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### Original Paper

## Efficacy of an inactivated Vaccine prepared from a new isolate of Newcastle Disease Virus

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### ABSTRACT

The incidence of Newcastle Disease virus (NDV) outbreaks in Egypt is still frequent even massive vaccination programs that due to viral mutation and appearing new isolates that need new vaccination program. Inactivated Newcastle Disease virus (NDV) vaccine was prepared using a new local isolate (NDV/Ch/Giza2014), from Giza Governorate, Egypt, inactivated with binary ethylenimine (BEI) and emulsified with Montanide ISA70VG as an oil adjuvant. The prepared vaccine was evaluated in comparison with an inactivated imported vaccine. Chick groups vaccinated with either prepared or imported vaccines showed high serum antibody titers from the third week post-vaccination until reached the maximum titer at the 9<sup>th</sup> week post-vaccination using Haemagglutination Inhibition (HI) test. The prepared and imported vaccines gave 95-100% of protection against the local and the classical strain in chicks, during challenge period, there no clinical signs or lesions on examination. In conclusion, the locally prepared inactivated NDV vaccine can protect chicken against either homologous or heterologous challenging viruses.

## 1. INTRODUCTION

Newcastle disease (ND) remains one of the most important diseases in poultry production worldwide and still a major constraint against the development of both industrial and village poultry production (Al-Garib et al., 2003; Balachandran et al., 2014). The Outbreaks of ND in commercial poultry and wildlife bird populations can cause high mortalities reached 100%. Clinical disease has many forms as respiratory, intestinal and neurological forms (Saif et al., 2003). The ND outbreaks are occurring frequently in Egypt and the source of the virulent NDV in these outbreaks is anonymous (Mohamed et al., 2009).

Newcastle disease virus (NDV) is a member of Avula virus genus, family Paramyxoviridae. Paramyxoviruses are single-stranded RNA, with a genome size of about 15 kb with a genomic arrangement of six genes coding NDV has a wide host species variety, including about 241 species of 27 orders, out of known 50 orders of birds (Madadger et al., 2013). Most commonly affected species include chickens, turkeys, ducks, pigeons (Zhang et al., 2011). NDV spreads via direct contact with secretions of infected birds; principally via ingestion (faecal/oral route) and inhalation (Alexander, 1988 & 2004).

The envelope of the NDV has two surface glycoproteins; Haemagglutinin-Neuraminidase (HN) which is responsible for attachment of the virus to the host cell receptors and fusion (F) protein which is responsible for fusion of viral envelope with the cellular plasma membrane. Both these two glycoproteins are the antigenic

components against which neutralizing antibodies are directed (Yusoff and Tan, 2001). Because NDV is RNA virus which characterized by the high rates of mutation and replication and large population sizes, so RNA viruses evolve rapidly (Domingo and Holland, 1997).

The NDV local isolates from Giza 2014 showed a pattern of 112R/K-R-Q-R/K-R F117 F protein cleavage site motif characteristic to velogenic NDV strains and the NDV local isolates from Qualubiya 2014 showed a pattern of 112G/E-K/R-Q-G/E-R L117 F protein cleavage site motif characteristic to lentogenic NDV strains (El-Bagoury et al., 2015; El-Habbaa et al., 2017).

Phylogenetic analysis of these isolates showed that NDV Giza 2014 was in a separate branch independent from other Egyptian isolates of NDV and it is more related to NDV Lasota strain (genotype II NDV) and NDV Clone 30 vaccinal strain, while NDV Qalyubia 2014 was grouped more related to genotype I Ulster NDV strain and Australian isolates originating from the same ancestral node but it is distantly related to other Egyptian NDV strains 2005 and 2006 grouped together with a common ancestral node but on a separate branch (El-Habbaa et al., 2017).

