



## Evaluation of Antiviral Activity of Allium Cepa and Allium Sativum Extracts Against Newcastle Disease Virus

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### ABSTRACT

Newcastle Disease Virus (NDV) is the causative agent of the most devastating diseases affecting poultry especially in large scale production causing severe economic losses due to high mortality which may reach 100% in very virulent strains of NDV. In the present study, NDV-suspected samples were isolated in 10 days-old Embryonated chicken Eggs (ECEs) via allantoic cavity for three passages. Viral isolates were identified by Haemagglutination inhibition (HI) test, electron microscope and reverse transcriptase polymerase chain reaction (RT-PCR). The antiviral effects of both onion and garlic extracts were studied on field isolates and reference strain of NDV. The antiviral effects involve the gross lesions changes of the embryo in inoculated ECEs, Haemagglutination (HA) test and Infectivity test (EID<sub>50</sub>%). The obtained results showed that most of the isolated viruses expressed subcutaneous Haemorrhage and death of the embryo at the fifth day post inoculation via allantoic cavity at the third passage of ECEs, while the field sample number (10) and the reference strain showed extensive haemorrhage and rapid death of embryos. The isolated viruses were identified as NDV of the susceptible to be infected samples by HI using hyperimmune serum against NDV prepared in rabbit. The Mean Death Time (MDT) of the field sample consisting of (10) and the reference strain which showed that the tested samples are velogenic strains was calculated. Electron microscopy investigation of some selected samples (of high HA titers) identified the virus as pleomorphic. Some selected samples were analyzed by RT-PCR and gel electrophoresis to express clear bands by using primers against the F-gene of NDV. Brown leaves of onion (*Allium Cepa*) and bulbs of garlic (*Allium sativum*) were separately extracted. These extracts were tested for their antiviral activity against the previously isolated and identified NDV. Investigating the antiviral effects of both onion and garlic extracts on some field samples showed absence of virus activity after treatments by extracts, while samples number (10) and reference strain showed the reduction of virus in HA test, subcutaneous hemorrhage and EID<sub>50</sub>%. The reduction of NDV infectivity may be due to the blocking of the attachment of the virus with the cell reports.

### 1. INTRODUCTION

The causative agent of Newcastle disease (ND) is Avian Paramyxovirus type-1 (APMV-1) called Newcastle disease viruses (NDV). It is a member of the family Paramyxoviridae of the genus Avulavirus. There are ten serotypes of avian Paramyxoviruses designated from APMV-I to APMV-10. NDV has also been categorized into five pathotypes based on viral virulence in infected chickens; viscerotropic velogenic,

neurotropic velogenic, mesogenic, lentogenic or respiratory and asymptomatic enteric NDV (Alexander, 1998, Alexander, 2003, Huang et al., 2004 and Orsi et al., 2009). One of the major threat viral diseases affecting poultry industry is ND causing great economic losses not only in Egypt but also worldwide. ND affects the respiratory, nervous, digestive systems with partial or complete drop in egg production of

chicken and other birds causing variable mortalities that can be as high as 100%. ND is one of Zoonotic diseases that causes conjunctivitis in humans (Mase et al., 2002, Numan et al., 2005 and Charabarti et al., 2007).

The NDV is an enveloped, pleomorphic generally appears as rounded to a filamentous particle of 100-500nm in diameter; poses a non-segmented, negative-sense, single-stranded RNA which is approximately 15.2 kb. The genome encodes for 6 different proteins, i.e. nucleoprotein (NP), phosphoprotein (P), fusion protein (F), matrix protein (M), hemagglutinin-neuraminidase (HN), and RNA large polymerase (L) protein. The F and HN proteins are the surfaces glycoproteins; the NP, P and M proteins encompass the viral inner surface whereas the L protein constitutes the viral nucleocapsid together with NP and P proteins (OIE, 1988, Hines and Miller, 2012 and Munir, 2012). Although ND can be diagnosed based on symptoms and postmortem findings, the clinical signs and gross lesions cannot accurately be distinguished between ND, Avian Influenza, Infectious bronchitis virus and other related respiratory viral diseases, so the tentative diagnosis of ND requires laboratory confirmation. The nasal discharge, fecal droppings, blood or internal organs (liver, kidneys, spleen, trachea, lungs, heart and intestinal contents) of morbid or freshly dead birds can be collected and processed for isolation of ND virus in the laboratory. Virus isolation and identification is regarded as the confirmatory test for ND (Alexander et al., 1984, Alexander, 2003, Alexander and Senne, 2008 and Cattoli et al., 2011).

