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Improvement of Chicken's Immunity Against Velogenic Newcastle Virus-Associated Feeding on Probiotics

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ABSTRACT

Newcastle virus is one of the risk factors of the chicken industry in Egypt and the world. The affected chickens have a significant death rate. The current study's major goals are to isolate and characterise Newcastle disease virus isolates, as well as to optimise chicken-associated feeding with probiotics.The sample of white leghorn Chicken organs was done from diseased Chickens collected from different commercial Chickens farms of Qaluybia, n=25, El Sharkia, n=20 El Gharbia , n=15 and El Monofia, n=15 governorates depending on distinct NDV like viral symptoms from January to May 2019 and 2020. NDV isolates were isolated and identified using non-NDV vaccinated chicken eggs that were 9-11 days old. The findings show that both velogenic neurotropic and velogenic viscerotropic strains of NDV are common. In hens utilising hemagglutination inhibition, seroprevalence shows strong antibody titers against NDV (HI). The probiotics as adaptive to feed healthy and NDV velogenic infected and chickens due to improving the immunity by increasing the cells immunity of differential leukocyte blood cell profile, Eosinophil, Lymphocyte, Monocyte cell count and Blood protein serum values, (Total Protein, Albumin and Globulin). While differential leukocyte blood cell profile and Blood protein serum values were decreased in NDV velogenic infected chickens compared to standard blood. Except total neutrophils were increased in infected chickens blood compared decreased in Healthy and infected chickens' blood with Standard blood. In conclusion, in Egypt, a number of velogenic NDV strains are currently circulating, and the inclusion of probiotics in chicken feed improves their resistance to NVD infection.

INTRODUCTION

Newcastle disease virus spreads all over the world. It is one of the most widespread infectious viral illnesses that affect poultry, resulting in high economic losses in the industry of poultry because of the increased rates of mortality among the affected flocks, which leads to the sharp decline in the production of meat and eggs (Alexander, *et al*.,1985).

The same results in Egypt (Samer, 2016 & Asmaa, *et al*., 2020) . Controlling NDV necessitates a number of factors, including frequent vaccination, slaughter, and compensation for damaged farmers. (Alexander, *et al.*,1985).

NDV is a member of the family paramyxoviridae and genus Avula virus and its genome is -ssRNA and about 15 Kb in length (De Leeuw and Peeters, 1999). The RNA-dependent RNA polymerase (L), fusion (F) protein, hemagglutinin neuramindase (HN), phosphoprotein (P), and nucleoprotein (N) are the six proteins encoded by the NDV genome (N) (Munir, *et al*., 2012). NDV was classified according to their virulence into three pathotypes (Adi, *et al*., 2010). Based on the tropism into viserotropic and neurotropic, the velogenic classified highly virulent (Piacenti, *et al.*, 2006), mesogenic (moderately virulent) and lentogenic (less virulent) strains (Ecco, *et al.*, 2011).

Many other species of birds can be also infected by NDV including pigeons and doves (Maged, 2013 and Pchelkina, *et al*., 2013). The presence of a certain amino acid sequence was recently found to be accompanied by the virulence of NDV strains infection due to the cleavage of the NDV-F protein by some host cell proteases (Yuasa, *et al*., 2012). Detection of NDV infection is usually conducted by many conventional diagnostic assays such as HA, HI, AGIDT, and SNT (Cho, *et al*.,2007 & He, *et al*., 2012). However, these procedures have a number of drawbacks, including the fact that they are time-intensive and, in most situations, lack sensitivity and specificity (Gopinath, *et al*., 2011). Enzyme-linked immunosorbent assay, Dot-ELISA, Reverse Transcriptase polymerase chain reaction (RT-PCR), and real-time PCR are some of the techniques used to diagnose NDV (Li, et al. 2009 &. Pham, *et al*., 2005).

Bacteriocins are antimicrobial proteins produced by bacteria that have antibacterial activity against both gramme positive and gramme negative microorganisms (Ouda *et al.,* 2014 and Saher *et al*., 2016). The selection and identification of a bacteriocin produced by *Lactobacillus* strains can be used as a probiotic bacterium to inhibit other bacterial pathogens (Enan *et al*., 2013 and Chikindas *et al*., 2017). These techniques are sensitive and specific for detecting NDV in a variety of samples in a short period of time. (Wise, *et al*., 2004 & Zhang, *et al* ., 2013).

The current study's main purpose was to identify the most common NDV strains in Egypt. Meanwhile, probiotics have been linked to increased immunity in hens against the velogenic Newcastle virus.

MATERIALS AND METHODS

This study was conducted during the period from 2019-2020 in labs of Dept. of poultry production, and Dept. of Microbiology, Virology, labs, Fac. of Agric., Ain shams Univ., and Cairo, Egypt.

Collection of Organs Diseased Chickens:

The diseased white leghorns Chickens were collected from different commercial Chickens farms of Qaluybia (n=25), El Sharkia (n=20), El Gharbia (n=15) and El Monofia (n=15) governorates depending on distinct NDV like viral symptoms (table, 1). The Chicken organs (kidney, liver, lung and spleen) were done in the morbid stage of the diseases as well as from apparently healthy Chickens. The collected organs were submitted to the virology laboratory Dept. of Microbiology Fac. of Agric. Ain Shams Univ. and stored at -20°C till further use.

Table 1: Number and localities of naturally NDV infected Chickens and History collected from different locations.

