Propagation of oncolytic Newcastle Disease Virus in Embryonated Chicken Eggs and its Research Applications in Cell lines

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Abstract

Newcastle Disease Virus (NDV) can modulate cancer cell signaling pathways and induce apoptosis in cancer cells. The laboratory-based studies of the oncolytic NDV requires a reliable protocol for the propagation of the oncolytic NDV. A comprehensive protocol is provided for virus propagation in fertile chicken eggs, which consistently yields high titer viral stock. Aim: Propagation of oncolytic NDV AMHA1 attenuated strain in Embryonated Chicken Eggs (ECE) and tissue culture infective dose 50% ($TCID_{50}$) determination protocol of the virus. Method: Specific pathogen-free fertilized chicken eggs were incubated at 37 °C and 55-60% humidity for 9 – 10 days. Over this period, embryo death was monitored using an egg candle regularly. Virus inoculation is carried out by injection of the diluted virus stock into the allantoic cavity using a needle. embryo death was recorded every two hours and the egg rushed to the refrigerator and fluids collected after four to six hours. Hemagglutination assay (HA) was used to determine the preliminary titer of the virus to collect the high titer egg fluids only which is about 128 to 256HAU. The Vero cell line was exposed to NDV at tenfold serial dilutions to determine $TCID_{50}$ of the virus. The number of viruses in 1 ml of allantoic fluid was measured of embryonated chicken eggs. Results: NDV Iraqi virulent strain has the ability to kill all the chicken embryos through (24-72) h of inoculation. A high titer of NDV was achieved from the infected eggs. Conclusion: Oncolytic NDV propagated in embryonated chicken eggs in high titers as indicated by $TCID_{50}$ value.

Keywords: Vero cell line, TCID50, Hemagglutination, cell culture

1. Introduction

Cancer incidence increased due to several factors, mainly environmental pollution (1). Cancer therapy aims at killing cancer cells while minimizing harm on normal cells, which is the selectivity and safety features (2). Newcastle disease virus (NDV), which has a non-segmented, single-stranded, negative-sense RNA genome, is a member of the genus avulavirus, in the family of paramyxovirus. NDV strains are classified into three major pathotypes: lentogenic, mesogenic, and velogenic, based on their pathogenicity in chickens. NDV can be classified into two classes based on genome length and the sequence of the F gene; Class I viruses are less genetically diverse, and Class II can be further divided into nine genotypes (3). It causes highly infectious diseases in birds of various species worldwide and induces significant

economic loss (4). NDV previously proved to have promising antitumor activity with great safety in lab animals (5). Apoptosis is characterized by a number of characteristic morphological changes in the structure of the cell, together with a number of enzymes- dependent biochemical processes. Failure of apoptosis and the accumulation of damaged cells in the body can result in various forms of cancer. An understanding of the pathways is, therefore, important in developing efficient therapeutics (6). Oncolytic virotherapy divided into two main groups, oncolytic wild naturally occurring viruses with preferential replication in human cancer cells and gene-modified viruses engineered to achieve selective oncolysis (7). Oncolytic NDV attenuated AMHA1 is a naturally oncolytic NDV strain that possesses broad-spectrum antitumor activity (8). NDV selectively replicates in human cancerous cells, and it interferes with several pathways in cancerous cells such as glycolysis (9). The replication of NDV has been tested on various human cancers (10). When NDV infects a cancer cell, it replicates speedily and infects neighboring tumor cells through the discharge of progeny virions, which are noticeable 3 hrs post- inoculation, and plaques develop within two days post-inoculation (11). Scientists are interested in NDV because it can replicate more quickly in human tumor cells than in normal cells and has oncolytic ects (12). Newcastle disease virus (NDV) is an oncolytic virus that may interact with radiotherapy to enhance treatment against cancer cells (13). It is found to enhance different chemotherapeutic agents such as doxorubicin and rituximab (14). The current work aimed to report the proper protocols that help to propagate the oncolytic NDV for preclinical studies.

2. Materials and Methods

2.1 Preparation of MEM (Minimum Essential Medium)

It was prepared by dissolving 11g of MEM (with L-glutamine) (US biological, USA) powder in ~600 ml of TDW and adding sodium bicarbonate powder (2.2 g), 10 ml of 1 M HEPES buffer, 0.5 ml Ampicillin 100 µg/ml, 1 ml Streptomycin 50 µg/ml, and Fetal Bovine Serum (FBS) 100 ml (10%). The volume was completed to 1 L with TDW, and the medium was sterilized using Nalgene 0.22 µm filter unit.

2.2 Preparation of RPMI-1640 medium powder (with HEPES buffer and L-glutamine)

RPMI-1640 medium powder (10.4 g) was dissolved in ~600 ml of TDW (US biological, USA). Sodium bicarbonate powder 2.2 g, 1 ml/L Ampicillin 100µg/ml, 0.5 ml/L Streptomycin 505 µg/ml, and FBS 100 ml. (10%) were added. The volume was completed to 1 L with TDW, and the medium was sterilized using a 0.22µm Nalgene filter unit as recommended by the manufacturing company.