The confirmation of ND virus can be done through electron microscope, Reverse- Transcriptase Polymerase chain reaction (RT-PCR), ELISA, etc. The pathogenicity of ND virus can be checked through intra-cerebral pathogenicity index (ICPI), intra-venous pathogenicity index (IVPI) or mean death time (MDT) (Alexander et al., 1984).

Despite routinely easy-used vaccination regimes for controlling of ND, many economic losses are still coming by ND. Trials are attempted to recognize novel materials of antiviral activities from natural or synthetic resources. Natural products are one of the relevant sources for antiviral substances. Therefore, many screening efforts have been made to identify antiviral substances from natural sources with high efficacy, low toxicity and minor side effects.

Onion (*Allium Cepa*) and Garlic (*Allium Sativum*) are widely distributed plants are used in all parts of the world as spices food as well as popular remedies. Both

plants are characterized by a strong sulphurous odour. The sulphur compounds in onion and garlic have potential antibiotic and flavour properties (Stoll and Seebeck, 1947). Onion (*Allium Cepa*) is a bulbous plant widely that is cultivated in every country of the world. Onion bulbs possess numerous organic sulphur compounds including Trans-S-(1-propenyl) cysteine sulfoxide, S-methylcysteine sulfoxide, S-propylcysteine sulfoxides and cycloallicin, flavinoids, phenolic acids, sterols including cholesterol, stigma sterol, b-sitosterol, saponins, sugars and a trace of volatile oil compounds mainly of sulphur compounds. Most of the plant parts contain compounds with proven antibacterial, antiviral, antiparasitic, antifungal properties and have antihypertensive, hypoglycemic, antithrombotic, antihyperlipidemic, anti inflammatory and antioxidant activity (Goodarzi et al., 2013). The onion extract could experimentally stop the hemagglutination activity of Newcastle disease virus for Hitchner-B<sub>1</sub> and Lasota strains (Abo-Zeid, 1989).

The garlic (*Allium sativum*) seems to have antiviral activity, but the responsible substances have not been distinguished. Using direct pre-infection incubation assays help in determining the *in-vitro* virucidal impacts of fresh garlic extracts in which thiosulfinates were the active components, and virucidal to each virus tested. Allicin contains the most of thiosulfinate in fresh garlic extract which acts propably against non-enveloped virus by inhibition of its adsorption or penetration (Weber et al., 1992).

The oily extracts of onion and garlic plants have virucidal action on different strains of Newcastle disease virus through decreasing the EID<sub>50</sub> (Embryo Infective Dose fifty) titer when compared with control (non treated viruses). Field application of both extracts on naturally infected birds comparing with untreated groups showed that two groups treated with onion and garlic manifested gain in high weight and less mortality percentages (Nadia et al., 1991).

The safety doses of plant extracts were mixed with lasota vaccinal strain of NDV to determine the antiviral effect of each mixture on NDV. The best extract that inhibit CPE of virus was found in onion extract followed by garlic extract against virus (sabra, 2004).

The aims of the present study are: isolation of NDV from pooled organs (trachea, lung, liver, spleen and intestine) that are collected from clinically NDV - suspected chickens via allantoic route inoculation of embryonated chicken eggs (ECEs) in 10-days old. And, identification of the isolated NDV in collected field

samples by Haemagglutination inhibition test using hyperimmune serum prepared in rabbits. In addition, electron microscopy examination and molecular confirmation of selected samples by reverse transcriptase polymerase chain reaction (RT-PCR). As well, studying the antiviral effects of onion and garlic extracts on field isolates and reference strain of NDV using HA for counting viral particles and EID<sub>50</sub> for detecting viral infectivity.

## **2 MATERIALS AND METHODS**

### **2.1 Clinical specimens' collection and preparation:**

Collected organs (trachea- lung- liver- intestine-proventriculus and spleen) from 6 clinically diseased chickens in 26 broiler farms in Dakahlia governorate were pooled for each farm representing as one sample. Birds were showing high mortality rate, sever hemorrhages in trachea, lung, spleen, cecal tonsils and petechial hemorrhages in proventriculus. One of the apparently healthy chicken was euthanized where (trachea-lung-liver-intestine-spleen- proventriculus) were collected and considered as a negative control. Samples were collected from commercial broiler farms located in Dakahlia provinces in Egypt, from November 2014 to May 2016. Chicks at the age of 28-42 days were suffering from moderate to severe respiratory signs accompanied with lesions including haemorrhages in the trachea, intestine and proventriculus glands. Collected pooled samples (26) were homogenized by tissue homogenizer and using sterile PBS containing [Penicillin G sodium (100,000 IU), Streptomycin (1gm), kanamycin (1gm), Mycostatin (250-500 IU)] and centrifuged at 2000rpm in 10 minutes. Collected supernatants were kept on -20°C until use for virus isolation.