Governorates	Case history	Age days	Signs
Qalubyia n=25	Flock of 12.000, Vaccinated Lasota at 1 day, Avino at 15 days, Lasota at 30 days Mortality:15:40:80:100 dpi	35	Depression, greenish-diarrhea, paralysis, swollen head.
El Sharkia n=20	Flock of 1200 , No vaccination. Mortality:15:30 dpi	28	Depression, loss of weight, nervous signs developed later.
El Gharbia n=15	Flock of 1500, Vaccinated Lasota at 5 days.	30	Depression, loss of weight, paralysis
El Monofia n=15	Flock of 7500, Vaccinated at 15 days. Mortality: 35:50:110 dpi	35	Depression, greenish-diarrhea, paralysis, swollen head

Detection of NDV was depending on clinical signs of NDV and serological test by Chromatographic Immunoassay antigen ® Rapid Test Kit.

Isolation of NDV was done from clinical specimens showed typical clinical signs of NDV and gave positive serologically raped test The tissue specimens were grinded in a sterile mortar and homogenized to make a 10% w/v phosphate buffer saline (PBS) pH 7.2 and added 1/10 volume of an antibiotic mixture (containing 10,000 IU/ml penicillin, 1 mg/ml streptomycin and 1 mg/ml gentamicin sulphate) as described by Numan *et al.*, 2008. The extracts were centrifuged at 3000 rpm for 20 minutes. Supernatants were collected and filtered through a 0.45 µm syringe filter (Gelman, USA) and stored at (-20°C)**.**

Inoculation of Embryonated Chicken Eggs (ECE):

The infected tissue suspensions were injected into 9–11-day-old native chicken (non-NDV vaccinated) embryonated chicken egg obtained from Koom Oshiem, El-Fayoum, Egypt as described by Radwan, *et.al.*, 2013). A small hole was drilled into the sterilized shell by egg puncher and injected with 200 µl of each filtrated infectious supernatants (five eggs/sample) at a 45° angle as described by Swayne *et al.* (1998). The injected eggs were incubated vertically in an incubator for 7 to 10 days at 37°C and kept under 60-70 % humidity. The eggs were examined daily and the death of embryos was recorded. The allantoic fluids were collected from inoculated embryos in sterile falcon tubes by sterile 5 ml syringes. The collected allantoic fluids were centrifuged at 3000rpm for 10 minutes and stored at (-20) °C.

Identification of NDV Isolates:

The red blood cells (RBCs) were sediment of blood samples and washed three times by saline phosphate buffer (PBS). The packed RBCs were diluted at 0.5% in PBS and used in the Hemagglutination **(**HA**)** and Haemagglutination inhibition (HI) tests.

Hemagglutination (HA)was performed as described by Allan and Gough (1974), Adi, *et.al*. (2010) and Ke, *et. al*. (2010). Serial two-fold dilution was carried by 25 µl of the egg fluids containing the virus were added to each tube, then serial two-fold dilutions will be carried out. Each tube was then filled with 25µl of 0.5 % chicken RBCs. The tubes were incubated for 1 hour at room temperature before the results were read.

Haemagglutination Inhibition (HI) test was carried out according to Alexander and Senne, 2008 and Radwan, et.al. 2013). PBS (500 µl) was dispensed into each serological tube, The specific NDV polyclonal antibody kindly provided from veterinary serum and vaccine research institute, Abbasia, Cairo, Egypt, was placed (500 µl) in the first tube and two-fold dilutions were made across tubes. NDV virus/antigen (500 µl) was added to each tube and incubated for 20 minutes at the 37°C. 500 µl of 1% (v/v) chicken RBCs was added to each tube and gently mixed, The RBCs were allowed to settle for about 40 minutes at room temperature.

Determination of Biological Properties of NDV Isolates:

A- Pathogenicity Tests:

Intra Curable Pathogenicity Index (ICPI) was carried out on one-day-old white leghorns chicken as mentioned in the Terrestrial Manual of OIE as the following: Infected allantoic fluid of nine NDV isolates with titer $\geq 2^4$ is diluted 1/10 in PBS with any antibiotics 0.05ml Each diluted was injected intra curable into the one-day-old chicken (10 chicks/isolate). Control chickens received only sterile normal saline. The results were scored cumulatively for every day at 8 days post-inoculation. The results were recorded as zero if healthy chickens, 1 if sick chickens and 2 if dead chickens. The ICPI was calculated as the sum obtained was divided by a number of observations.

Intravenous Pathogenicity Index (IVPI) was carried out in 6 weeks-old white leghorns chickens inoculated intravenously with 0.1ml of 10^{-1} allantoic fluid dilution and then observed for 10 days. The results were scored as healthy chickens as zero, sick chickens as 1, paralyzed chickens as 2 and dead chickens as 3. The sum obtained is divided by 100.

Mean death time (MDT): Serial dilutions of allantoic fluid were prepared and 0.1ml of the last three dilutions (10^{-7}, 10^{-8}, and 10^{-9}) was inoculated into the allantoic cavity (9–10-day-old chickens embryonated eggs) using a minimum 10 eggs per dilution. The control egg received only sterile normal saline with antibiotics. The highest dilution at which all embryos died soon was considered as mean lethal dose (MLD) and the MDT is the average time at which the eggs inoculated with MLD died. Shorter the killing time was considered as the more virulent Strain.

Determination of NDV Concentration: A series of ten-fold dilutions are carried out on allantoic fluid of velogenic NDV. Five embryonated eggs are inoculated with 0.1 mL of each dilution and incubated for 4 days at 37°C. The HA was used to determine the virus has infected and multiplied in each of the eggs. The range of dilutions should include at least two ten-fold dilutions above and below the dilution expected to contain the endpoint.

