# ISOLATION IDENTIFICATION AND PATHOTYPING OF NEWCASTLE DISEASE VIRUSES FORM NATURALLY INFECTED CHICKENS IN IRAQI KURDISTAN REGION

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**ABSTRACT**

**Iraq has been endemic for Newcastle disease virus (NDV) with natural infection causing significant losses in the poultry industry since 1968. This study was designed to identify a natural infection of NDV occurred in three governorate in the northern part of Iraq (Erbil, Sulymaniyah and Duhok) among chicken flocks and estimate its virulence by the mean death times(MDT) and intra-cerebral pathogenicity index (ICPI). Ninety six flocks with high mortality were examined for post mortem lesions and the samples collected from suspected ND chicken flocks, either sick or dead showed characteristic clinical findings and show positive rapid test for NDV antigen. The collected samples were pooled, homogenized and centrifuged then the virus propagted in embryonated chicken eggs was confirmed by real time RT-PCR, hemagglutination (HA) test and hemagglutination inhibition (HI) test using anti-NDV hyper-immune serum. The results were isolation 9(12%) out of 96 samples where NDV was positive clinicaly. The MDT and ICPI revealed that the isolates were compatible to mesogenic (1/9) and velogenic (5/9) types. These results demonstrated that the NDV strains were virulent for chickens and the vaccination might not give enough protection.**

**Key words: circulating NDV Velogenic ICPI,MDT, Northern Iraq HI Virus isolation**



# عزل وتشخيص فايرس مرض النيوكاسل المتوطن في محافظات اقليم كردستان العراق من الدواجن المصابة طبيعيا وتحديد ضراوتهم

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**المستخلص**

**يعد العراق من البلدان المتوطنة فيه فايرس مرض النيوكاسل والذي يحدث اصابات و يسبب خسائرآ كبيرة لصناعة الدواجن منذ 1968. صممت الدراسة من اجل عزل وتشخيص فايرس النيوكاسل الذي يصيب قطعان الدواجن بين الحين والاخر في مناطق شمال العراق(اربيل وسليمانية ودهوك) ويمكن تحديد ضراوته من خلال اختبارات متوسط الوقت اللازم لقتل جنين الدجاج (MDT) والمؤشر المرضي عند الحقن في الدماغ (ICPI). من اجله جرى فحص 96 قطيعآ يعتقد اصابته بمرض النيوكاسل مع وجود نسبة هلاكات عالية، جرى تشريح الدجاج الهالك والمصاب التي عليها علامات سريرية مميزة ثم اجري اختبار سريع للتاكد من وجود مستضد الفايروس لمرض النيوكاسل جرى اخذ العينات العضوية وخزنها ثم نقلها الى المختبر وخلطت العينات المتشابه من قطعان الاقضية والنواحي تم قطعت وفصلت بجهاز الطرد المركزيز شخص الفيروس في السائل الالنتوي المحصود من البيض المحقون باستخدام اختبار التلازن الدموي واختبار تثبيط التلازن الدموي باستخدام مصل عالق المناعة مضاد لفايروس النيوكاسل وreal time RT–PCR جرى عزل تسعة (12%) عزلات من مجموع 96 عينة موجبة سريريا واظهرت نتائج اختباري (MDT) و(ICPI) اكدت النتيجة ان هناك خمس عينات ضارية و عينة واحدة متوسطة الضراوة و فشل في اللقاح.**