Prevention and control of NDV depends mainly on strict application of biosecurity measures and intensive vaccination programs that have been successfully used over the world for several years (Alexander, 2000). Nevertheless, in the last few years, NDV has caused several outbreaks in Egyptian domestic poultry flocks, resulting in massive economic losses (Osman et al., 2014). The frequent incidence of NDV infection, even in vaccinated

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birds, is not only related to improper vaccination or immune suppression but may also be due to viral mutation leading to changes in the genomic sequence of the virus, thus altering its biological properties and virulence (Ke et al., 2001; Kattenbelt et al., 2006).

The recent study aimed to prepare and evaluate of locally prepared binary ethylenimine inactivated oil emulsion NDV vaccine from the newly isolated NDV strain (NDV/Ch/Giza2014).

## 2. MATERIAL AND METHODS

### 2.1.1. Locally isolated NDV (NDV/Ch/Giza2014) (accession number KR535624):

It was locally isolated at Central laboratory for evaluation of veterinary biologics (CLEVB) in 2014 from Giza Governorate and was identified genetically. The Phylogenetic analysis of NDV Giza 2014 with other reference and vaccinal strains of NDV revealed it was in a separate branch independent from other Egyptian isolates of NDV.

The NDV local isolate from Giza 2014 showed a pattern of 112R/K-R-Q-R/K-R F117 F protein cleavage site motif that is cleaved by a variety of proteases, resulting in systemic infection characterized in velogenic and mesogenic antigens of NDV (It was identified under the Name of (NDV/Ch/Giza2014) with Accession Number KR535624). Its titer was  $10^{7.5}$  EID<sub>50</sub>/ml. It was used as the seed virus for vaccine preparation as well as a challenge virus for vaccinated birds. The challenge dose was adjusted to be  $10^6$  EID<sub>50</sub>/ml per bird and injected intramuscular.

### 2.1.2. Challenged NDV genotype VIIId:

It was obtained from strain bank department of CLEVB. Its titer was  $10^7$  EID<sub>50</sub>/ml. The challenge dose was adjusted to be  $10^6$  EID<sub>50</sub>/0.5ml per bird and injected intramuscular. It was used for challenging of both vaccinated and non-vaccinated birds.

### 2.2. Imported Inactivated Newcastle Disease (ND) Vaccine:

It was an oil emulsion vaccine contains inactivated ND virus (Lasota strain) (binary ethylene amine inactivated NDV vaccine in oil adjuvant emulsion and its titer was  $10^{7.5}$  EID<sub>50</sub>/ml). The vaccine was obtained from Intervet international company and was used in the local market in Egypt. It was administered IM in a dose of 0.5 ml/bird.

### 2.3. Antigens and antisera:

2.3.1. ND antigens for Lasota virus and the newly isolated NDV (NDV/Ch/Giza2014) were prepared (OIE, 2019) and their titers were  $2^7$  and  $2^{7.5}$  HA, respectively. They were used in HI test.

2.3.2. Standard ND antisera were obtained CLEVB (Its titer is 12 log<sub>2</sub>) and used as positive control for evaluation of tested ND vaccines.

### 2.4. Experimental Hosts:

#### 2.4.1. Embryonated Chicken Eggs (SPF-ECE):

Twenty Specific Pathogen Free (SPF-ECE), 9-10-day old, obtained from the SPF egg farm, Kom Oshim, EL-Fayoum, Egypt. The eggs were used for propagation and titration of ND viruses and confirmation of completion of virus inactivation of the tested inactivated ND vaccine.

#### 2.4.2. SPF Chicks:

Total number of 150, one-day-old SPF chicks were obtained from SPF poultry farm, Kom Oshim, EL-Fayoum, Egypt. The chicks were maintained at CLEVB in positive pressure isolators with continuous light for evaluation of the tested ND vaccines.

### 2.5. Preparation of inactivated NDV vaccine:

#### 2.5.1. Propagation of NDV in SPF-ECE (OIE, 2019):

The locally isolated (NDV/Ch/Giza2014) was propagated in SPF-ECE for preparation of an inactivated oil emulsion ND vaccine. The virus was in sterile physiological saline pH 7.2, (0.1ml) of virus (virus titer) was inoculated in to the allantoic sac of each of 10 days old SPF-ECE and incubated at 37°C with daily candling. Harvest the allantoic fluid of the inoculated eggs after 72 hrs. for examination of HA activity. The harvested allantoic fluid was titrated in ECE. The titer of the virus was adjusted to be  $10^6$  EID<sub>50</sub>/dose for vaccine preparation and tested for sterility against any bacterial, fungal and mycolasmal contamination.