### **2.2 Vaccinal and reference viral strains:**

Standard NDV "La sota" vaccine was kindly provided by Veterinary Serum and Vaccine Research Institute, Abbasia (VSVRI), Cairo. It is a live attenuated virus vaccine. Each vial is used for vaccination of 1000 bird. NDV La Sota strain was supplied from central veterinary laboratory weybridge. In addition, the recently isolated velogenic Newcastle disease virus reference strain (NDV-B7-RLQP-CH-EG-12), England (genotype VII D) is obtained from Veterinary Serum and Vaccine Research Institute, Abbasia, Cairo. National laboratory for veterinary control on Poultry Production Animal health Research institute. Its Haemagglutination titer is 2<sup>9</sup>.

### **2.3 Preparation of hyperimmune serum (HIS) against NDV:**

Hyperimmune serum against NDV was performed according to Samiullah et al., (2006), in which five rabbits were used; four of them were firstly inoculated by La Sota strain with complete Freund's adjuvant and the other one was kept as a control negative. Booster does was prepared by mixing equal quantity of incomplete Freund's adjuvant (Sigma) to the antigen. The inoculum was given subcutaneously 0.2 mL per rabbit for more three booster doses. Collected serum after the day (48) post inoculation was inactivated by 56°C for ½ hour.

### **2.4 Isolation of NDV from suspected to be infected samples:**

#### **2.4.1 Preparation of the collected samples expected to be infected by NDV:**

According to Terregino and Capua (2009) the portion of each collected ND suspected field samples (proventriculus, lung, trachea, intestine, spleen and liver tissues) were stored at -80°C until processed for inoculating in ECEs. Samples preparation was carried out by sterile forceps and scissors; small pieces of all tissues corresponding to 2 gm were collected and homogenized as a pool in a mortar then collected in a 15ml Falcon tube. The suspension was clarified by centrifuging at 2000 rpm for 10 min at room temperature (RT). Samples' supernatants were collected for cultivation in ECEs.

#### **2.4.2 Cultivation of ND suspected prepared samples in ECEs:**

It was performed according to Capua and Alexander (2009), 0.2 ml of the supernatant mixed with antibacterial and antifungal agents and incubated at 4°C then inoculated in triple 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 24h post inoculation were considered non-specific death. All the embryos that died after 24h or survived till the end of incubation period were chilled in refrigerator (4°C) overnight. The allantoic fluid (AF) was harvested, divided into aliquots and stored in sterile screw-capped vials at -20°C. Three egg passages were performed. The allantoic fluids of the third passage were collected and stored at -20°C till used in antiviral study of onion and garlic extracts against NDV.

### **2.5 Heamagglutination and heamagglutination inhibition tests for isolated virus:**

According to Capua and Alexander (2009), two-fold serial dilutions of the harvested allantoic fluids were prepared by using phosphate buffer saline (PBS) in V-

bottomed microtitre plastic plate and 1% freshly prepared chicken RBCs to each well. For positive HA activity, there was a fine layer of RBCs lining the entire bottom of the well (lattice shape). RBCs will settle at the center of the well in the shape of a button it is negative result (red-button shape). HA unit was calculated for each sample as the highest viral dilution caused complete HA by 1% chicken RBCs. Four HA units for each sample were used for HI test according to OIE (2004), in which suspected allantoic fluid of each sample was diluted to two-fold in a microtiter plate followed by addition of inactivated hyperimmune serum prepared in rabbits and 1% chicken RBCs for monitoring viral HA ability confirming that suspected virus is NDV.

#### **2.6 Main death time test for NDV isolate and reference strain.**

According to OIE (1988), in which a ten-fold serial dilution of fresh infective allantoic fluid in sterile saline for each dilution, 0.1mL were inoculated into the allantoic cavity of five ECEs (10 day-old) per each dilution and incubated at 37°C. Each egg is examined twice daily for 7 days and the times of any embryo deaths are recorded. The minimum lethal dose is the highest virus dilution that causes all the embryos inoculated with that dilution to die and MDT is the meantime in hours for the minimum lethal dose to kill those embryos. The MDT has been used to characterize the NDV pathotypes as follows: velogenic, less than 60 h; mesogenic, 60 to 90 h; and lentogenic, more than 90 h.