**كلمات مفتاحية: اصابات، عزل، تشخيص ، جنين.**

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## INTRODUCTION

Newcastle disease (ND) has been concerned as one of the most important devastating diseases of poultry because of its wide range host and worldwide distribution with severe economic losses in domestic poultry, especially in chickens (2). Newcastle disease (ND) is an acute infectious viral disease of domestic poultry and other species of birds regardless of variation in sex and age (8,28). Clinical signs are dependent on factors such as the virus strain, host species, age of the host, co-infection with other micro-organisms, environmental stress, and immune status (34) In chickens, the general symptoms are loss of appetite, listlessness, abnormal thirst, weakness, air sacculitis, tracheitis and conjunctivitis. Respiratory signs can include sneezing, gasping for air, nasal discharge and coughing, whereas a clear intestinal symptom is a greenish watery diarrhea. Nervous symptoms may consist of paralysis of wings and/or legs, twisting of head and neck or complete paralysis (10). The major clinical signs are respiratory distress, diarrhea, circulatory disturbances and impairment of the central nervous system (16). Gross lesions are primarily developed hemorrhage and ulcers with raised borders with central necrosis in the duodenum, ileum and cecum and hemorrhage of the proventriculus mucosa, pneumonic lungs, hemorrhages in trachea, air sacs, brain, cecal tonsils and lymphoid tissue lesions and the spleen is more susceptible to the necrotic effects of NDV (22). Newcastle disease 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 (*12). The detection and differentiation of NDVs are based on virus isolation using embryonated chicken eggs, there are several methods for pathogenicity and characterization of NDV, on the basis of invivo estimation of pathogenicity (7) .These in-vivo tests are mean death times (MDT) in embryonated eggs of chicken, Intracerebral pathogenicity index (ICPI) in 1 day old chicks, and Intravenous pathogenicity index (IVPI) in six weeks old chicks (28). Based on the clinical signs and course of disease strains all NDV isolates are grouped into five pathotypes based on severity of the disease in chickens; viscerotropic velogenic, neurotropic velogenic, collected mesogenic, lentogenic and asymptomatic enteric type (16). The first confirmed outbreaks of ND occurred in 1926 in Java, Indonesia and in Newcastle, UK (11). ND is fatal and still top ranked poultry disease. Annual losses caused by this disease worldwide are inmillions of dollars (33). The first isolated of NDV by V.R. Kascheulla and his co-workers from infected chickens at Abu Graib in Iraq, designated AG68 and confirmed by (35). Iraq has been considered as an endemic country by ND. In the successive years, an intensive vaccination program against NDV has been followed in the large-scale and small poultry farming. However, this virus showed the ugly face against poultry industry in north of Iraq causing severe outbreaks in poultry farms 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 natural infections especially in the north of Iraq. Therefore study was carried out to isolate and characterize the NDV strains among chicken flocks in the north of Iraq and determine its virulence nature.

## MATERIALS AND METHODS

**Samples Collection:** Ninety six flocks from Northern Iraq consulted among them selected 26 in Erbil(E1-E26), 14 in Sulymaniyah(S1-S14) and 6 in Duhok(D1-D6) Provinces. examined for clinical history and Post Mortem lesions and Samples collected from suspected ND chicken focks during January-Sept.2016 after necropsy of diseased chicken presented to YARA-Barz Company Laboratory section and veterinary centers. ND was diagnosed based on case history, clinical signs and post-mortem finding also tested with Antigen Rapid Test Card manufactured by Shenzhen Lvshiyuan Biotechnology Co. Ltd catalog No.(LSY-20025) for detection of ND virus antigen the positive NDv Flocks obtained the organ sample (proventriculus, lung, trachea, intestine, cecal tonsils, spleen and brain) Most sacrificed chickens had moderate mortality and greenish diarrhea, also showed circulatory disturbances and respiratory difficulties. These samples were collected properly in sterile plastic containers and stored in freezer (-18ºC)

transported in cool box to (Al-Nahdha Transboundary Animal Diseases Laboratory in Central Veterinary Laboratory and Research Department) in Baghdad which they stored in deep freeze (-20ºC) until processed and inoculated into embryonted chicken egg (ECE).