Probiotic Strain and Preparation Of The Cell-Free Spent Medium (CFSM): *Lactobacillus helveticus* EMCC 1654 was obtained from the Microbial Culture Collection, Microbiological Resources Centre, Ain-Shams University, Cairo, Egypt. The probiotic strain was culture on MRS agar (Oxoid) medium for 24 h at 37°C with 5% CO_2. To prepare CFSM, a single colony was transferred into MRS broth under the same growth incubation conditions for 24 h. as described by (Sevda, *et al*., 2015, Abdelhamid *et al*., 2018 & 2019) to an optical density at 600 nm of 1.6 which corresponds to 1 x 10^8 cells/ml. The cells were removed using centrifugation at 6000 rpm at 4°C for 10 min. The supernatant was filter-sterilized with a 0.2 mm–pore-size filter and referred to as CFSM. The pH of the supernatant was then adjusted to 7.4 and supplemented with 50 μg/mL penicillin and 50 U/mL streptomycins. The CFSM probiotic strain was used for the chickens study to investigate the protective effect against viral infection.

Amino Acid Composition was determined by (Csomos, *et al*.,2002) by HPLC with an AAA400 amino acid analyzer (IngosLtd., Czech Republic) equipped with an OstionL GANBion exchange column.

Determination of Minimum Inhibitory Concentration (MIC) of probiotics was determined by the broth microdilution method as approved by the guidelines of Clinical and Laboratory Standards Institute (NCCL/CLSI, 2007). The probiotic was serially diluted to yield concentrations of (100, 50, 25, 12.5, 6.25, 3.125, 1.562, 0.781 and 0.097mg/ml). Twenty microliters (20μL) of bacterial suspension (0.5 McFarland standards), were added to each well except the control wells (contained broth only and distilled water only). An automatic ELISA microplate reader (Sun Rise–TECAN, Inc. ®, USA) adjusted at (600nm) was used to measure the absorbance of the plates before and after incubation at 37 °C after 24h for bacteria. The absorbance was compared to detect an increase or decrease in bacterial growth and the values plotted against concentration.

Application of MIC Probiotic Food Additives:

Chickens and Feeding: The fourteen white chickens were obtained from a local hatchery in Qalubyia governorate. The chickens were weighed and randomly divided into experimental four treatments: Healthy (Control), infected NDV Velogenic, feeding feed additives, Feeding feed additives and infected NDV Velogenic. The chickens were housed in cages, in a room with automatically controlled temperature; humidity and air exchange were provided with continuous light. During the 4-week experiment, all chickens were fed on a diet with additives. starter 1 up to 7 days were left to adapt to the new environment.

Diet was supplemented with 8g/Kg (MIC) probiotic of the cell-free medium of *L. helveticus*.

Virus Doses: The infectivity titre of MDV velogenic isolate suspension 10^{-12} were diluted 1:100 in drinking water and inoculated (0.1 ml per chicken) and left chicken drank all amount of water.

Body Weights (BW) of chickens of each treatment were recorded per week.

Clinical and Postmortem Signs And % Mortality were observed daily and recorded for 30 days.

Analysis of Blood Chemical: Whole blood was collected for blood chemical analysis plasma from the wing vein of the chickens by using a syringe (20 unit/ml) on the 21 days from each treatment. The dead and live chickens were taken to obtain the virus in their tissues and organs.

Total Serum Protein (TSP) was determined using direct Biuret reagent containing (NaOH, potassium iodide, copper sulphate, sodium, potassium tartarate) and Standard containing aqueous solution of protein, equivalent to 6 g/dL (50 g/L) were used.

The total proteins were calculated as follows: Total Serum protein (g/dL) = absorbance of sample / absorbance of standard X 6.

Serum Albumin Level (SAL) was determined using acetate buffer reagent containing bromocresol green (pH 4.1) and detergent. The standard containing aqueous solution equivalent to 4 g/dL (50 g/L) of albumin was used. The absorbance rate was read at 578 or 623 nm of sample and standard against reagent blank within 30 min.

Serum Albumin (g/dL) = absorbance of sample / absorbance of standard X 4.

Total proteins (g/dL) = Total Serum protein + Serum albumin (g/dL)

Serum Globulin Fractions (SGF) was calculated as the difference between total serum proteins and serum albumin level;

SGF (g/dL) = TSP – SAL.

RESULTS

1- Probiotic of *L. acidophilus*:

The total nitrogen and protein contents were 4.2575 and 18.252 mg in cell-free spent medium (CFSM) respectively. The amino acid compositions were identified as follows, 15 amino acids (ASP, THR, SER, GLU, GLY, ALA, VAL, ILE, LEU, TYR, PHE, HIS, LYS, ARG and PRO). The Glutamic acid was rich by 0.19 mg/100 ml followed by Aspartic acid 0.10 and Proline 0.12 mg/100 ml (table,2). Bacteriocin consisted of one polypeptide chain with a molecular weight of 4.435 KDa.

Table 2: The amino acids composition in Bacteriocin from *L. acidophilus*.