2.3 Newcastle Disease Virus

The attenuated Iraqi AMHA1 strain of NDV was supplied by Experimental Therapy Department, Cell Bank Unit, Iraqi Center for Cancer and Medical Genetic Research (ICCMGR), Mustansiriyah University. A stock of infectious virus was propagated in embryonated chicken eggs (180 eggs) supplied by Al-

Khaleel hatchery for embryonated chicken eggs production (Baghdad, Iraq). NDV is quantified by a hemagglutination test (HA) in which one hemagglutination unit (HAU) is defined as the smallest virus concentration leading to visible chicken erythrocyte agglutination. The virus was stored at −86°C. Viral titer was determined by 50% tissue culture infective dose (TCID50) titration on monkey kidney (Vero) cells according to standard.

2.3.1 Virus stock preparation

NDV Iraqi strain was provided as a frozen allantoic solution. It was directly thawed, and the sample was centrifuged at 3000 rpm for 30 min at 4oC to remove any debris. 10µl the antibiotics Penicillin 100 unit/ml, and Streptomycin 100 µg/ml were added to the virus sample. The virus was diluted by the ratio (1:10) with sterile and cooled PBS (Phosphate buffer saline). The virus sample is ready now for injection into 9 or 10 days embryonated chicken eggs (15).

2.3.2 Propagation of NDV in Embryonated Chicken Eggs (ECE)

Embryonated Chicken Eggs of 9-days-old were incubated in a special incubator at 37^{o}C and on suitable humidity. The eggs were turned twice daily, and after one day of incubation, the eggs were transilluminated with an egg-candling lamp to ensure the viability of the embryo. All non-fertile eggs and those containing a dead embryo were discarded, and active embryos were proceeded for incubation. For the second candling before inoculation, the air sac was marked off, and a suitable site of injection was marked on the eggshell. Where on important blood vessels were running away from the embryo, the eggshell was sterilized with iodine at the inoculation site to sterilize the outer surface, where a small hole was drilled about 0.4 cm above the air sac border (16). Figure (1)

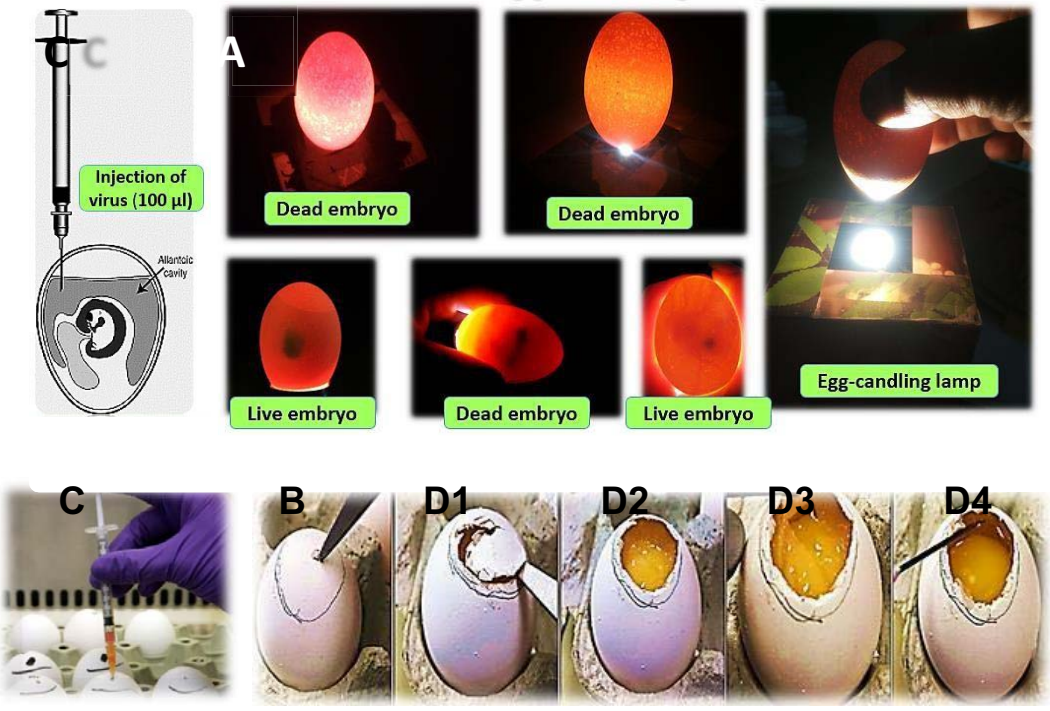


Figure (1) (A)- The eggs were illuminated with an egg-candling lamp to insure the viability of the embryo. (B)-The air sac was marked off and a suitable site of injection was marked on the eggshell, small hole was drilled above the air sac border. (C)- 100 µl of prepared virus solution was injected into each ECE with a fine needle. (D 1-4) The egg shell was cut by scissors to harvest virus.

100 µl of prepared virus solution was injected into each embryonated chicken egg with a fine needle, and the puncture holes were covered with paraffin. The eggs were incubated at 37^{o}C in a humidified incubator for further 24 hrs. Up to 24-hr dead embryos were discarded. The embryos were observed daily every 2-3 hours for viability. Any loss of viability, the egg will immediately be transferred into the refrigerator (4^{o}C) and death time was recorded. Live embryos were returned to the incubator.