**Virus Propagation in ECE:** Isolation of virus was carried out at Virology department /Al-Nahdha Veterinary Laboratory and Research Department in Baghdad under a Biohazard safety cabinet class II, USA started with orgranize the samples according to sub district Valleys After thawing frozen organs at room temperature and pooled each regarding sub-districts (while trachea with lung , spleen and intestinal with ceacal tonsil samples were processed separately) using sterile forceps and scissors, small pieces of all tissues corresponding to 2 gm were collected and Fine grained with 3-5 ml maintenance essential media using Mortar and pestle till homogenization the suspension was centrifuged the suspension was centrifuged (2000/10minute at 4ºC) a thirty percentage of suspension filtered with 0.2 filter divided in two part 1ml of suspension for real time RT-PCR technique and rest for ECE Inoculation. The positive sample of NDv by real time RT-PCR inoculated in ECE.. Since Specific Pathogenic free(SPF) eggs are not available, commercial eggs can be used. These eggs will probably contain antibodies to ND, but the antibodies will be confined to the yolk sac until around day 15 of incubation (30). These eggs were incubated in a clean environment for another 1-2days then 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 5days post incubation were chilled in refrigerator (4ºC) overnight. The allantoic fluid (AF) was harvested and stored in sterile screw-capped vials at -80°C till further use.

**Preparation of hyper-immune serum:**

Twenty one day-old non-vaccinated chickens were vaccinated using killed-oil vaccine at first day subcutaneous and lasota NDV vaccine at day 7th, 14th , 21st 28 and 42 day. Hyper-immune serum was separated from the blood collected from vaccinated chickens and preserved at -20°C until further use. **(**18**)**

**Serological Identification of NDV:** The presence of NDV in AF was determined by slide HA test, micro-titer plate HA and HI tests as other scientist's (15). 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 room temperature. 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 room temperature 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.

**Molecular Identification of NDV**

**Viral RNA preparation and real time RT-PCR**: The NDV RNA was extracted directly from allantoic fluid using a QIAamp Viral RNA Mini Kit (Qiagen, USA) according to manufacturer's recommendations.

**Primers and probes: S**ets of primer combinations designed for amplification of matrix genes were used in real time RT-PCR. A velogenic specific primer probe designed by (35) to amplify a wide range of Velogenic NDVs was used to detect in this study (Table 2B).

**Real time RT PCR used:** Molecular diagnosis of extracted RNA NDv using a comersial Genekam biotechnology (Genekam Germany) according to manufacturer's recommendations. First labeling micro tube according the number of samples then adding ( 7µl from A + 10µl from B + 1µl from Y = 18µl) per reaction. from which you can take 18µl and add to each labeled micro tube then add 2µl of extracted RNA with sterile pipette-tip with filter to each leabled micro tube. label

except D1(+v)e and D2 (-ve) control (avoid touching the wall). Running via PCR program 3600 seconds at 42°c for one cycle, 600 seconds at 70°c, annelling 15 seconds at 95°c for 40 cycles extension 60 seconds at 52°c then using the software for analyzing the graphics. The negative control should run along with the bottom and positive control must give a curve in the software graphics

**Pathotyping determination via MDT and ICPI**

**Mean Death Time (MDT):** Tenfold ($10^{-6}$ to $10^{-9}$) dilutions of fresh infective AF in sterile phosphate-buffered saline (PBS) were prepared From each dilution, 0.1 mL was inoculated into the allantoic cavities of five 9 days old embryonated chicken eggs another five eggs inoculated with 0.1ml of PBS. The inoculated eggs were incubated at 37°C, examined twise daily for 7 days and the times of the embryo deaths were recorded. The MDT has been used to characterize the NDV pathogenicity as follows: velogenic, less than 60 hrs; mesogenic, 60 to 90 hrs; and lentogenic, more than 90 hrs.(6**)**

**Titration of NDV isolates**

The median embryo infective dose 50 (ELD50) of each isolate was determined using the method of Reed and Muench (29). To determine the ELD50, 10 fold serial dilutions of AF sample were used $10^{-1}$, $10^{-2}$, $10^{-3}$,$10^{-4}$, $10^{-5}$ and $10^{-6}$. Each of these dilutions was inoculated to 5 embryonated chicken eggs. Mortality was recorded upto five days post-inoculation, The dilution of inoculum producing 50 percent dead of embryonated egg was determined. The Reed Muench formula was used to calculate:

$$\text{Index} = \frac{\%\text{ infected at dilution immediately above 50\%} - 50\%}{\%\text{ infected at dilution immediately above 50\%} - \%\text{ infected at dilution immediately below 50\%}} =$$

The index was calculated by applying this equation to the dilution that produced lethal rate directly more than 50%.