Amino acids composition					
Amino acids		g/100 ml	Amino acids		g/100 ml
ASP	Aspartic	0.10	LEU	Leucine	0.08
THR	therionine	0.04	TYR	Tyrosine	0.03
SER	[i]Serine	0.04	PHE	Phenylalanine	0.05
GLU	Glutamic	0.19	HIS	Histidine	0.03
GLY	Glycine	0.08	LYS	Lysine	0.08
ALA	Alanine	0.08	ARG	Argnine	0.03
VAL	Valine	0.09	PRO	Proline	0.12
ILE	Isoleucine	0.05			

Detection and Isolation and of NDV Isolates:

Prevalence and Detection of NDV:

The clinical signs were related to the risk factors, age, mortality %, programmer of vaccination, clinical and postmortem signs were recorded 19 diseased chickens out of 95 collected samples in table (3) like depression, greenish-diarrhea, paralysis, swollen head and nervous. Prevalence of chicken virus disease in Qalubyia were (5 / 25), El Sharkia (5 / 15), El Monofia (7 / 35), and El Gharbia (5 / 25) governorates .. Five samples showed depression, loss of weight and paralysis in Qalubyia and El Gharbia. Seven samples showed mild respiratory signs from El Monofia and Qalubyia governorates. Two samples showed depression, greenish diarrhea and paralysis from El Sharkia governorate. (fig.1). According to the Chromatographic immunoassay test, it was found that 9 NDV samples from 19 were given positive results (Table 3).

Table 3: History of the collected samples from diseased birds used for isolation of Newcastle disease virus (NDV).

Governorates	Case history	Age days	Signs	Prevalence %	Serological *test N=19
Qalubyia n=25	Flock of 12.000 Vaccinated Lasota at 1 day, Avino at 15 days, Lasota at 30 days Mortality:15:40:80:100 dpi	35	Depression, greenish-diarrhea, paralysis, swollen head.	5/25	2
El Sharkia n=20	Flock of 1200. No vaccination. Mortality: 15:30 dpi	28	Depression, loss of weight, nervous signs developed later.	2/20	2
El Gharbia n=15	Flock of 1500. Vaccinated Lasota at 5 days.	30	Depression, loss of weight, paralysis	5/15	2
El Monofia n=15	Flock of 7500 Vaccinated F vaccine at 15 days. Mortality: 35:50:110 dpi	35	Depression, greenish-diarrhea, paralysis, swollen head	7/35	3

*= Chromatographic Immunoassay

Fig. 1: Photograph showing clinical symptoms and postmortems signs on the naturally infected chickens such as greenish diarrhea, paralysis, dead and severe haemorrhages on the proventriculus.

Virus Isolation and Propagation on Chicken Embryo:

Hemagglutination (HA) test:

Aallantoic fluids in the chicken embryo eggs were subjected to the HA test using 0.5% chicken RBCs to detect NDV in these samples (table 4). The nine NDV isolates (namely ND1, ND2, ND3, ND5, ND6, ND7, ND9, ND10 & ND13) were cultivated on allantoic fluids in the chicken embryo eggs at 9-11 days gave positive results in HA test. The virus ND2, ND5 &ND6 (severe isolates) due to death 5/5 the chicken embryo at 72, 54 & 84 hrs, ND1, ND7, ND8, ND9 & ND10 (Moderate severe isolates) due to death 4/5 the chicken embryo at 84, 94,85&95 hrs and ND3, & ND13 (Mild severe isolates) 3/5 the chicken embryo at 104 &148 hrs the incubation period respectively. The dead embryo was examined for the internal signs (fig2) to confirm that the death was due to NDV. As shown in (fig.2) severe hemorrhages were observed around the embryo and in the brain region. The other virus isolates No. (ND 4, ND 8, ND 11, ND 12, ND 14 & ND 15) inoculated in the chicken embryo gave negative results in HA test.

Table 4: Results of inoculation of suspected field samples in SPF chicken embryo

Isolates	Incubation period (h)	Died embryo (n=5)	*HA test .	Isolates	Incubation period (h)	Died embryo (n=5)	*HA test
Control	-	0	-	ND_8	-	0	-
ND_1	84	4	+	ND_9	85	4	+
ND_2	72	5	+	ND_{10}	95	4	+
ND_3	104	3	+	ND_{11}	-	0	-
ND_4	-	0	-	ND_{12}	-	0	-
ND_5	54	5	+	ND_{13}	148	3	+
ND_6	84	5	+	ND_{14}	-	0	-
ND_7	94	4	+	ND_{15}	-	0	-

*Hemagglutination **(HA)**

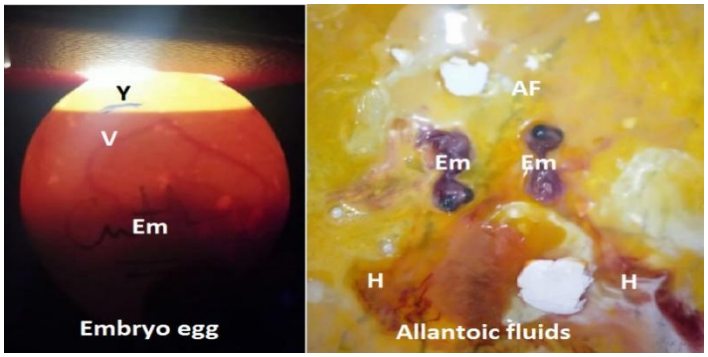

Fig. 2: Photograph showing (a) The amnionic cavity, and free of large blood vessels (b) the dead embryos (Em), severe haemrrhages (H) around the embryo and on brain region of the embryo.Yac sac(Y) , Vein =V **B-2-** NDV virus titration **.**

NDV Isolates Titration:

All nine isolates gave positive results with different titers. The highest titer was 128 HAU recorded by ND2 & ND5, 64 HAU by ND6 isolates. The moderate titter was 32 HAU recorded by ND7 & ND9 and 16 HAU recorded by ND1, ND3 & ND10 isolates. The lowest titter was 8 HAU recorded by ND13 isolate (Table,5).