#### 2.5.2. Inactivation of the propagated NDV:

The harvested infected allantoic fluid was treated with binary ethylene amine (BEI obtained from Veterinary serum and vaccine research institute) at a final concentration of 0.001 M (1% v/v), with continuous stirring at 37 °C (for 18 hours) during inactivation process according to Bahnemann (1990). A minimum of three samples of the virus were withdrawn every 2 hrs and titrated by inoculation in chicken embryo eggs using five eggs for each dilution at the rate of 0.2 ml/egg. All eggs were sealed with wax and incubated for 5 days at 37 °C. Eggs were candled daily. Allantoic fluid from surviving embryos was testing for HA activity. The time required must be sufficient to ensure freedom from live virus. After complete inactivation of virus, the inactivation was stopped in each virus sample by adding 20% sodium-thiosulphate solution to a final concentration of 2%.

#### 2.5.3. Preparation of the vaccine emulsion:

It was prepared as water in oil emulsion (W/O) using Montanide™ ISA70 VG (SEPPIC, France batch No. 948400) at a ratio of 3/7 (v/v) according to the standard protocol of manufacture instruction.

#### 2.5.4. Comparative evaluation of the prepared and imported inactivated NDV oil emulsion vaccines:

The quality control of the prepared and imported inactivated NDV vaccines were tested by sterility and safety and were carried out according to Egyptian standard regulation for veterinary Biologics (2009) and OIE (2019).

##### 2.5.4.1. Sterility test:

It was done for ensuring that the prepared and the imported ND inactivated vaccines were free from any bacterial and fungal contamination. Samples from the tested vaccines inoculated into nutrient agar and thioglycolate broth media then incubated at 37°C for detection of any bacterial contamination. Other samples were cultured on Saburaoud agar media and incubated at 25°C for detection of any fungal contamination. Daily inspection of the inoculated media for any possible growth.

#### 2.5.4.2. Safety test in chicks:

Groups of 3 weeks old chicks were inoculated S/C with double the field dose (0.5 ml) of the tested vaccines. Another group of chicken were kept unvaccinated as control. All the chicks were observed for 21 days for any signs of local reaction or appearance of any clinical signs of NDV.

#### 2.5.4.3. Potency of the prepared vaccine:

Groups of SPF chickens (3 weeks old) were vaccinated S/C with the field dose (0.5 ml) of the tested ND vaccines. Blood samples were taken weekly for serological analysis of antibodies against NDV using HI test. Three weeks post vaccination, the vaccinated and the control chicken were challenged with  $10^6$  EID<sub>50</sub> /0.5ml of both the imported NDV-genotype VIIId and the newly isolated (NDV/Ch/ Giza2014) viruses intramuscular. All the dead and the clinically infected birds were recorded during the observation period (2 weeks) for detection of the protection %.

#### 2.6. Experimental Design:

In this study (150) SPF chicken were used to evaluate the efficacy of locally prepared and imported inactivated ND vaccines. The vaccinated chicken groups were divided to 2 groups.

The first group (50 bird) was vaccinated with locally prepared inactivated ND vaccine and the second group (50 birds) was vaccinated with the imported inactivated ND vaccine. While the control group (50 birds) of chicken. Each of the 3 groups was subdivided in to 3 subgroups. The 1<sup>st</sup> & 2<sup>nd</sup> subgroups (20 bird/each) were challenged with the newly isolated NDV/Ch/Giza2014 & (NDV-genotype VIIId) virus, respectively, the 3<sup>rd</sup> subgroup (10 birds) kept for serological analysis. The control groups were subdivided into 3 subgroups, the 1<sup>st</sup> & 2<sup>nd</sup> subgroups (20 bird/each) were infected with the same previously mentioned challenge viruses and the 3<sup>rd</sup> subgroup (10 birds) was kept for control negative serum as shown in (Table 1).