**2. ICPI for the NDV Isolates:** The ICPI was performed to evaluate the pathogenicity of NDV isolates. The test was applied on 70 one-day old chicks which were divided in to 7 groups are 10 chicks/group according to international OIE standards (8) Fresh infected AF obtained during passage three the NDV in ECE with a HA titer log2 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 route(I/C) in the caudal part of the brain in the middle of universal line between two eyes in 10 chicks each group , as well as one group (10 chicks) was injected with 0.05 ml PBS in same route as control. The birds were observed for the clinical symptoms every 24h/ 8 days for 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 was characterized as a velogenic strain, 0.5-1.5 as a mesogenic strain; while an ICPI below 0.5 indicated a lentogenic strain.

**RESULTS ANDDISCUSSION**

**Isolate NDV from infected chickens from the North of Iraq**

A total of 26 in Erbil (E1-E26), 14 in Sulymaniyah (S1-S14) and 6 in Duhok (D1-D6) out of 96 examined flocks exhibited ND signs clinically and the mortality rate ranged from 16-50%, death occured within 24-96 hrs after the onset of clinical signs. Thirty two organ samples were positive inthe rapid test for viral isolation. Clinically, the most common clinical signs were greenish diarrhea, incoordination, swelling of eyelids and head, respiratory sign, torticollis and death. The most commonly observed post mortem lesions were marked hemorrhagic ulcers in the intestinal wall and cecal tonsils, pin point hemorrhages at the tip of proventriculus glands, hemorrhagic lungs, tracheitis with congestion and catarrhal exudates showing in (Figure2 A,B and C) respectively and (table 1).

**Virus Identification:** Thirty two ND-suspected field samples were pooled regarding their sub-districts in nine tubes were homogenized and suspension was confirmed for presence of ND virus via real time RT-PCR. The sample positive in real time PCR were propagated in 9 -11 days old ECE via allantoic sac. In all positive cases embryos died within 24 to 96 hrs post inoculation, nine showed positive rapid HA within few seconds indicating that the isolates were hemagglutinating viruses. All HA positive embryo died within 40 to 96 hrs post-inoculation. Confirmation of ND virus via real time RT-PCR of samples was positive for

NDV showing (Figure 1). The rapid HA positive samples were titrated using micro HA test; the titers ranged from 1:32 - 1:512 (Table 2A).Nine positive AF samples, were inhibited by anti-NDV hyper-immune serum using HI test except three which indicated that include other than NDV (Table 2A). The HI titers of the positive viruses were from 1:16 - 1:256 (Table 2A).