Table 5: Titration of HA virus from the allantoic fluid by HA test.

NDV isolates	HA results	End point	HA titer/mL (HAU)
Control	(-)	---	---
ND_1	(+)	1 /16	16
ND_2	(+)	1 /128	128
ND_3	(+)	1 /16	16
ND_5	(+)	1 /128	128
ND_6	(+)	1 /64	64
ND_7	(+)	1 /32	32
ND_9	(+)	1/32	32
ND_{10}	(+)	1/16	16
ND_{13}	(+)	1 /8	8

(-) = Rosetta shape , (+) = Button shape

Heamagglutination Inhibition Activity (HIA):

The inoculated nine NDV isolates in chicken embryos gave positive results in HIA test using a polyclonal antiserum of NDV. The NDV titers of the nine isolates (ND1, ND2, ND3, ND5, ND6 ND7 ND9, ND10, ND13) 4, 64, 8, 64, 32, 4, 8, 4 and 4 HIU respectively (table,6). The highest Endpoint and virus titer were 1/64 & 64 HIU recorded by ND2 & ND5, 1/32 & 32 HIU by ND6. The moderate Endpoint and titer were, 1/8 & 8 HIU by ND3, ND9. The lowest Endpoint and titer were 1/4 & 4 HIU by ND1, ND7, ND10, ND13 isolates.

Table 6: Titration of NDV from allantoic fluid by Heamagglutination Inhibition activity.

NDV isolates	HIA results	End point	HIU titer/ml
Control	(-)	---	---
ND_1	(+)	1 /4	4
ND_2	(+)	1 /64	64
ND_3	(+)	1 /8	8
ND_5	(+)	1 /64	64
ND_6	(+)	1 /32	32
ND_7	(+)	1 /4	4
ND_9	(+)	1/8	8
ND_{10}	(+)	1/4	4
ND_{13}	(+)	1 /4	4

(-) = Rosetta shape , (+) = Button shape

Biological Properties of NDV Isolates:

1- Intra curable Pathogenicity Index (ICPI):

Intra curable Pathogenicity Index (ICPI) **o**f the NDV isolates were 1.56, 1.83, 0.21, 1.96, 1.82, 1.23, 1.15, 1.38 and 0.46 for ND1, ND2, ND3, ND5, ND6, ND7, ND9, ND3 and ND13 respectively. In comparison with the reference value of different pathotypes of NDV isolates (table,7). The virulent of NDV isolates were classified into three pathotypes: pathotypes-1, high virulent (Velogenic), isolates ND2, ND5, ND6, pathotypes-2, moderator virulent (mesogenic) isolates ND1, ND7, ND9, ND10 and pathotypes-3, low virulent (lentogenic) isolates ND3, ND13, NDV pathotypes.

Table 7: ICPI determination of pathotypes of NDV isolates

NDV isolates	ICPI	Reference values	Pathotypes
Control	0.0	0.0	--
ND1	1.56	1.5: 2.0	Mesogenic
ND2	1.83	1.5: 2.0	Velogenic
ND3	0.21	0.2: 0.5	Lentogenic
ND5	1.96	1.5: 2.0	Velogenic
ND6	1.82	1.5: 2.0	Velogenic
ND7	1.23	1.0- 1.50	Mesogenic
ND9	1.15	1.0: 1.5	Mesogenic
ND10	1.38	1.0: 1.5	Mesogenic
ND13	0. 46	0.2: 0.5	Lentogenic

2- Intravenous Pathogenicity Index (IVPI):

The intravenous pathogenicity index (IVPI) was assayed based on the result of ICPI, HA and HI tests. The virus isolates could be classified three NDV pathotypes, velogenic (ND5), mesogenic (ND7) and lentogenic (ND13) isolates and appeared symptoms illustrated in figure (5) and their IVPI were 2.25, 0.42, 0.12 for ND5, ND7 , ND13 and comparison with the reference value 2.00:3.00, 0.00:0.5, 0.00:0.5 respectively (Table 8).

Table 8: IVPI determination of Pathotypes of NDV.

NDV isolates	IVPI	Reference values	Pathotypes
Control	0.00	_____	_____
ND5	2.25	2.00:3.00	Velogenic
ND7	0.42	0.00:0.5	Mesogenic
ND13	0.12	0.00: 0.5	Lentogenic