The collected eggs were harvested after 3-6h cooling in the refrigerator at 4^{o}C., the eggshell was cut by scissors, and the allantoic fluid was harvested using sterile syringes, (unclear or bloody allantoic fluids were rejected) purified from debris by cold centrifugation at 3000 rpm for 30 min at 4°C, filtered by syringe filter (0.45µm), and distributed in Eppendorf tubes. It is measured by HA immediately (figure (2, 3)), before pooling and filtration where later $TCID_{50}$ of NDV will be calculated (figure (12)), and it was then stored at -86°C (15 and 17).

2.4 Chicken Red Blood Cells (CRBC)

Chicken RBC were collected from brachial veins under the right-wing of chicken by using a heparinized syringe to draw about 2 ml of blood, which was then used in carrying out a hemagglutination test (HA) to determine the titer of the virus.

2.4.1 Preparation of chicken RBC solution

The chicken RBC solution was prepared by collecting blood from a healthy bird into a heparinized tube. Chicken blood (2ml) was added into an anticoagulant test tube (heparinized tube) and then transferred into a centrifuge test tube where 7 ml cold PBS was added and mixed using a centrifuge (1000 rpm, 4^{o}C, and 10 min) to wash the blood, washing it three times with PBS. The supernatant was discarded, the pellet (RBC) was collected, and then taking 0.1 ml of the washed RBCs, which was completed to 10 ml with PBS to prepare 1% RBC solution for the Hemagglutination test (18).

2.5 Hemagglutination assay (HA)

NDV was quantified using the hemagglutination assay. PBS (50 µl) was added to all wells in a 96-well microplate u-shape (7 lines for 7 eggs from A to G and H was the control). Virus allantoic fluids (50 µl) was taken from each tube and added to the tissue culture plate (U-shape), 2-fold serial dilutions were done, (The micropipette tips were replaced between each dilution). RBC solution (50 µl) (1%) was added to all wells in the 96-well microplate u-shape (8 lines) and mixed by agitating the plate gently. The 96-well microplate was incubated at room temperature for 20 minutes and observed for agglutination network

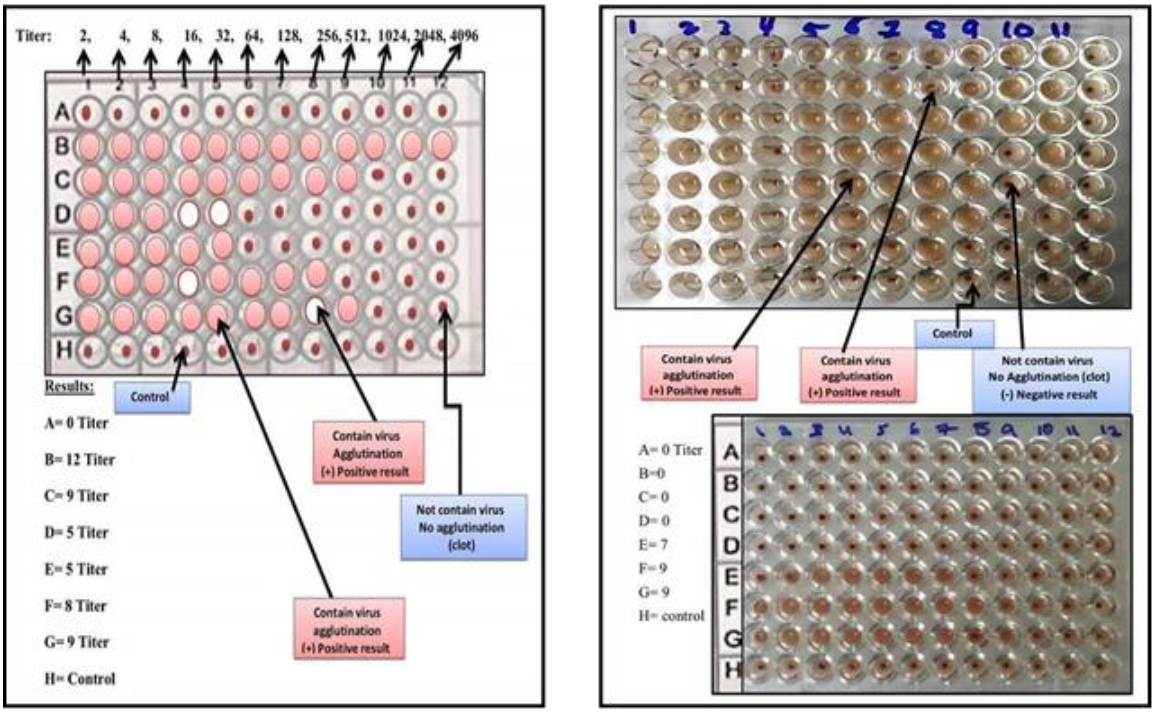


Figure (2): Calculation of titer of NDV by HA test in 96-well microplate

NDV titer was calculated for all virus allantoic fluids according to network formation. The allantoic fluids tubes (Known HAU titer) were centrifuged at 3000rpm/min ^{o}C and collected. allantoic fluids tubes with high titer were pooled and filtrated using Nalgene filters (0.45 μm) and aliquoted to several Eppendorf tubes and were stored at deep freeze (-86 °C). (figure (2, 3, 4)), (19).