**Pathotyping of NDV Isolates in Chicken Using MDT and ICPI:** To characterize the isolates biologically and clarify whether they are avirulent (vaccine strains) or virulent strains, the pathogencity of NDV isolated from the chickens was assessed by Mean Death Times(MDT) and ICPI test. The MDT observed in emberyonated chicken eggs was 59hr for (E1, E2) and 60-61.8hrs for(S1, S2) post inoculation chickens showed that the diffusely red those subcutaneous tissue of the head was filled with blood and the blood vessels over the body were prominent. Concerning D1,D2 Chicken embryos the MDT was observed 60-64 hrs post inoculation, showing diffuse congestion and hemorrhage in the whole body with edema in the head region. In the C control non-inoculated NDv chickens. To determine the ELD50, 10 fold serial dilutions of AF samples were used i.e. $10^{-1}$, $10^{-2}$, $10^{-3}$,$10^{-4}$, $10^{-5}$ and $10^{-6}$. Each of these dilutions was inoculated to 5 embryonated chicken eggs. Mortality was recorded upto seven days post-inoculation. The calculated ELD50 of AF sample isolate was $10^{-8.159}$ to $10^{-8.839}$ /0.1ml of virus (Table 3). Clinically, chicks in all groups except control group showed clinical signs ranged from general non-specific signs of depression, closed eyes, off intacking and lateral recumbence to nervous in nature including paresis and paralysis of legs (one or both) and wings (one or both), twisting of the neck, muscle tremors and incoordination. Intracerebral pathogenicity index (ICPI) is considered by OIE standard manual as a gold standard for measuring viral virulence for ND strains(3)..The ICPI was calculated the results showed that 5 samples out of 6 samples of the NDV isolates had an ICPI value >1.5 (Table 3), which were congruent to velogenic NDV isolates, whereas one samples out of 6 samples had value 1.325 for mesogenic isolates (Table 3).

**Table 1. Investigation data of the NDV among chickens flock in (Erbil, Sulymaniyah, and Duhok Provinces) during 2016**

| Location | Flocks(ND) Number | Age/ days | suspected Total capacity | Mortality% | PM lesion |
|---|---|---|---|---|---|
| Erbil | E1 | 28 | 10000 | 15.0 | Congestion of lung, trachea, and spleen, enteritis, hemorrhages and ulcer on the tips of the proventriculus glands, cecal tonsils enlarged, thickened and hemorrhagic |
| | E2 | 32 | 8000 | 9.0 | |
| | E3 | 30 | 10000 | 10.0 | |
| | E4 | 34 | 7000 | 8.0 | |
| Sulymaniyah | S1 | 36 | 12000 | 15.0 | Congestion of lung, trachea, and spleen |
| | S2 | 30 | 8000 | 10.0 | enteritis, hemorrhages and ulcer on the tips of the proventriculus glands, cecal tonsils enlarged, thickened and hemorrhagic |
| | S3 | 30 | 10000 | 7.0 | |
| Duhok | D1 | 35 | 7000 | 5.0 | cong. lung, trachea, and spleen Enteritis, hemorrhages and ulcer on the tips of the proventriculus glands, cecal tonsils enlarged, thick |
| | D2 | 28 | 8000 | 8.0 | cong. lung, trachea, and spleen Enteritis, hemorrhages and ulcer on the tips of the proventriculus glands, cecal tonsils enlarged, thick |

**Table 2A. Isolation and identification of ND Viruses from naturally infected chickens in (Erbil, Sulymaniyah and Duhok) during 2016**

| Location | Sample | Type of chicken | Age (day) | No. passage | (log2) HA | (log2) HI | Real time -PCR | CT value |
|---|---|---|---|---|---|---|---|---|
| Erbil | E1 | Broiler | 28 | 3 | 128 | 64 | Positive | 19.1 |
| | E2 | Broiler | 32 | 3 | 128 | 64 | Positive | 18.9 |
| Sulymaniyah | S1 | Broiler | 36 | 3 | 32 | 16 | Positive | 19.3 |
| | S2 | Broiler | 30 | 3 | 32 | 16 | Positive | 18.8 |
| Duhok | D1 | Broiler | 35 | 3 | 512 | 256 | Positive | 18.5 |
| | D2 | Broiler | 28 | 3 | 128 | 64 | Positive | 18.6 |

**Table 2B. Oligonucleotides and probes used in the Real Time based PCR**

| Primer Name | Target gene | Size (bp) | Sequence | Ref |
|---|---|---|---|---|
| APMV1F | M+4100 | 121 | 5'-AGT GAT GTG CTC GGA CCTTC-3' | ( 23) |
| APMV1R | M-4220 | 121 | 5'-CCT GAG GAG AGG CAT TTG CTA-3' | ( 23) |
| APMV1-LNA | prob | - | 5'HEX-TTC TCT AGC AGT GGG ACA GCC TGC-BHQ-1'-3 | ( 23) |