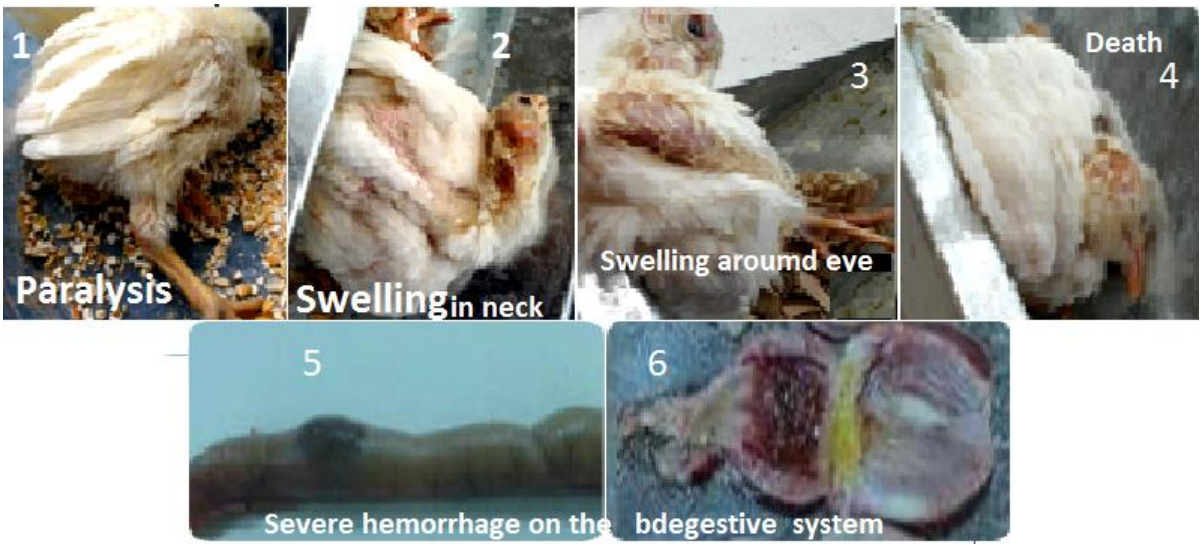

Fig. 5: Photographers the clinical signs on infected chickens showing: paralysis (1), swelling in the neck (2), swelling around the eye (3) death (4) postmortem signs included lesions and severe hemorrhages on the digestive system organs (5&6).

3-Mean Death Time (MDT):

Mean death times of NDV pathotypes (velogenic, mesogenic and lentogenic) were assayed in the embryonated eggs. MDT was 51, 78, and 96 hrs of NDV pathotypes respectively (Table,9). Related to the reference value three NDV of pathotype.

Table 9 : MDT of Pathotypes of NDV.

NDV isolates	MDT (hr)	Reference values	Pathotypes
Control	0.0	_____	_____
ND5	46	Less than 60 hr.	Velogenic
ND7	82	From 60:90 hr.	Mesogenic
ND13	103	More than 90 hr.	Lentogenic

Purified ND5, ND7, ND13 NDV isolates were propagated in ECE with high concentrations, 52, 45 & 35 mg of ND5, ND7, ND13 respectively in allantoic fluid the purified NDV isolates by the alternative low and high-speed centrifugation.

UV spectra of purified NDV isolates differed among NDV isolates using a spectrophotometer at a wavelength from 200 to 300 nm. The obtained results showed that, A_{min} at 235, 230, 225 nm, A_{max} at 260, 258, 256 nm, A_{260}/A_{280} were 1.329, 1.434, 1.313 for the ND5 (velogenic) , ND7(mesogenic) and ND13 (lentogenic) respectively (table10 & fig. 6) . The values of A_{260}/A_{280} ratio indicate to good purified virus particle for three NDV isolates. The concentration of the three NDV isolates was determined and the dilution of inoculums producing 50 percent infection of eggs was calculated by Read Muench formula. The EID_{50} was $10^{-9.7}$, $10^{-8.6}$ and $10^{-6.5}$ / ml for velogenic, mesogenic and lentogenic isolates, respectively.

Table 10: UV Spectrum of purified NDV isolates

NDV Isolates	UV Spectrum					EID_{50}
	A_{min}	**A_{max}**	**A_{260}**	**A_{280}**	**$A_{260}/_{280}$**	
ND5 (velogenic)	235	260	0.565	0.425	1.329	$10^{-9.7}$
ND7 (mesogenic)	230	258	0.552	0.385	1. 433	$10^{-8.6}$
ND13 (lentogenic)	225	256	0.545	0.415	1.313	$10^{-6.5}$

Impact of Probiotic Food Additives on NDV Infected Chickens:

Reduction of NDV Infectivity:

The probiotics as diet additives were reduced the mortality or NDV infectivity percentage from 100% to 32.25%. fed infected chicken with ND5 isolate (velogenic) done in drinking water. As well as belated the time required for showed the distinct viral clinical signs to ten days compared with infected chicken (control) 4 days for showed the distinct viral clinical signs.

Improvement the Bodyweight of NDV Infected Chickens:

The probiotics as food additives were significantly improved the bodyweight of both healthy and NDV infected chickens at four weeks, 362.45 and 290.46 g compared with healthy 250.80 g and significantly decreased of NDV infected chickens 125.62 g non-fed on probiotics. Improvement growth with a high rate of healthy (25.29 g) and NDV infected chickens (21.19g) fed on probiotic food additives compared with control 20.03g per day. On the contrary, it was a low rate (12,37 g) per day of NDV infected chickens (Table,11).

Table 11: Effect of Probiotic food additives on body weight (gm) in infected NDV chickens.

Treatments / Growth period		Chickens fed on probiotics				LSD 5%
		Unfed on Probiotic		fed on Probiotic		
		Healthy	Infected	Healthy	Infected	
1(week).		35.45	32.12	40.853	37.542	1,2
2(week).		98.95	76.45	104.24	101.14	3,2
3(week).		175.62	112.16	200.45	164. 12	5.8
4(week).		250.80	125.62	362.45	290.46	8,6
LSD 5%		12,4	6.5	15.7	15.8	
Growth rate	per week	140.20	86.58	176.99	148.32	
	per day	20.03	12,37	25.29	21.19	

Improvement of NDV Velogenic Infected Chickens Immunity:

Total Protein Serum Values:

Probiotic food additives supplemented diet was raised the Blood protein serum values, (total protein, Albumin and Globulin) with 40.04, 63.42 and 48.59 % compared with control respectively. As well as infected chickens with the ND5 isolate and fed on Probiotic supplemented diet with 47.95, 53.60 and 30.30% compared with infected and non-fed chicken on Probiotic respectively. The result of serum albumin in chicken has not hoed a significant difference in all groups treated with additive or infected by NDV or both infected and fed on a supplemented diet (Table, 12).