Table 1 Experimental design of the study

Group ID	Group No.	Subgroup		Treatment
		ID	No	
A	50	1	20	Vaccinated with prepared NDVEI* & challenged with NDV/Ch/Giza2014 virus.
		2	20	Vaccinated with prepared NDVEI & challenged with NDV-genotype VIIId virus.
		3	10	Vaccinated with NDVEI & unchallenged.
B	50	4	20	Vaccinated with prepared NDVF* vaccine & challenged with NDV/Ch/Giza2014 virus.
		5	20	Vaccinated with prepared NDVF vaccine & challenged with NDV-genotype VIIId virus.
		6	10	Vaccinated with prepared NDVF vaccine & unchallenged
C	50	7	20	Vaccinated with NDVI* vaccine & challenged with NDV/Ch/Giza2014 virus.
		8	20	Vaccinated with NDVI vaccine & challenged NDV-genotype VIIId virus.
		9	10	Vaccinated with NDVI vaccine & unchallenged
Control	50	10	20	Unvaccinated and challenged with NDV/Ch/Giza2014 virus.
		11	20	Unvaccinated and challenged with NDV-genotype VIIId virus.
		12	10	Unvaccinated & Unchallenged

NDVF\*: formalin inactivated NDV. NDVEI\* binary ethylenimine inactivated NDV. NDVI\*: imported inactivated NDV

### 3. RESULTS

#### 3.1 Inactivation effectiveness of BEI against NDV:

To conform the complete inactivation of the virus, the BEI-inactivated NDV/Ch/Giza2014 was inoculated into allantoic cavity of 10-day-old SPF-ECE. All chicken embryo eggs still alive after 7 days following three passages, and no residual active NDV was detected by HA test.

#### 3.2 Sterility test:

By examination of the nutrient agar, thioglycolate broth media and Sabouraud agar media with the tested inactivated ND vaccines, it didn't show presence of any bacterial & fungal contamination.

#### 3.3 Safety test:

The chicks didn't show any local or adverse systemic reactions due to any viral diseases during the observation period (21 days) after inoculated S/C with double field dose of the tested vaccines.

#### 3.4 Results of potency test:

The post vaccination antibody response showed detectable HI antibody titers by 1<sup>st</sup> week post vaccination (WPV). The mean HI antibody titers of the tested

inactivated ND vaccines used for vaccination of chicken were explained in (Tables 2 & 3).

It was observed from (Table 2) that in case of locally prepared ND inactivated vaccine, the mean HI antibody titre increased from 0 at pre-vaccination time to (3.6 log<sub>2</sub>) at 1<sup>st</sup> weeks post vaccination (WPV) and was increasing to 9<sup>th</sup> WPV (10.4 log<sub>2</sub>) when using (NDV/Ch/Giza2014) Ag. While the mean Ab titer of chicken vaccinated with inactivated imported ND vaccine increased gradually from (3.5 log<sub>2</sub>) at 1<sup>st</sup> WPV to reach (10.3 log<sub>2</sub>) at 9<sup>th</sup> WPV when using the same Ag.

It was observed that the Ab titers were increased gradually from 2<sup>3.4</sup> & 2<sup>3.6</sup> at 1<sup>st</sup> WPV to become 2<sup>10.2</sup> & 2<sup>10.3</sup> at 9<sup>th</sup> WPV for inactivated locally prepared & imported Lasota vaccines, respectively (Table, 3).

#### 3.5 Efficacy of ND Vaccines:

Results of challenge test of chicken groups vaccinated with local & imported ND vaccines using the isolated (NDV/Ch/Giza2014) virus (Table 4). It was observed that by challenging the immunity of chicken groups vaccinated with both the locally prepared & the imported ND vaccines, (100% & 95%) of the chicken of each group, respectively were protected against the disease for 10 days post challenge in comparison to the control group (0% protection). We observed the same result after challenging with NDV-genotype VIIId virus (Table 5 and Figure 1).