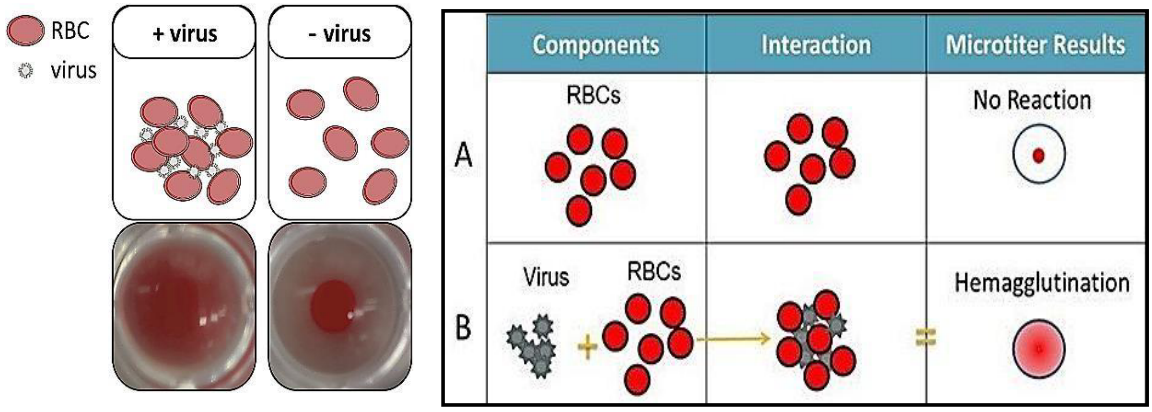


Figure (3): Hemagglutination (the clumping together of red blood cells) by NDV (20)

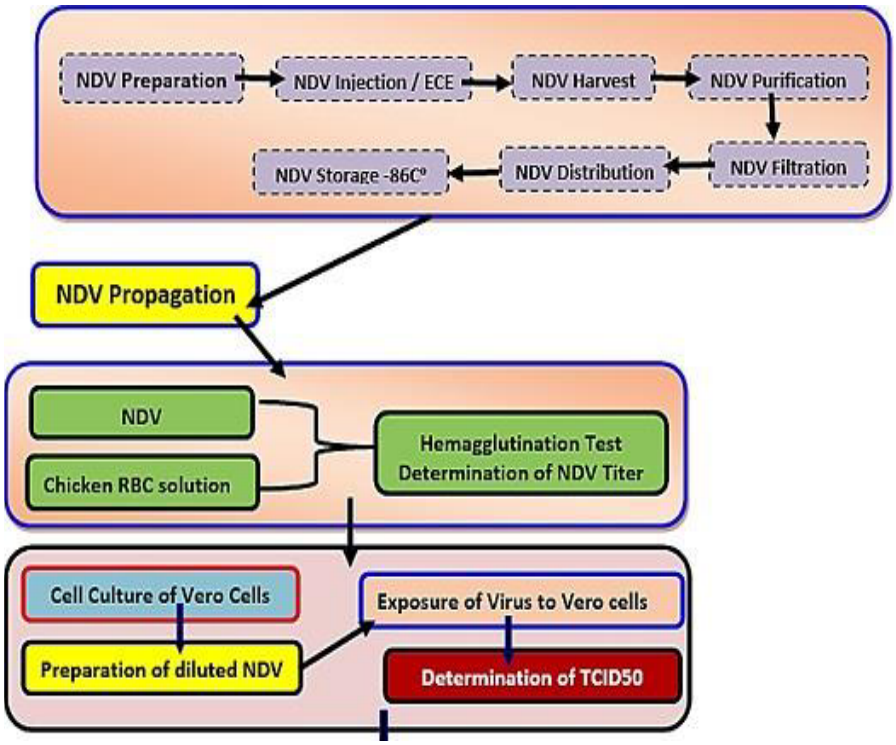


Figure (4) Study Design

2.6 Cell culture of Vero cell line

Vero cells are kidney epithelial cells isolated from an African green monkey (21). These cells are used to calculate virus titer in $TCID_{50}$ assay. Vero cell line maintained using MEM media supplemented with 10% fetal bovine serum (FBS) and antibiotics as described earlier. After the cell became a monolayer (figure (5)), complete media was discarded, and trypsin was used to detach the cells from the

tissue culture flask. Trypsinized cells were incubated for 3 min at 37 ^{o}C. Detached cells should be round-shaped and free-floating in the trypsin solution as soon as they detach (figure (6)). Complete media was added to the trypsinized Vero cells to inhibit trypsin. 10 µl solution was taken from the Vero cells suspension to a hemocytometer to calculate the number of the cells by light microscope (figure-7).