Table 12: Effect of Probiotic food additives on Blood values in infected NDV chickens.

Treatments / Protein blood	**Chickens fed on probiotics**					
	Healthy			**Infected**		
	Control	**Probiotic**	**Increasing rate**	**Virus**	**Probiotic+virus**	**Increasing rate**
Total protein	4.82	6.75	40.04%	2.92	4.32	47.95%
Albumin	1.75	2.86	63.42%	1.25	1.92	53.60%
Globulin	2.84	4.22	48.59%	1.65	2.15	30.30%

Complete Blood Profile:

The probiotics as adaptive to feed NDV velogenic infected and healthy chickens due to improving the immunity by increasing the complete blood profile included red blood cell and cells immunity, differential leukocyte blood cell profile, except total neutrophil, were increased in infected chickens blood compared with Standard blood (Tables 13 &14).

Complete red blood cell profile was increased of NDV velogenic infected chickens feeding on probiotic were, 4.1 $x10^{12}$/L, 9.8 g/dI , 32.1% , 69 fI , 22.1 pg , 31.6/dI , 195 $x10^{9}$/L . As well as, decreased in NDV velogenic infected chickens 2.16 $x10^{12}$/L, 2.9 g/dI , 9.6% . 65 fI , 19.2 pg , 22.1/dI , 86 $x10^{9}$/L while increased in healthy chickens , 4.5 $x10^{12}$/L , 13.4 g/dI , 42.3% , 85 fI , 28.4 pg, 32.3g/dI , 375 $x10^{9}$/L of Red cell count , Hemoglobin , PCV , MCV , MCH , MCHC and Platelets count respectively compared with Standard blood (Table ,13).

Table 13: Effect of probiotics as adaptive to feed NDV velogenic infected and healthy chickens on complete red blood cell profile.

Treatments / Complete blood profile	Chickens fed on probiotics				
	Healthy blood		Infected blood		Standard blood
	Unfed on Probiotic	Fed on Probiotic	Unfed on Probiotic	Fed on Probiotic	
Red cell count /L	4.5 $x10^{12}$	6.2 $x10^{12}$	2.2 $x10^{12}$	4.1 $x10^{12}$	4.5 -6.0$x10^{12}$
Hemoglobin g/dI	13.4	12.9	2.9	9.8	11.5-16.5
Packed cell volume %	42.3	52 %	9.6%	32.1%	40-54
Mean cell volume(MCV) fI	85	90 fI	65 fI	69 fI	80 – 92
Mean cell Hemoglobin (MCH) pg	28.4	30.2 pg	19.2 pg	22.1 pg	27-32
Mean cell Hemoglobin conc. (MCHC) g/dI	32.3	33.1/dI	22.1/dI	31.6/dI	30-35
Platelets count /L	325 $x10^9$	375 $x10^9$	86 $x10^9$	295 $x10^9$	150-400 $x10^9$

The probiotics as adaptive to feed NDV velogenic infected and healthy chickens due to improving the immunity by increasing the cells immunity of differential leukocyte blood cell profile were 5.1 $x10^9$/L, 32 %, 6% NDV velogenic infected and 6.5 $x10^9$/L, 45 %, 8% healthy chickens of total leukocyte, Eosinophil, Lymphocyte, Monocyte cell count, respectively. while differential leukocyte blood cell profiles were decreased in NDV velogenic infected chickens compared to Standard blood (Table, 14). Except total neutrophil were increased in infected chickens blood 77% (Staff neutrophil 2% & Segmented neutrophil, 75%) , compared decreased in Healthy and infected chickens blood 62 & 61% (Staff neutrophil 2% & Segmented neutrophil , 60& 59 %) respectively with Standard blood (Table 14) .

Table 14: Effect probiotics as adaptive to feed NDV velogenic infected and healthy chickens on differential leukocyte blood cell profile.

Treatments / Differential leukocyte blood cell	Chickens fed on probiotics				
	Healthy blood		Infected blood		Standard blood
	Unfed on Probiotic	Fed on Probiotic	Unfed on Probiotic	Fed on Probiotic	
Total leukocyte cell count /L	6.5 $x10^9$	7.5 $x10^9$	2.95 $x10^9$	5.1 $x10^9$	4 -11$x10^9$
% Total neutrophil	62	59	77	61	40-75
%Staff neutrophil	2	2	2	2	-
%Segmented neutrophil	60	56	75	59	-
%Total Eosinophil	2	2	1	2	1-6
%Total Basophil	0	0	0	0	0-1
%Total Lymphocyte	45	52	18	32	20-45
%Total Monocyte	8	9	4	6	2-10