Table 2 HI test of vaccinated chicken groups by using NDV/Ch/Giza2014 Ag

Vaccine	No. of chicken	Mean HI titre / WPV								
		1	2	3	4	5	6	7	8	9
Locally prepared vaccine	10	2 <sup>3.6</sup>	2 <sup>5.8</sup>	2 <sup>7.8</sup>	2 <sup>8.2</sup>	2 <sup>8.8</sup>	2 <sup>9.3</sup>	2 <sup>9.8</sup>	2 <sup>10.2</sup>	2 <sup>10.4</sup>
Imported vaccine	10	2 <sup>3.5</sup>	2 <sup>5.3</sup>	2 <sup>7.5</sup>	2 <sup>7.8</sup>	2 <sup>8.5</sup>	2 <sup>9</sup>	2 <sup>9.5</sup>	2 <sup>9.9</sup>	2 <sup>10.3</sup>
Control	10	0	0	0	0	0	0	0	0	0

Table 3 HI test of vaccinated chicken groups by using Lasota virus

Vaccine	No. of chicken	Mean HI titre / WPV								
		1	2	3	4	5	6	7	8	9
Locally prepared vaccine	10	2 <sup>3.4</sup>	2 <sup>5.6</sup>	2 <sup>7.7</sup>	2 <sup>8.1</sup>	2 <sup>8.6</sup>	2 <sup>9.2</sup>	2 <sup>9.6</sup>	2 <sup>10</sup>	2 <sup>10.2</sup>
Imported vaccine	10	2 <sup>3.6</sup>	2 <sup>5.4</sup>	2 <sup>7.6</sup>	2 <sup>7.9</sup>	2 <sup>8.5</sup>	2 <sup>9.2</sup>	2 <sup>9.6</sup>	2 <sup>10</sup>	2 <sup>10.3</sup>
Control	10	0	0	0	0	0	0	0	0	0

Table 4 Challenge test of vaccinated chicken and challenged with NDV/Ch/Giza2014 virus:

Vaccine type	No. of chicken	Daily observation of chicken										Total no. of dead bird	Protection %
		1	2	3	4	5	6	7	8	9	10		
Locally prepared vaccine	20											0	100
Imported vaccine	20					1						1	95
Control	20			12	3	3	2					20	0

Table 5 Challenge test of vaccinated chicken and challenged with NDV-genotype 7D virus:

Vaccine type	No. of chicken	Daily observation of chicken										Total no. of dead bird	Protection %
		1	2	3	4	5	6	7	8	9	10		
Locally prepared vaccine binary	20											0	100
Imported vaccine	20					1						1	95
Control	20			15	3	2						20	0

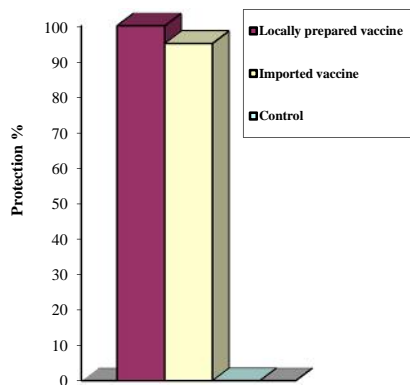


Fig. 1 Chart describing result of challenge test of vaccinated chicken

4. DISCUSSION

In Egypt, NDV outbreaks are occurring frequently and the epidemiology of the virulent NDV Isolates from these out breaks was elucidated. (Radwan et al., 2013). Vaccination was used in Egypt as a routine tool to prevent or decrease losses due to ND infection (Abd El Aziz et al., 2016). Also, vaccination strategy has an important role in the limitation of viral shedding and subsequently, minimize the spread of infection to the surrounding environment (Miller et al., 2010). A variety of vaccines are used to control the disease in chicken as live attenuated and inactivated ND vaccines to control the outbreaks caused by virulent ND viruses (Allan et al., 1973).

In this study, an inactivated ND vaccine was prepared using the locally isolated ND virus (NDV/Ch/Giza2014), then its efficacy was compared with that of the imported vaccine for protection of chicken against the ND infection.