**Table 3. Pathotyping of isolated NDV from naturally infected chickens in (Erbil, Sulymaniyah and Duhok) during 2016**

| Location | Sample | Type of chicken | Age (day) | No. passage | ELD50 | MDT (hrs) | ICPI | Pathotype |
|---|---|---|---|---|---|---|---|---|
| Erbil | E1 | Broiler | 28 | 3 | -8.839 | 59.3 | 1.550 | Velogenic |
| | E2 | Broiler | 32 | 3 | -8.839 | 59.2 | 1.525 | Velogenic |
| Sulaymanhia | S1 | Broiler | 36 | 3 | -8.167 | 60.25 | 1.600 | Velogenic |
| | S2 | Broiler | 30 | 3 | -8.75 | 61.8 | 1.562 | Velogenic |
| Duhok | D1 | Broiler | 35 | 3 | -8.159 | 64.25 | 1.325 | Mesogeni |
| | D2 | Broiler | 28 | 3 | -8.160 | 60.55 | 1.562 | Velogenic |

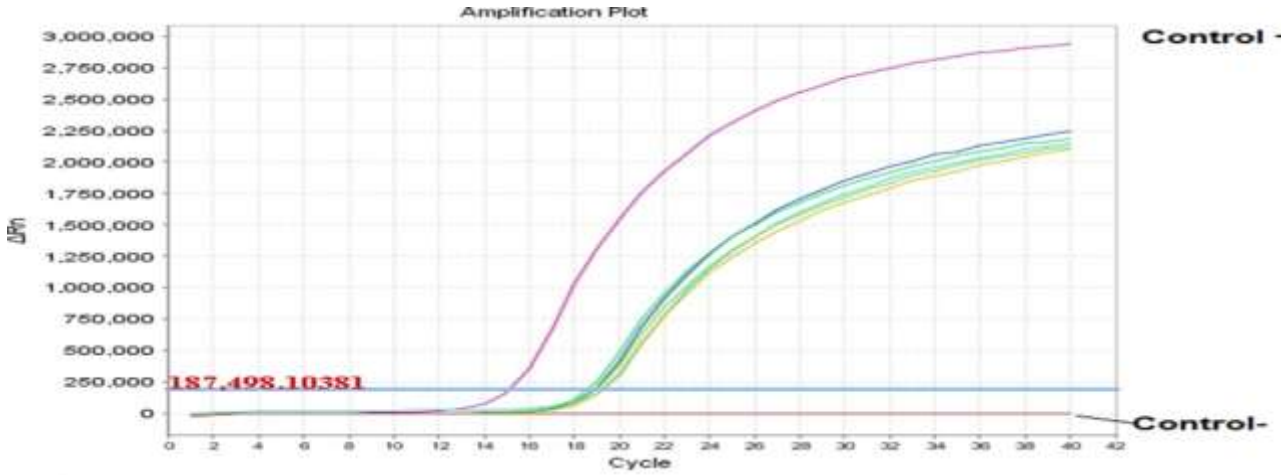


**Figure 1. Real Time PCR finding of NDV in the clinical specimens: Results of the Real Time PCR of the tested samples from chickens using the designed F gene oligonuclotiesds. (A) The amplification curve of the tested samples,**