DISCUSSION

NDV genotypes have increasingly become the most common strains in chicken farms, resulting in significant economic losses for the poultry industry. Despite the use of numerous chicken vaccination regimens, the disease becomes widespread in commercial chicken farms. (Munir *et al*., 2012 and Dimitrov *et al*., 2016). The sample of white Chicken organs was done from diseased Chickens collected from different commercial Chickens farms of Qaluybia,

n=25, El Sharkia, n=20 El Gharbia, n=15 and El Monofia, n=15 governorates depending on distinct NDV like viral symptoms with clinical signs including respiratory signs as nasal discharge and gasping, and, periorbital edema, nervous signs characterized by torticollis and also greenish colored diarrhea. from January to May 2019 and 2020. NDV isolates were isolated and identified using non-NDV vaccinated chicken eggs that were 9-11 days old. The virus was isolated using ECE, which is an extraordinarily sensitive approach for isolating NDV (Alexander, 1989 and OIE, 2008). Megahed, *et al*. (2018) found hemorrhagic dead embryos 48-96 hours PI after first passage in the prepared samples injected through the allantoic sac method. The presence of the virus was determined using a quick plate HA test, which revealed that all infected samples showed positive hemagglutination activity in the first passage's harvested allantoic fluid. These samples were sent in for HI testing with an NDV-specific antibody. This result was almost identical to the previous one (Farooq, *et al*., 2014). Our findings revealed a significant incidence of velogenic neurotropic and viscerotropic NDV strains.

Asmaa, *et al*. (2020) discovered that NDV class II genotype VII was circulating in Qalybia chicken flocks. In hens utilising hemagglutination inhibition, seroprevalence shows strong antibody titers against NDV (HI). NDV isolates were inoculated in specific chicken eggs, identified by hemagglutination (HA) and hemagglutination inhibition (HI) techniques. There were 9/19 (47.37%) of the selected suspected farm that was positive for NDV With HI test. Several virulent NDV strains are currently circulating in Egypt. Globally, extensive vaccination programs, hygiene measures, and quarantine procedures have all been used to combat NDV in the chicken sector (Claassen, *et.al*., 2004). However, many NDV outbreaks continue to be recorded on a regular basis. This could be attributable to a variety of factors, including the use of intense vaccines at random, frequent NDV mutations, and the emergence of new NDV pathotypes (Pchelkina, *et.al.*,2013). The implementation of excellent management plans, as well as precise diagnosis of the circulating strain and evaluation of the flocks' immunological status against NDV, are the mainstays of science-based control methods. NDV has been diagnosed using a variety of procedures since its discovery. Comparing the sensitivity of some traditional methods for detecting NDV, such as (HA, isolation by ECE, and HI). This is in agreement with other studies (Phan, *et al*.,2013) that developed recombinant based NDV-N based ELISA using the baculovirus expression system. The serological test by Chromatographic Immunoassay antigen ® Rapid Test Kit was also reported to be able to detect reactive animals to NDV in HI negative tested samples in this study (Phan, *et al*. ,2013). The identification of the causal NDV in specific outbreaks is an important aspect. Previously, pathotyping of NDV was mostly based on the Pathogenicity Index (ICPI) or the Intravenous Pathogenicity Index (IVPI), as well as the mean death time (MDT) (Mishra, *et al*., 2001). These procedures are specific and have been accepted for several years, although they are little specific, labour intensive, and time-consuming (Chen, *et al.*, 2013). Much other research that used a similar strategy for pathotyping NDV and found that this approach was feasible (Pham, *et al.*, 2005). In Egypt, a seroprevalence investigation employing both HI and PCR techniques showed a significant seroprevalence of IBV. The probiotics as adaptive to feed healthy and NDV velogenic infected and chickens due to improving the immunity by increasing the cells immunity of differential leukocyte blood cell profile, Eosinophil, Lymphocyte, Monocyte cell count and Blood protein serum values, (total protein, Albumin and Globulin). While differential leukocyte blood cell profile and Blood protein serum values were decreased in NDV velogenic infected chickens compared to Standard blood. Except total neutrophil were increased in infected chickens blood compared decreased in Healthy and infected chickens blood with Standard blood. Finally,

multiple velogenic NDV strains are circulating in Egypt right now.

The total nitrogen and protein contents of probiotic of *L. acidophilus* used in this study were 4.2575 and 18.252 mg in cell-free spent medium (CFSM) respectively. The amino acid compositions were identified as 15 amino acids. *Lactobacillus* strains produce a bacteriocin that can be utilized as a probiotic bacterium to suppress other bacterial infections. In this case, an inhibitory chemical known as a bacteriocin was created (Enan *et al*., 2013 and Chikindas *et al*., 2017). Bacteriocins are antibacterial proteins produced by bacteria that may kill both gramme positive and gramme negative microorganisms. (Ouda *et al.*, 2014) . Bacteriocin consisted of one polypeptide chain with molecular weight 4.435 KDa. The probiotic protein acted as an antiviral agent, preventing the virus from spreading and suppressing its release. According to *Zhang et al.* (2013), the antiviral mechanism of Mx protein is still unknown, despite the fact that the triple GTP-binding region is a key element of Mx protein and GTP activity is required for it to perform its antiviral function (Pitossi *et al*., 1993). Negative-strand RNA viruses are inhibited by Mx proteins. The antiviral specificity of different Mx proteins varies, as do the antiviral processes. In mice, the Mx protein found in the nucleus can stop the influenza virus from replicating. Mx A, on the other hand, can prevent viral nucleocapsid from entering the nucleus and hence impede virus reproduction by blocking virus entry into the cytoplasm.

Conclusions*:*

In Egypt, several aggressive NDV strains are currently circulating. Develop more specific diagnostic assays and vaccines that represent the local field strains. Probiotic is considered a promising antiviral substance when added to chicken food.

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