All the tested inactivated ND vaccines ensured that they were safe, sterile, pure and valid for use (Zou et al., 2016; Monir et al., 2018; OIE, 2019).

The HI test considered the most suitable serological method for detection of the immune response against AIV and NDV vaccines (Tang et al., 2005).

In the current study, the ability of locally prepared and imported inactivated ND vaccines in induction of good protective immune response for chicken were tested. Immune response of both ND vaccines was determined passing on the serology performed weekly after vaccination using both ND Ags (NDV/Ch/Giza2014 and NDV genotyping VIIId).

The current study and (Sarcheshmei et al., 2016) recorded that the mean HI titer of all vaccinated groups was higher than 7.5 log<sub>2</sub> on day of challenge. While, Kapczynski and King (2005) and Boven et al. (2008) recorded that the mean HI titer of all vaccinated groups was higher than 5 log<sub>2</sub> on day of challenge and remained high until the end of the experiment.

The current study and (Courtney et al., 2012) observed that the locally prepared and the imported ND vaccines produce nearly the same antibody titers when examined by the local and standard strains (NDV/Ch/Giza2014 and NDV genotyping VIIId) in chicken and showed that the antigenic similarity is shared among all NDV strains and isolates will cross-protect against other NDV isolate.

There were no detected clinical signs of disease or mortality due to the vaccine strain during the monitoring period of the safety trial. This result is similar to the findings of Atul et al. (2012) and Igwe and Eze (2016). The efficacy of the inactivated local and imported ND vaccines examined by challenge tests was cleared in (Tables 4 and 5). The protection % of local ND vaccine was 100% and 95% against the local and the standard challenge viruses in chicken host that agree with findings of Monir et al. (2018). While, Abdu et al. (2012) recorded

that the protection % of chicks vaccinated with Lasota vaccine in water are 90%.

The previous results demonstrated that the inactivated ND vaccines (either locally prepared or imported) induced a sufficient effective protection for chicken against both local and standard challenge viruses and this confirmed the findings of (Hu et al., 2011), who reported that the heterologous vaccines can prevent infection and viral transmission if sufficient time is allowed for bird to mount a proper immune response beside the use of homologous Ags. Furthermore, it must focus on ways to accelerate speed of the immune response evoked beside the use of homologous Ags. Also, when flock immunity increases, even low level of Ab titers may be sufficient to prevent infection depending on the challenge dose (Miller et al., 2010). In addition to Miller et al. (2013) reported that Lasota vaccines induce the lowest pre-challenge Ab levels, however there was in most cases 100% protection against mortality and clinical signs but not effective in protecting against viral replication and transmission.

Eventually, virulent NDV continues to be endemic in Egypt and many countries around the world despite massive vaccination programs. Also, it is necessary to note that the increase in variability in the HN protein compared with the F protein can affect the cross protection. So, the selection of vaccinal Ags must be applied according to the cross-protection studies with live animals.