#### **2.7 Characterizing the morphology of identified NDV by negative staining of electron microscopy method:**

To study the morphological characteristics of the field isolates, three allantoic fluids of isolates were selected and placed for 3 minutes on a copper - carbon coated grid (400 mesh) which previously was washed with 0.01 M potassium phosphate buffer. The grid was washed 3 times with double distilled water and was dried with Whitman tissue paper followed by staining with 2% aqueous uranylacetate (w/v) according to Morales et al. (1990). The grid was examined with transmission electron microscope (JEOLJEM-2100, Japan) in the Electron Microscope Unit, Faculty of Agriculture, Mansoura University, Mansoura, Egypt.

#### **2.8 Molecular identification of field Newcastle Disease Virus by RT-PCR:**

The viral genomes from allantoic fluids of the third passage of four- selected ECE samples and the reference strain were extracted by using the QIAamp viral RNA mini kit (QIAGEN, Hilden, Germany) according to the manufacture's protocol. Primers for amplification of F gene of NDV were used according to Nanthakumar et al., (2000), Forward primer (NDVF) is 5'-GCAGCTGCAGGGATTGTGGT-3' (nucleotide position 158-177) and reverse (NDVR) 5'-TCTTTGAGCAGGAGGATGTTG-3' (nucleotide position 493-513) with correct size (356bp). Briefly, 25 µl of master mix were added to 1 µl of forward primer, 1 µl of reverse primer, 5 µl of DNA (cDNA of NDV) and 18 µl of Nuclease-free water making volume 50 µl. The used thermal conditions were: initial cycle of 94°C for 2 min. followed by 40 cycles of 94°C for 15 sec., 48°C for 30 sec., 72°C for 30 sec., and a final elongation step of 72°C for 7 min. 5µl of each PCR products were separated in 1.5% agarose gel in TAE buffer stained with 3µl ethidium bromide and loaded by 1µl loading dye of the individual wells of agarose gel. In addition, 2 µl of molecular mass marker (100 bp DNA marker) add to it 2 µl loading dye in signal well to be used as DNA ladder and compared the PCR product with DNA ladder and visualized by ultraviolet (UV) transillumination. The gel was photographed.

#### **2.9 Plant extraction and safe doses determination:**

Red onion extract was prepared according to Abozeid (1989) in which; oven-dried brown leaves were ground in an electric mill to obtain a fine plant powder, 100 gm of plant powder were mixed with 500ml ethyl alcohol (70%). The mixture was kept for about 8-10 hours at about 30°C for extraction. Supernatants were collected and filtered by cheesecloth and whatman filter paper No.1. The filtrate was concentrated by exposure to air current of a fan and by using an electric oven at 50°C. The oven dried extract was then dissolved in 10ml PBS, then tested for sterility and kept at refrigerator till use.

The safe does of onion extracts was determined for ECE using a ten-fold serial dilution from onion extract. Each dilution was inoculated into four ECEs. The lowest dilution of the onion extract at which all inoculated ECEs remained alive was determined and considered as the safe dose of the concerned plant extract.

Garlic extract was prepared according to **Ismail et al., (1989) and Mohammed and Aattar (2001)**, as follows: Aqueous Garlic extract was prepared by grinding the bulbs of garlic in blender using sterilized distilled water (1:1 w/v), the bulbs were pressed through two layers of cheesecloth, and then the fluid extract was centrifuged at 1000 rpm for 30 min. The supernatant was collected and stored at -20°C. Egg embryo safe dose of garlic extracts was accessed as that of onion extract.

### **2.10 The Antiviral effect of onion and garlic extracts on field isolates and reference NDV.**

Studying the antiviral effect of both onion and garlic extracts with different dilutions of virus were performed by mixing one ml of the safe dose from each plant extract to equal volume of different dilutions of used virus from  $10^{-6}$  to  $10^{-9}$ . These mixtures were left for about one hour at room temperature before inoculation. One ml from each mixture was inoculated into five ECEs. The inoculated eggs were incubated at 37°C for 5 days, daily examined, and the embryo index and Subcutaneous (s/c) hemorrhage was carried out according to Shojai et al., (2016). Also virus residual was investigated in the collected allantoic fluids by HA according to Abo-Zeid, A. (1989) and Mohammed and Al-Attar (2001) and infectivity determination by EID<sub>50</sub> according to Smit and Rondhuis (1976), Young et. al., (2002) and Ibraheem et. al., (2012).

