vaccination program, protected birds from morbidity and mortality.

Conclusion

Finally it could be concluded that inactivated Newcastle vaccines, Inactivated Newcastle plus H5 avian influenza vaccines, inactivated ND VII vaccine, and live clone vaccines provide protection against Egyptian field ND VII isolates and the post with live vaccine may add advantage in increasing protection.

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Conflict of Interest

The authors declare no conflicts of interest to the current manuscript.

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الملخص العربي

فيروس مرض النيوكاسل (النمط الوراثي السابع) في قطعان الدجاج بمحافظة الدقهلية وفعالية بعض اللقاحات

منال علي حياكة^{1*} مني محرز علي^{1*} محمد عبدالعزيز ليد^{2*} كامل ابراهيم ابو العزم^{3*}
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 بعد اختبار خمسة عشر مزرعة دواجن تعاني من أعراض تنفسية وأعراض عصبية ونفوق عالية في القطعان تصل الي 50 بالمائة وهذه الأعراض تتشابه مع أعراض مرض النيوكاسل لذلك للتأكد من وجود أو عدم وجود إصابات بفيروس النيوكاسل وذلك باستخدام تفاعل البلمرة المتسلسل (PCR) عشرة مزارع كانوا مصابين بالنيوكاسل وللتأكد ولمعرفة ما إذا كان فيروس النيوكاسل وحده هو السبب الرئيسي والمسؤول عن الإصابات والناقق في هذه المزارع. لذلك تم اختبار العينات باستخدام تفاعل البلمرة المتسلسل للتأكد من وجود فيروس النيوكاسل والتأكد من خلوها من انفلونزا اتش 5 وانفلونزا اتش 9 وانفلونزا اتش 7 وايضا التهاب القصبة الهوائية الفيروسي.

وقد سبق لهذه المزارع التحصين ضد مرض النيوكاسل. وقد تبين بعد التحليل الجيني للجين المسؤول عن الضراوة الخاص بمعزولتين أن هاتان المعزولتان تحتوي على صفات الفيروسات شديدة الضراوة كما أن هذه الفيروسات تعتبر نيوكاسل جينوتايب 7 بعد التأكد من خلوها من باقي الفيروسات الأخرى السابق ذكرها.

كما اتضح بعد التصنيف الجيني لمعزولات النيوكاسل أنها أقرب في تركيبها الجيني لتلك الفيروسات المعزولة من الشرق الأوسط.

وبعد عمل دراسات معملية علي التحصينات لتقييم القاحات المستخدمة في السوق لمعرفة اذا ما كانت اللقاحات تصد ضد الإصابة بفيروس نيوكاسل جينو تايب 7 تبين ان اللقاحات المستخدمة في التجربة المعملية سواء كانت لقاحات حية او لقاحات ميتة او استخدام القاحات الحية مع الميتة فإنها تعطي مناعات كافية لصد العدوي بنسبه تتراوح بين 86,7% و100% ذلك عن طريق التنافس مع الفيروس وأيضا عن طريق قياسات مناعات الدم باستخدام إختبار منع تلتزن الدم