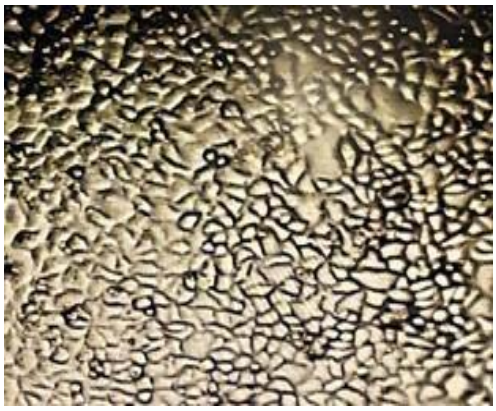


Figure (5): Vero cells confluent monolayer after Storage at -86°C

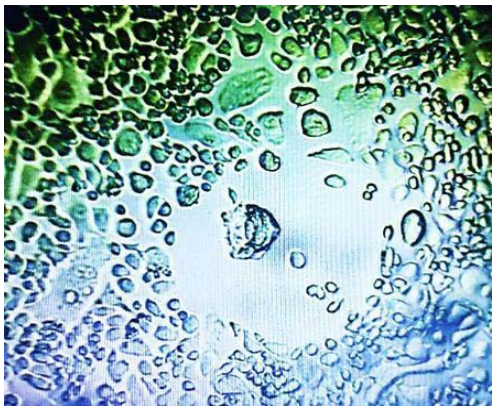


Figure (6): Trypsinization to detach cells from the wall in cell culture

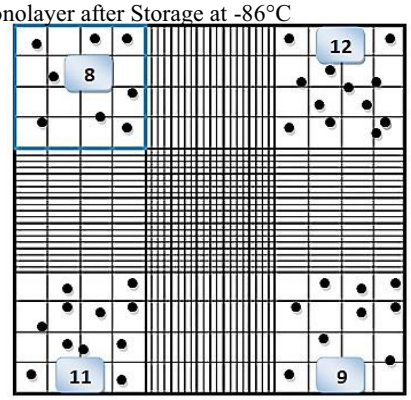


Figure (7): Calculation number of the cells in sample by hemocytometer under light microscope

Figure (8): Vero cell ready for use to calculate $TCID_{50}$

2.6.1 Calculation of the cells number

The number of cells in each square (4 squares) was calculated, and then the average was calculated (10 cells). The average was multiplied by 10^4 to find the number of cells in 1 ml. The cells were examined by an inverted microscope. 10000 cells/well in 50 µl were seeded in the 96 multi well microplate (flat bottom).

2.7 Preparation of serial NDV dilutions

Ten sterile Eppendorf tubes (1.5ml) were labeled and numbered in sequence, SFM was prepared and 900 µl SFM was aliquoted in to all Eppendorf tubes, 100µl stock allantoic virus suspension (known HAU titer =256-512) was added to first tube and mixed well, later 100 µl was taken from first tube and tenfold serial dilutions were prepared, after replacing the tip each time.

2.8 Exposure of NDV to Vero cells line

Vero cell lines were seeded at 10^4 cell/well in 96- well microplate flat bottom, and after 24 hrs or when confluent monolayer was achieved, growth media was discarded, and virus suspension (ten-fold serial dilution) was added, 1/10, $1/10^2$, $1/10^3$, $1/10^4$, $1/10^5$, $1/10^6$, $1/10^7$, $1/10^8$, $1/10^9$, and $1/10^{10}$ were added to columns 1 to 11. The cells were inoculated with 50 µl/well of NDV with 8 replicates for each dilution, an amount of 50 µl SFM only was added to column number 12 in 96- well microplate as a control. The plate was covered with a sterile adhesive paper and incubated for 2 hrs at room temperature to allow virus penetration. Each column represents replicates to each dilution. later, 96- well microplate was incubated at 37°C for 5 days. The titer value of the virus was read under inverted microscope for ($TCID_{50}$) determination.

2.9 Determination of Tissue Culture Infective Dose 50% ($TCID_{50}$) of NDV

$TCID_{50,}$ which means the amount of a pathogenic agent that will cause infection in 50 % of cell cultures inoculated. NDV titer was calculated on Vero cell lines in comparison with the control to determine infection dose of 50% in cell cultures as described by (22), in which the titer was determined as the dilution that causes 50% cytopathic effect in the incubated well. The following formula is used to calculate the $TCID_{50}$.

$$\log_{10} (TCID_{50} /ml) = L + d (S - 0.5) + \log (1/v)$$

L = negative $\log_{10}$ of the most concentrated virus dilution tested, in which all wells are positive (no. of positive columns completely). L = 5

d = $\log_{10}$ of dilution factor. d = log 10= 1 (1:10 dilution)

S= sum of individual proportions (pi)

Pi = calculated proportion of an individual dilution

v = volume of inoculum (ml/well)

Pi = Amount of positive wells / total amount of wells per dilution

V= volume of inoculum (ml/well) = 50 µl /ml = 50 µl / 1000µl = 0.05

3. Results

3.1. Propagation of Newcastle Disease Virus in Embryonated Chicken Eggs

Embryonated chicken eggs (ECE) were used in virus propagation. The infected embryos were characterized with hemorrhage in comparison to control embryos (figure (9)). Results showed that NDV Iraqi virulent strain was able to kill all embryos (9 or 10 days old) through (24-72) h of inoculation. 8 embryonated chicken eggs were killed before 24h.