## **3 RESULTS**

### **3.1 Isolation of the field collected samples inoculated in 10 days old ECEs via Allantoic cavity:**

The inoculated chicken embryos showed diffuse congestion, hemorrhage in head and toes and over the whole the body, filled subcutaneous tissue with blood and prominent blood vessels over the body by 26 inoculated samples especially sample number (10, 19, 21 and the reference strain) **Figure (1)**.

### **3.2 Haemagglutination activity of the isolated samples in ECE for three serial passages:**

After three serial passages of the collected allantoic fluids in ECEs, the collected allantoic fluids of the first passage showed positive (+ve) reactions for 20 samples from the tested 26 samples, after the 2<sup>nd</sup> passage, the +ve HA samples increased to be 24 of the tested 26 samples. The samples number 10 and 25 showed high HA titers so no further passages were done for these two samples. The 3<sup>rd</sup> passage of inoculated samples showed higher titers by HA test for almost all tested samples. Twenty-five from the tested 26 collected allantoic fluids were positive by HA test. **Table (1)** shows nine samples of high HA titers ranging from  $\log 2^{-6}$  to  $\log 2^{-10}$ , ten samples of moderate HA titers ranging from  $\log 2^{-4}$  to  $\log 2^{-5}$  and six samples of low HA titer ranging from  $\log 2^{-1}$  to  $\log 2^{-3}$ . Reference strain was tested in all passages as a control positive sample.

### **3.3 Haemagglutination inhibition Test (HI) for the positive HA isolates.**

Twenty-two samples from the twenty-five +ve HA samples and the reference strain had positive results by HI test. 9 samples showed high HI titer ranging from  $\log 2^{-6}$  to  $\log 2^{-10}$ , 6 samples gave moderate HI titer which ranged from  $\log 2^{-4}$  to  $\log 2^{-5}$  and 7 samples showed low HI titer ranging from  $\log 2^{-1}$  to  $\log 2^{-3}$  and 3 samples Showed negative (-ve) result. Reference strain was used as a control positive sample.

### **3.4 MDT for NDV isolate and reference strain.**

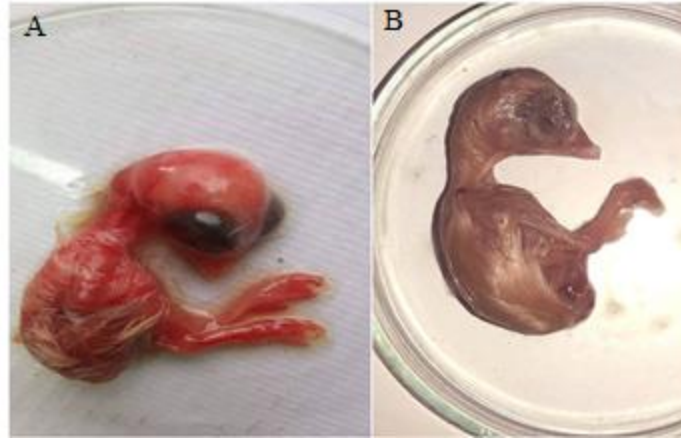
Isolate number (10) and reference strain were analyzed for MDT for detecting their pathogenicity. About 58 hours post inoculations were needed for the death of all inoculated ECEs at the dilution  $10^{-6}$ .

### **3.5 Morphological characterization of the isolated NDV by Electron Microscopy**

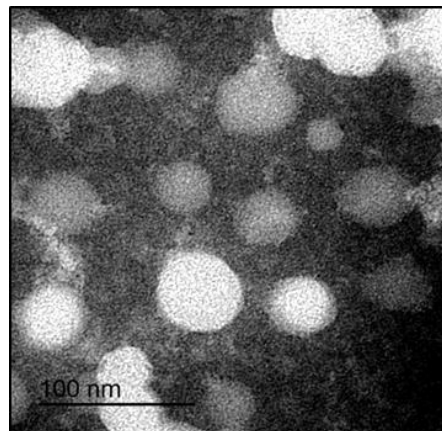
Negative staining of electron microscopic investigation of some isolated NDV in three selected samples appeared the virus as pleomorphic in shape (100 nm) **Figure (2)**.