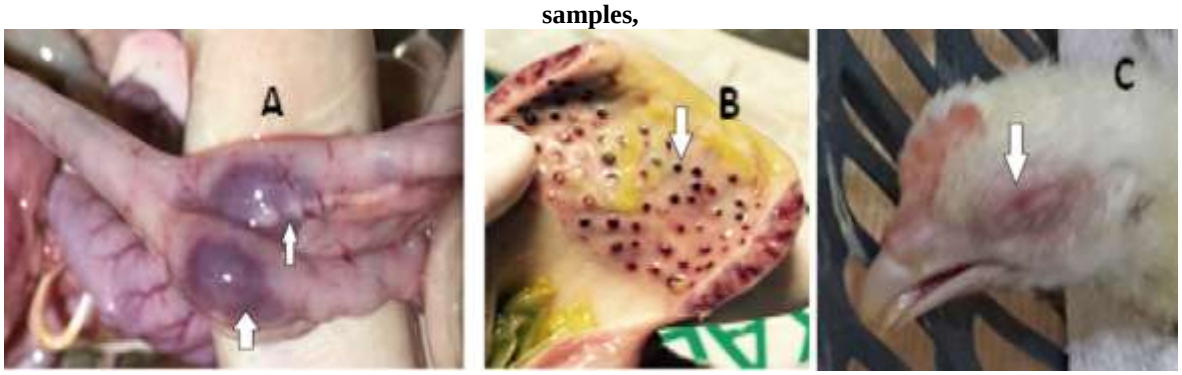


**Figure 2. Clinical signs and gross lesions in chickens suspected to be naturally infected with NDV A) Hemorrhages on cecal tonsils, B) Hemorrhages on proventriculus gland tips, C) Broiler chicken of 28 days infected with NDV showing edematous black eye**

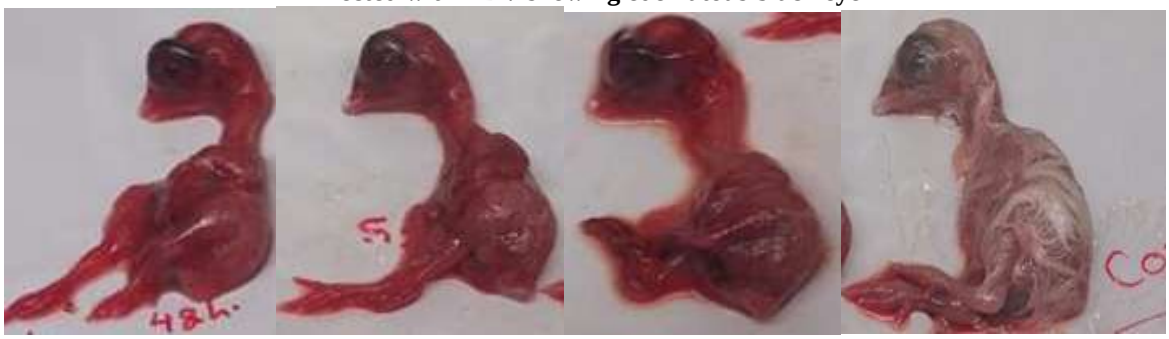

**Figure. 3: Effect of NDv on inoculated ECE. .E and S Chicken embryo (59-60 hours Post inoculation) is diffusely red and the subcutaneous tissue of the head is filled with blood and the blood vessels over the body were prominent. D. Chicken embryo (60-62 hours Post inoculation), showing diffuse congestion and hemorrhage in the whole body and edema in the head region. C.Control non-inoculated NDv chicken**


**Figure. 3. Clinica features of Newcastle disease virus (NDV) in chicks used for ICPI test. A. Paralysis of both legs with lateral recumbency with twisting of the head and neck. B. Depression and off intaking**