## 5. REFERENCES

1. Abd El Aziz, M.N., Hatem S. Abd El-Hamid, Hany F. Ellkany, Soad A. Nasef, Sherif M. Nasr, Ahmed R. El Bestawy (2016): Biological and Molecular Characterization of Newcastle Disease Virus Circulating in Chicken Flocks, Egypt, During 2014-2015. *Zagazig Vet. J.* 44 (1): 9-20.
2. Abdu, p. A., musa. U., joannis, t. M., sa'idu, l., mera, u. M, salami-shinaba, j.o. and haruna, S. (2012): Vaccination of Chickens Against Newcastle Disease with La Sota and V4 Vaccines Using Brans, Ground Grains and Water as Vehicles. *Vom Journal of Veterinary Science* 9: 1 – 10.
3. Alexander, D. J. (1988): Newcastle disease, p. 114-120. In H. G. Purchase, L. H. Arp, C. H. Domermuth, and J. E. Pearson (ed.), *A laboratory manual for the isolation and identification of avian pathogens*. 3rd ed. American Association for Avian Pathologists, Inc., Kennett Square, Pa.
4. Alexander, D. J. (2000): Newcastle Disease and other avian paramyxoviruses. *Rev. Sci. Tech.*, 19 (2): 443 – 462.
5. Alexander, D. J. (2004): *OIE Manual of Diagnostic Tests and Vaccines for Terrestrial Animals*. Office of International Des Epizooties, Paris. Pp. 161.
6. Al-Garib, S. O., Gielkens, A. L. J. and Koch, G. (2003): Review of Newcastle disease virus with particular references to immunity and vaccination. *World's Poul. Sci. J World's Poul. Sci. J.* 59: 185-200.
7. Allan, W.H., Lancaster, J.E. and Toth, B. (1973): *The production and use of Newcastle disease vaccine*, Italy. Pp. 53.
8. Bahnemann, H.G. (1990): Inactivation of viral antigens for vaccine preparation with particular reference to the application of binary ethylenimine. *Vaccine* 8 (4): 229-303.
9. Balachandran, P., Srinivasan, P., Sivaseelan, S., Balasubramaniam, G. A. and Gopala Krishna Murthy, T. R. (2014): Isolation and characterization of Newcastle disease virus from vaccinated commercial layer chicken. *Veterinary World* 7(7): 457-462.
10. Boven, M.V, Bouma, A., Fabri, T.H.F., Katsma, E., Hartog, L. and Koch, G. (2008): Herd immunity to Newcastle disease virus in poultry by vaccination. *Avian Pathology* 37(1):1-5.
11. Courtney, S.C., Susta, L., Gomez, D., Hines, N., Pearson, J.E., Brown, C.C., Miller, P.J. and Afonso, C.L. (2012): Highly divergent virulent isolates of Newcastle Disease Virus from the Dominican Republic are members of a new genotype that may have evolved unnoticed for two decades. *J. Clin. Microbiol.* 51(2): 508-517.
12. Domingo, E. and Holland, J.J. (1997): RNA virus mutations and fitness for survival. *Annu Rev Microbiol.* 51:151-78.
13. Egyptian standard regulations for evaluation of veterinary Biologics (2009): *The Egyptian standard regulations for the central laboratory of veterinary Biologics*, 1, 2<sup>nd</sup> Ed.
14. El-Bagoury, G.F., El-Adaway, S.F., El-Habbaa, A.S. and El-Mahdy, S.S (2015): Isolation, identification and pathotyping of Newcastle disease virus from chickens in Egypt. *Benha Veterinary Medical Journal*, 29 (1):196–204.
15. El-Habbaa, A.S., El-Bagoury, G.F, El-Adaway, S.F., and ElMahdy, S. S. (2017): Isolation, pathotyping and genotyping of Newcastle disease virus from broiler chickens in Egypt. *J. Virol. Sci.* 1: 114-122.
16. Hu, Z., Hu, S., Meng, C., Wang, X., Zhu, J. and Liu, X. (2011): Generation of aa genotype VII Newcastle disease virus vaccine candidate with high yield in embryonated chicken eggs. *Avian Dis.* 55: 391-397.
17. Igwe, A. O. and Eze, D. C. (2016): Evaluation of the Efficacy of Inactivated Oil-Emulsion Newcastle Disease Komarov Vaccine against Clinical Disease, Lesions and Immune Response, Following Challenge with Velogenic Newcastle Disease Virus in Laying Chickens. *Nigerian Veterinary Journal* Vol. 37 (1): 32-44.
18. Kapczynski, D.R. & King, D.J. (2005): Protection of chickens against overt clinical disease and determination of viral shedding following vaccination with commercially available Newcastle disease virus vaccines upon challenge with highly virulent virus from the California 2002 exotic Newcastle disease outbreak. *Vaccine* 23: 3424–3433.
19. Kattenbelt, J.A., Stevens, M.P. and Gould, A.R. (2006): Sequence variation in the Newcastle disease virus genome. *Virus Research* 116: 168–84.
20. Ke, G.M., Liu, H.J., Lin MY, Chen, J.H., Tsai, S.S. and Chang, P.C. (2001): Molecular characterization of Newcastle disease viruses isolated from recent outbreaks in Taiwan. *Journal of Virological Methods* 97: 1–11.
21. Madadgar, O., Karimi, V., Nazaktabar, A., Kazemimanes, M., Ghafari, M.M., Dezfouli, S.M.A. and Hojjati, P. (2013): A study of Newcastle disease virus obtained from exotic caged birds in Tehran between 2009 and 2010. *Avian Pathol.* 42(1): 27-31.
22. Miller, P. J., Afonso, C. L., Attrache, J.El, Dorsey, K.M., Courtney, S.C., Guo, Z. and Kapczynski, D.R. (2013): Effects of Newcastle disease virus vaccine antibodies on the shedding and transmission of challenge virus. *Dev. Comp. Immunol.* 41: 505-513.
23. Miller, P.J.; Decanini, E.L. and Afonso, C.L. (2010): Newcastle disease: evolution of genotypes and the related diagnostic challenges. *Infection, Genetics and Evolution* 10: 26-35.
24. Mohamed, M.H., Kumar, S., Paldurai, A., Megahed, M.M., Ghanem, I.A., Lebdah, M.A. and Samal, S.K. (2009): Complete genome sequence of a virulent Newcastle disease virus isolated from an outbreak in chickens in Egypt. *Virus Genes* 39: 234-237.
25. Monir, N. M., El-Bagoury, G.F., Omar, L. M. and Sharawi S. A. (2018): Evaluation of Locally Prepared Inactivated Newcastle Disease Virus Vaccine. *BVMJ* 34(2): 278-290.
26. OIE, (2019): Newcastle disease. *OIE manual of standards for diagnostic tests and vaccines*. Version adopted by the World Assembly of Delegates of the OIE in May 2019.
27. Osman, N., Sultan, S., Ahmed, I.A., Ibrahim, R.S., and Sabra, M. (2014): Isolation and Pathotyping of Newcastle Disease Viruses from Field Outbreaks among Chickens in the Southern Part of Egypt 2011–2. *Global Veterinaria* 12: 237-243.
28. Radwan, M. M., Darwish, S. F., El-Sabagh, I. M., El-Sanousi, A. A. and Shalaby, M. A. (2013): Isolation and molecular characterization of Newcastle disease virus genotypes II and VIII in Egypt between 2011 and 2012. *Vir. Gen.* 47: 311–316.