### **3.6 Confirming of the isolated NDV by RT-PCR:**

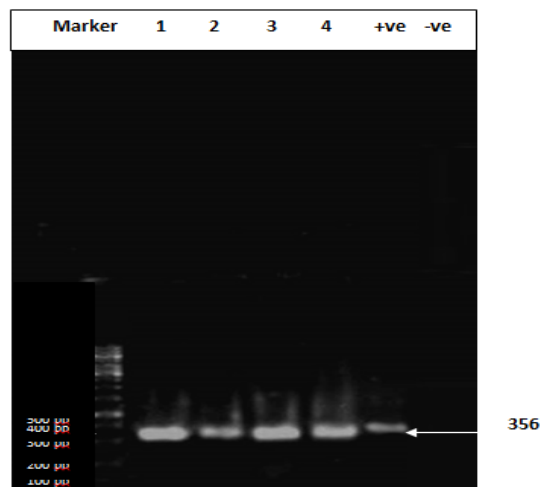
Four selected samples of allantoic fluids, of higher HA titers after three serial passages in ECEs, and reference strain were analyzed for RT-PCR technique against F-gene of NDV. The RT-PCR products run by gel electrophoresis revealed that all samples showed clear bands at 356 pb **Figure (3)**.



**Fig. (1):** Chicken embryos inoculated by samples suspected to be infected by NDV of ECE. A: Chicken embryo shows diffuse congestion, hemorrhage in head, toes and over whole the body, filled subcutaneous tissue with blood and prominent blood vessels over the body, B: Control non inoculated chicken embryo.



**Fig. (2):** Negative staining of electron microscopic investigation of the isolated NDV in three selected samples showed the virus as pleomorphic in shape (100nm).



**Fig. (3):** Gel electrophoresis of four selected PCR products (lanes from 1-4 represented field samples No. 1, 10, 19 and 21) and Control +ve sample (Genotype VII).

**3.7 Detection of the safe doses of onion and garlic extracts by ECE:**

For detection of the safe dose of onion extract in inoculated ECEs, tenfold serial dilutions of the stock extract were tested for the gross lesions in embryos and found that the dilution (10<sup>-2</sup>) showed no diffuse congestion, no hemorrhage in the whole body, no edema in head region and normal size of head. While the safe dose of garlic extract for the ECEs using four different concentrations (25, 50, 100 and 200 mg/ml) of the extract revealed that only the 200mg/ml and 100mg/ml concentrations of extract had toxic effects on CEE, in a dose- dependent way. Whereas the other two concentrations of the extract, the 50mg/ml and 25 mg/ ml, had no toxic effect in all inoculated eggs.

**3.8 The antiviral effect of onion and garlic extracts expressed by reduction of gross lesions (embryo index) and subcutaneous heamorrhage:**

The inoculated virus mixed with plant extracts showed the absence of haemorrhage and retarded growth of embryos appeared by all field and reference samples in ECEs after treated by onion extracts. Samples number (1, 19 and 21) have no gross lesions in ECEs after treated by garlic extract, while reduced haemorrhage

keeping the normal size of embryos by sample number (10) and +ve control sample **Table (2)**.

**3.9 The antiviral effect of onion and garlic extracts expressed by reduction of Haemagglutination activity:**

The virus mixed with onion extracts showed complete absence for HA activity of all field and reference samples after treated ECEs, while the garlic extracts appeared antiviral effect against NDV by stopping HA activity (samples No. 1, 19 and 21) or reducing HA activity (Samples No. 10 and Control +ve sample) **Table (3)**.

**3.10 The antiviral effect of onion and garlic extracts expressed by reduction of the infectivity titer (EID<sub>50</sub>%):**

By onion extract as shown in **Table (4)** .Analysis of the infectivity titers (EID<sub>50</sub>%) of four field isolates and reference NDV of non-mixed after mixing with garlic and onion extracts revealed the absence of infectivity (samples No. 1, 19 and 21) or reduced infectivity (samples No. 10 and control +ve sample). It is treated ECEs by garlic extract and only reference (+ve control) after mixing ECEs

**Table (1):** shows the sum of +ve HA allantoic fluid of inoculated field samples to the total inoculated samples at each ECE passage, as well, the total +ve HI samples.

Lab. Tests	HA titer			HI test
	1 <sup>st</sup> passage	2 <sup>nd</sup> passage	3 <sup>rd</sup> passage	
+ve samples/total inoculated samples	20/26	24/26	23/24	22/25
		Samples No.10 and 25 no further passages		

**Table (2):** Gross lesions changes (Subcutaneous hemorrhage and growth rate of Embryos) in non-mixed and mixed by onion and garlic extracts and challenged by NVD isolates.