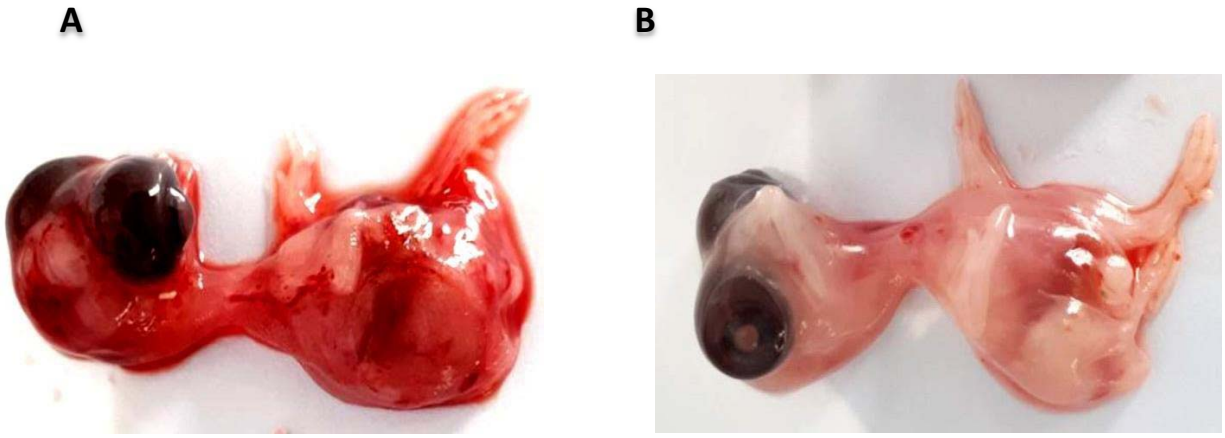
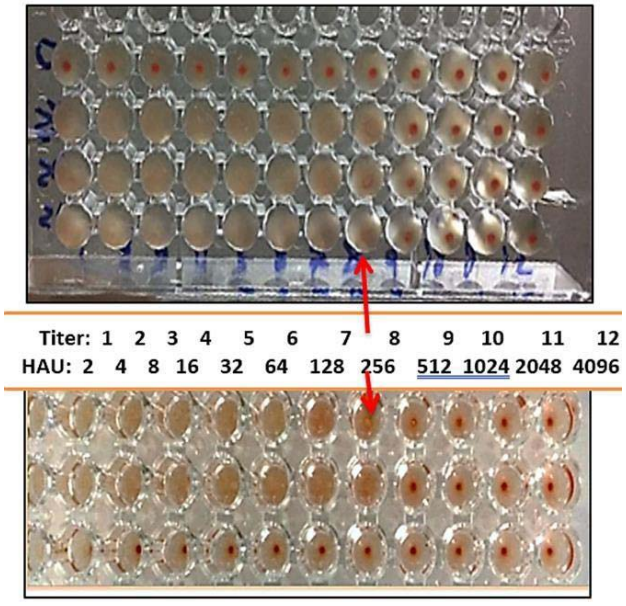


Figure: (9) Inoculation of NDV in Embryonated chicken eggs: (A) Skin hemorrhage in ECE after inoculated with NDV (B) ECE control.

3.2 Hemagglutination Test

Infected chicken embryonated eggs showed marked hemorrhage as compared to the uninfected control embryos. The collected virus was purified, tittered, and quantified by the hemagglutination test. Titer and HAU of NDV were measured (HAU = 256) (figure (10).



Figures (10) Hemagglutination test, show the agglutination (positive result) of RBC forms a distinctive mesh at the bottom (titer 8). The negative hemagglutination is characterized by a sharp red dot in comparison to the control. HAU = 256 was shown

3.3 Tissue Culture Infectious Dose ($TCID_{50}$)

It showed that NDV formed plaques in number of infected wells, while uninfected control cells showed confluent monolayer (figure-11). The NDV $TCID_{50}$ was 124.6 x 10^6 virus/ml (table-1 and 2) (figure-12).