29. Saif, Y.M, Barnes, H.J. and Fadly, A.M et al, (2003): Diseases of Poultry; Blackwell Publishing Company, Iowa, USA.
30. Sarcheshmei, M., Dadras, H., Mosleh, N. and Mehrabanpour, M.J. (2016): Comparative evaluation of the protective efficacy of different vaccination programs against a virulent field strain of the Newcastle Disease virus in broilers. Brazilian Journal of Poultry Science 18(3): 363-370.
31. Tang, Y., Lee, C.W.J., Zhany, Y., Senn, D.A.J., Dearth, R. and Byram, B. (2005): Isolation and characterization of H3N2 influenza A virus from turkeys. Avian dis. 49: 207-213.
32. Yusoff, K. and Tan, W.S., (2001): Newcastle disease virus: macromolecules and opportunities, Avian Pathol. 30: 439-455.
33. Zhang, S., Wang, X., Zhao, C., Liu, D., Hu, Y., Zhao, J. and Zhang, G. (2011): Phylogentic and pathological analysis of virulent Newcastle disease viruses isolated from domestic ducks in China. J. PLoS one 6(9): 1-9.
34. Zou, Y.J., Liu, Y., Wang, Y.Q., Wu, J., Gong, F.L. and Ma, G.H. (2016): Immune Effect of HTCC Hydrogel Microspheres and the Blending Emulsion of Oil and Microspheres as New Adjuvants of Combined Vaccine against Newcastle Disease and Avian Influenza. Available at: <http://www.jproeng.com/EN/abstract/abstract2719.shtml>