Number of sample	Sample non-mixed The embryos showed	Sample mixed by garlic The embryos showed	Sample mixed by onion The embryos showed
1	Moderate hemorrhage, reduced growth	No hemorrhage, normal size	No hemorrhage, normal size
10	Sever hemorrhage, reduced growth	mild hemorrhage, normal size	No hemorrhage, normal size
19	Moderate hemorrhage, reduced growth	No hemorrhage, normal size	No hemorrhage, normal size
21	Moderate hemorrhage, reduced growth	No hemorrhage, normal size	No hemorrhage, normal size
Reference (+ve control)	Sever hemorrhage, reduced growth	mild hemorrhage, normal size	No hemorrhage, normal size

**Table (3):** Hemagglutination activity of non-mixed and mixed ECEs by garlic and onion extracts and challenges by field and reference NDV isolates.

Number of sample	Titer of NDV non mixed	Titer of NDV mixed by garlic	Titer of NDV mixed by onion
1	2 <sup>9</sup>	-	-
10	2 <sup>9</sup>	2 <sup>3</sup>	-
19	2 <sup>6</sup>	-	-
21	2 <sup>6</sup>	-	-
Reference (+ve control)	2 <sup>9</sup>	2 <sup>6</sup>	2 <sup>1</sup>

**Table (4):** The viral infectivity titers (EID50%) of some selected samples of NDV inoculated in ECEs and calculated by Reed and Muench formula for non-mixed and mixed ECEs by onion and garlic extracts.

Number of sample	Titer of virus non-mixed ECEs	Titer of Virus mixed by garlic extract of ECEs	Titer of Virus mixed by onion extract of ECEs
1	10 <sup>4</sup> EID <sub>50</sub> /0.1 ml	No infection	No infection
10	10 <sup>7.6</sup> EID <sub>50</sub> /0.1 ml	10 <sup>2.8</sup> EID <sub>50</sub> /0.1 ml	No infection
19	10 <sup>5.5</sup> EID <sub>50</sub> /0.1 ml	No infection	No infection
21	10 <sup>6</sup> EID <sub>50</sub> /0.1 ml	No infection	No infection
Reference sample	10 <sup>8.6</sup> EID <sub>50</sub> /0.1 ml	10 <sup>5.3</sup> EID <sub>50</sub> /0.1 ml	10 <sup>1</sup> EID <sub>50</sub> /0.1 ml

#### 4. DISCUSSION

APMV-1 belongs to genus Avulavirus in family of Paramyxoviridae causing a serious, highly contagious and fatal disease affecting the respiratory, nervous, and digestive systems, of poultry and other birds called Newcastle disease (Mase et al., 2002, Chakrabarti et al., 2007, Hassan et al., 2010, Rezaeianzadeh et al., 2011, Chukwudi et al., 2012 and Kapczynski et al., 2013). In Egypt, Newcastle disease virus (NDV) causes endemic, continuous long-lasting outbreaks with significant losses in the poultry industry since 1948 (Osman et al., 2014). In this study, laboratory isolation and identification of NDV from infected chickens were carried out using 26 samples collected from different farms, whereas each collected field sample included five pooled organs (trachea, lung, liver, spleen and intestine) after homogenization. Isolation of the virus was done using ECEs and identification was performed by HI, electron microscope and RT-PCR. Some of these samples were selected for studying the antiviral effect of garlic and onion extracts. The most important post mortem lesions were hemorrhagic

and/or necrotic lesions in proventriculus, caeca and small intestine. Sometimes necrotic foci were observed in the pancreas, oedematous, haemorrhagic, necrotic and ulcerative lymphoid tissue in the gut wall, pneumonic lungs, haemorrhages in trachea, air sacs, brain, cecal tonsils and spleen. These lesions were in agreement with that observed by (Capua and Alexander, 2009, Osman et al., 2014). Twenty-six pooled samples were collected from farms NDV-suspected sample and homogenated for virus isolation via allantoic cavity route of 10 days old ECEs, three serial passages were done and the embryo lesions were observed. The embryos of inoculated ECEs by the inoculated virus showed diffuse congestion, hemorrhage in head, toes and over whole the body, subcutaneous tissue filled with blood and blood vessels over the body were prominent especially in sample number 10. These results were similar to the results observed by (Capua and Alexander, 2009, OIE, 2013 and Osman et al., 2014). Identification of the isolated ND virus through allantoic cavity route of inoculated ECEs (after 3<sup>rd</sup> passage) was carried out by HA; HI, EM and PCR test. The isolated



virus haemagglutinated chicken RBCs (1%) in which, 25 samples out of 26 tested samples showed positive results. These results were similar to the results observed by (Capua and Alexander, 2009, Osman et al., 2014) whose described the using of HA test for diagnosis of NDV.