Newcastle Disease struck the poultry industry in the North part of Iraq causing severe economic losses. However, many governmental and private poultry farms were established intensively in Iraq in the last two decades. These farms suffered from ND and circulating ND virus, also for our knowledge no recent studies about the NDVs in the northern part in compare to some studies about NDV in the central and southern part. All commercial chicken farms, the common vaccination schedule against ND used in the area of study was live LaSota virus given in different routes at 5 -8 days of age, booster dose at 18-19 days of age and repeated at 28 day of age. Inactivated vaccine had been used in some flocks at 3-5days of age. So, this study was carried out to isolation and pathotyping of the field virus among chickens and characterizes the isolates according to its virulence in this area. The most commonly observed post mortem lesions were marked hemorrhagic ulcers in the intestinal wall and cecal tonsils(Figure 2 A), pin point hemorrhages at the tip of proventriculus glands (Figur 2 B), hemorrhagic lungs, tracheitis with congestion and catarrhal exudates(Figure 2 C, Table (1). These findings agree with the findings (27). Confirmation of ND virus via real time RT-PCR of samples was positive for NDV showing Figure (1) agreed with finding (32) The rapid HA positive samples were titrated using micro HA test; the titers ranged from 1:32 - 1:512 (Table 2A).Nine positive AF samples, were inhibited by anti-NDV hyper-immune serum using HI test except three which indicated that include other than NDV (Table 2A). The HI titers of the positive viruses were from 1:16 - 1:256 (Table 2). Partialy agreed with finding (21). Newcastle disease is recognized as one of the foremost threats and causes serious economic losses in the poultry industry despite the intensive vaccination regimes applied against this disease. In Iraq, ND is still recognized to be endemic in many parts. Conventional diagnosis of ND via virus isolation on embryonated eggs followed by serological identification in hemagglutination inhibition test is laborious and time consuming. The speed of the diagnosis has been increased by using methods based on molecular biology used as Reverse Transcriptase- PCR (2,14,19). Reverse Transcriptase- PCR for the detection of NDV was first described by Jestin & Jestin in 1991 (25) and to date it has been successfully developed in different modifications (2). using universal primers to detect all NDVs (14). Śmietanka and co warkers (2006) have applied RT-PCR method for the detection and identification of NDV in allantoic fluids of infected embryonated eggs.(31) In this study, real time RT-PCR method was applied for the detection and identification of NDV in pooled NDv samples in three provinces at northern part of Iraq Erbil, Sulaymaniyah and Duhok. Positive samples inoculated in to 9 day old embryonated egg, alantoic fluid gave positive reaction in HA test. The hemagglutination activity was inhibited by NDV hyper immune serum using HI assay to confirmed as applied by (5) and this result agreed with finding of Spackman et al., showing real time RT PCR correlated with virus isolation and much faster but cannot detect nucleic acid in all

samples.(32). The isolation and pathotyping of NDVs from the field of chickens is critical for the control of ND and vaccination evaluation because the commercial chickens were vaccinated, almost all flocks were accompanied by high mortality (25-50%), (36). Clinical and PM findings of ND (Table1). In addition the isolation of NDV from the organ samples collected from different localities was confirmed by HA and HI tests and real time PCR (Table2A). It was determine clinical sing and gross lesion for this field NDV in area. The virulence of NDV was measured by MDT, which varied from 59.2-64.25 hrs showing virulence of field NDV according to OIE international standards the definitive in vivo assessment of virus in addition to the ICPI test, which is regarded as the most sensitive and widely used test for measuring virulence (13,25). The ICPI show five of the identified isolates the value was more than 1.5(>1.5) revealed the virulence of NDv to chickens producing characteristic clinical and PM features of this virus in infected chicks (Fig. 3 ,Table3) and the higher ICPI score was probably due to a higher susceptibility of day old chicks to NDV infection (7) 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 that do not protect vaccinates from infection along with viral shedding. Also, parental immunity contributed to vaccination inefficiency in young chicks (20). In conclusion, our results indicated that the NDV isolates among vaccinated chickens were virulent and associated with endemic ND in poultry farms in northern Iraq in spite of extensive vaccination program. The NDV caused severe economic losses in most of these farms due to vaccination failure, inefficient vaccination or virus genetic drift should be considered to draft the efficiency of the commercial available vaccines against these isolates and multiple sources of vaccine. Finally Continuous checking of the flocks' immunological status should be carried out after each vaccination to evaluate the antibody response to administrated vaccines with proper biosecurity practice.

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