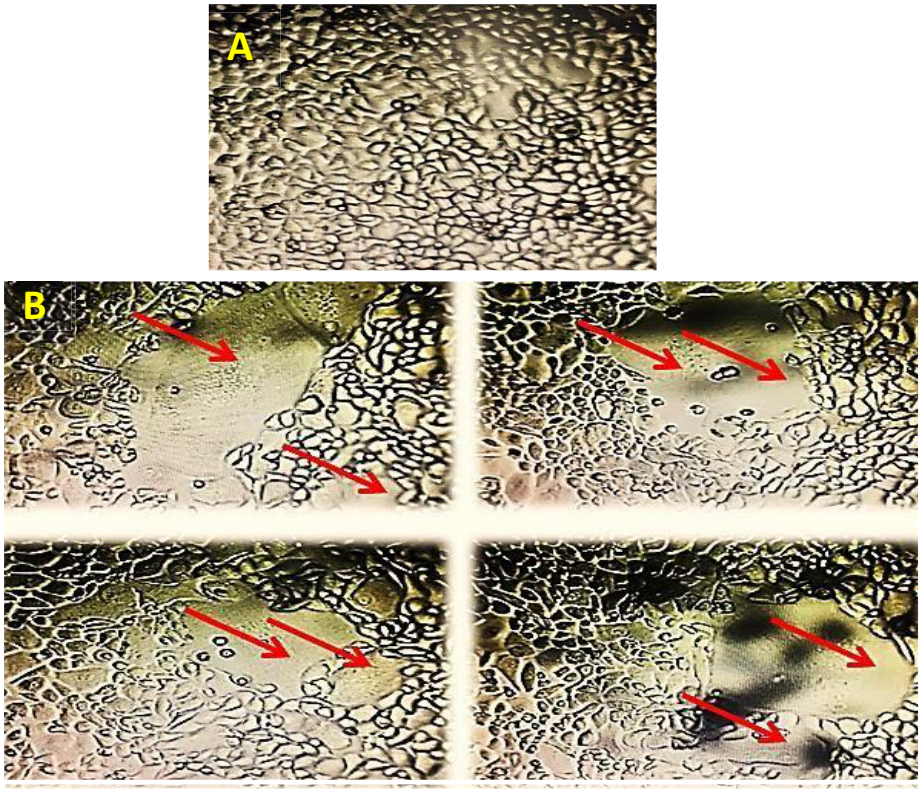


Figure (11) TCID50 assay Vero cells treated with tenfold serial dilutions of NDV: A- Vero cells untreated as .control, B- Vero cells treated with NDV showed plaques cells. (Red arrows refer to plaques)

Table (1) Results of Vero cells were treated with NDV to determine $TCID_{50}$

A			B			C		
log_{10} virus dilution	Ratio of infection	Proportion (pi)	log_{10} virus dilution	Ratio of infection	Proportion (pi)	log_{10} virus dilution	Ratio of infection	Proportion (pi)
-1	8/8	1	-1	8/8	1	-1	8/8	1
-2	8/8	1	-2	8/8	1	-2	8/8	1
-3	8/8	1	-3	8/8	1	-3	8/8	1
-4	8/8	1	-4	8/8	1	-4	8/8	1
-5	8/8	1	-5	8/8	1	-5	8/8	1
-6	6/8	0.750	-6	5/8	0.625	-6	5/8	0.625
-7	3/8	0.375	-7	3/8	0.375	-7	4/8	0.500

-8	**1/8**	**0.125**	**-8**	**1/8**	**0.125**	**-8**	**2/8**	**0.250**
-9	**0/8**	**0.0**	**-9**	**1/8**	**0.125**	**-9**	**0/8**	**0.0**
-10	**0/8**	**0.0**	**-10**	**0/8**	**0.0**	**-10**	**0/8**	**0.0**