The Haemagglutination of the isolated virus was inhibited using anti-NDV hyperimmune sera in which, 22 samples out of 25 tested samples showed positive results. These results were similar to the

results reported by (Capua and Alexander, 2009, Osman et al., 2014), whose described the using of HI test for identification of + HA tested ND samples. The impact of NDV isolates is related to its virulence, the laboratory method developed by Hasnson and Brandly (1955) which proposed the classification of NDV isolates upon allantoic cavity inoculation using MDT for both field sample number (10) and the reference strain. MDT test showed that both tested samples are velogenic in their pathogenicity. The isolated virus was examined by negative staining method of electron microscopy appearing pleomorphic in shape; roughly spherical with diameters around 100 nm. These results were in agreement with the results revealed by (OIE, 1988, Yusoff and Tan, 2001 and Capua and Alexander, 2009). Molecular techniques are widely used to detect ND virus in specimens. In the current study, the molecular confirmation of NDV using reverse transcription-polymerase chain reaction (RT-PCR) for the F gene of the virus, revealed the presence of positive bands after the gel-electrophoresis. Four field isolated samples from positive HI test samples were selected for RT-PCR. These results agreed with (Haque, 2012, OIE, 2012, Getachew et al., 2015) whom recommend RT-PCR and gel electrophoresis for diagnosis of NDV. Different concentrations of garlic extracts were tested for detecting the safe doses in ECEs, 10 and 25 mg/ml concentrations were found to be the safe sides of garlic extract, this came in accordance with Semple et al., (1998) in which they suggested that the antiviral activity of crude garlic extracts should be detected in at least two subsequent dilutions of the maximum non-toxic concentration to ensure that the activity is not directly correlated with

the toxicity of the garlic extract. The antiviral effects of the used concentrations of garlic extracts showed more anti-viral effects on viruses of mesogenic type comparable to velogenic used samples (Sample number 10 and reference velogenic strain). How this happens is still unknown. It would be of interest if further studies are needed to clarify whether one or more concentrations are present in this preparation that give its antiviral effect and whether or not it is effective on the other genus of the paramyxoviruses.

The EID<sub>50</sub> of the used mixed garlic extract and some selected isolates showed inhibition in some samples (1, 19, and 21), while reduced viral infectivity in samples number 10 and the reference strains. This inhibitory effect to virus growth by this preparation may be brought about by preventing the attachment of the virus to the cell- receptors, due to its interaction with the viral is justified to conclude that the concentrations that were effective on the virus but not toxic on the cells were attributed solely to the direct envelope components leading to its destruction, the way for that to is still unknown to happen Mohammed and Al-Attar (2001).

Ten folds serial dilutions of onion extracts were examined for safety dose determination using ECEs. The selected safety doses were applied for studying the antiviral effect of the onion extract on NDV. The obtained data of mixing of safe dose of onion extract with different dilutions of selected samples of NDV. The antiviral effect of onion extracts was investigated by observing the viability of embryos in inoculated ECEs, HA, and EID<sub>50</sub> tests. The results showed by onion extracts were the same as garlic extracts. From the previous data, these results confirm the antiviral effect of the used onion extract.

The certain plant extracts inhibit virus haemagglutination and might be applied as useful protective antiviral agents Macpherson et al., (1953). Early before, Francis (1947) introduced two different and interesting explanations for the inhibitory effect of plant extracts. The first type of inhibition acts on the cell and blocks the receptor sites in the cell which required for the invasion of a number of viruses. The second, inhibitors react with the virus- producing a typical neutralizing effect,

some of these agents are polysaccharides which effectively neutralize virus even in egg infection. Despite that our results indicated the efficiency of garlic and onion extracts as inhibitors for NDV, none the less further investigations should be carried out including: Studying the mechanism of the antiviral effect during the different stages of the replication cycle of the virus on tissue culture system, screening fractionation of onion extract either into defined phytochemical group or into purified chemical constituents to define the active principle, studying the antiviral effect of onion and garlic extractions before and after virua inoculation and the field application of the plant extracts on poultry farms is required.

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