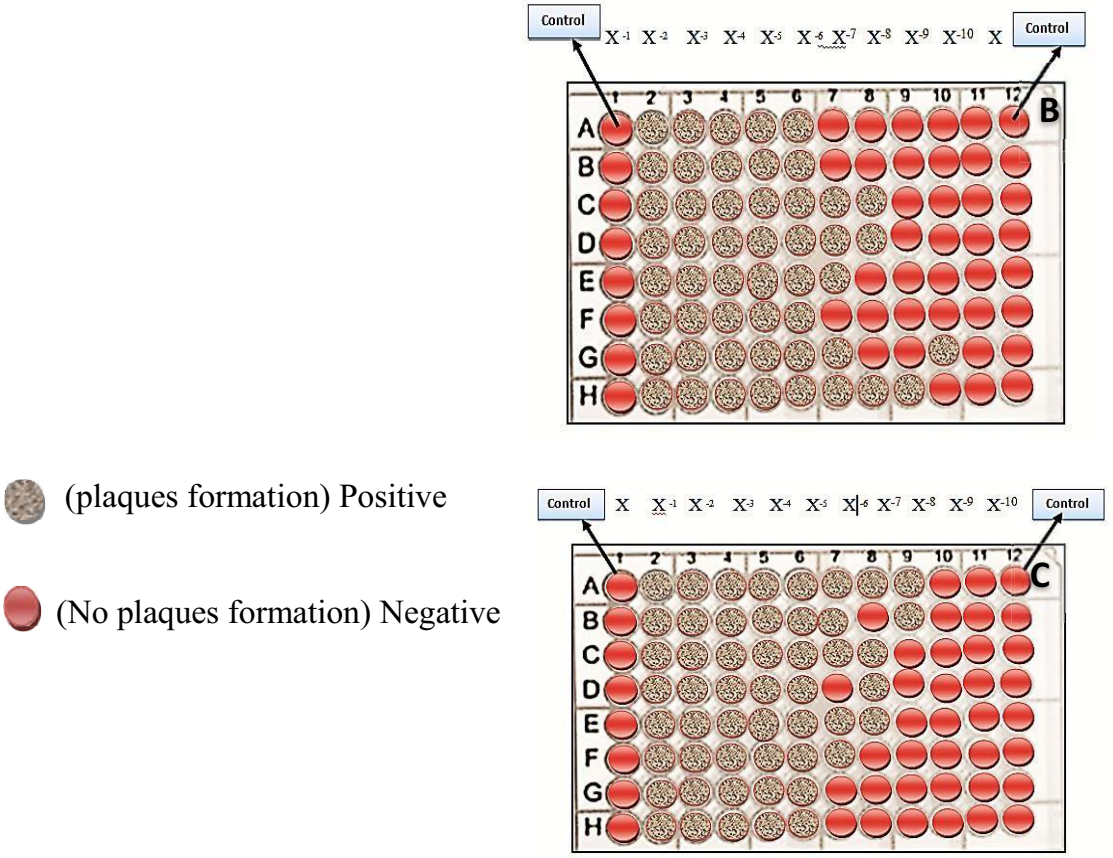


Figure (12) NDV infection to Vero cell line to measure $TCID_{50}$: It was showed plaques formation which considered were treated with the virus showed plaques formation, a positive result represents

plaques formation table (1- A) and figure (12-A), the second experiment table (1- B) and figure (12-B), and the third experiment table (1- C) and figure (12-C). The final values for $TCID_{50}$/ml for exposure of NDV to Vero cell line in 96-well microplate, which represents the number of viruses for each 1ml, are shown in table (2).

Table (2) Final value for TCID50/ml for exposure of NDV to Vero cells

No. of Exp	$TCID_{50}$/ml	Average $TCID_{50}$/ml
1	**1.122×10^8/ml**	**1.246×10^8 /ml**
2	**1.122×10^8/ml**	
3	**1.496×10^8/ ml**	**124.6×10^6/ml**

4. Discussion:

The aim of the current short study is showing the propagation protocols of oncolytic NDV in Embryonated Chicken Eggs and the determination of Tissue Culture Infective Dose 50% of the virus. Explain the method using Vero cell line. Newcastle disease virus Iraqi strain is an interesting oncolytic agent with promising antitumor properties. One of the major antitumor properties is selective replication in transformed cells (19).

The results showed that the attenuated Iraqi attenuated strain of NDV has the ability to kill most chicken embryos within (24-72) h, as described by (23) that NDV strains can be categorized as velogenic (highly virulent), mesogenic (intermediate virulence), or lentogenic (non-virulent). Mesogenic strains in 60–90 hrs and lentogenic strains in more than 90 hrs. The isolated virus showed Syncytia formation, which is a hallmark of NDV infection in the infected Vero cells. It was reported by (3 and 24) that syncytia are a typical cytopathic effect caused by the virus and can lead to tissue necrosis and that it might also be a mechanism of virus spread. Newcastle disease virus was quantified by the hemagglutination assay. Hemagglutination (adhesion of RBC) test showed a positive result as a typical hemagglutination mesh pattern of chicken red blood cell 256 HAU; the virus is able to agglutinate human or animal red blood cells (25). The virus induces syncytia by fusing infected cells with neighboring cells leading to the formation of enlarged multinucleated cells.

This event is induced by the surface expression of a viral fusion protein that is fusogenic directly at the host cell membrane. Syncytia is an ideal cytopathic effect caused by the virus, which leads to tissue necrosis. Usually, these syncytia are the result of the expression of a viral fusion protein at the host cell membrane during viral replication; it is the mark of the mechanism of virus spread (26). NDV Iraqi strain is a broad-spectrum oncolytic agent that can destroy tumor cells demonstrated that NDV Iraqi strain exhibits oncolytic activity to a wide range of tumor cells., this was also confirmed by previously (27).

$TCID_{50}$ is an assay used to determine the titer of most viruses (28). According to (29), $TCID_{50}$ refers to the ability of a certain virus dilution to infect 50% of the cell cultures inoculated. Therefore, a titer is defined as a number of infectious virus units per unit volume. The $TCID_{50}$ titer provides qualitative information on dose or dilution that causes cytopathic effect (CPE) in 50% of the sample, instead of giving the quantitative amount of virus infection that is present in a sample. NDV formed plaques on Vero cell line and normal tissue bird such as chick embryo fibroblast in vitro. It did not form plaques on any normal fibroblast cell line from other sources (30).

The gated of $TCID_{50}$ ($124.6x10^6$) used in the present study ensure the high virulent of NDV. When each infected cell produces a virus and eventually lyses, only the immediately adjacent cells become infected. Each group of infected cells is referred to as a plaque that is surrounded by uninfected cells. After several infection cycles, the infected cells in the center of the plaques begin to lyse, and then the virus moves to the uninfected cells gradually (31). The oncolytic NDV AMHA1 strain was isolated as virulent strain (32)

and after years of passaging in embryonated chicken eggs its attenuated (5).

Conclusion:

Oncolytic NDV propagated in high titer in embryonated chicken eggs, and tittered by HA test, and induce cytopathic effect on Vero cell line through TCID**50** value. NDV has the ability to induce cytopathic effect in Vero cells and can be used as permissive cells for NDV.

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