Short Communication

Persistence of Newcastle Disease Virus Genotype II and Shedded Genotype VII in Poultry Farm Environment

Fady Samir[1], Rania F. El Naggar[2], Mohamed M. Hamoud[3], Manal M. Zaki[1], Abdulrhman M. Gamal[1], Samah E. Laban[1], Shaimaa A. E. Nasr[1], El Shaimaa Ismael[1], Osama K. Zahran[1]*

[1]Department of Animal Hygiene and Veterinary Management, Faculty of Veterinary Medicine, Cairo University, Giza, 12211, Egypt; [2]Department of Virology, Faculty of Veterinary Medicine, University of Sadat City, Sadat, 32897, Egypt; [3]Department of Poultry and Rabbit diseases, Faculty of Veterinary Medicine, Cairo University, Giza, 12211, Egypt.

Abstract | Despite intensive vaccination programs in many countries, including Egypt, Newcastle disease still being a significant threat to the poultry industry. Bad management for poultry byproducts (e.g. manure) might be a reason for environmental pollution which contaminate the environment either chemically through haphazard usage of medications and or biologically through massive use of homologous/heterologous vaccines especially the live ones. The aim of the current work is to estimate the persistence time for velogenic Newcastle disease virus (NDV) of genotype VII in poultry manure in compare to LaSota vaccine strain as a model of genotype II. Velogenic NDV of genotype VII are a major threat to the Egyptian poultry industry since 2011 onwards with continuous evolution and diversity. In the present study, manure from specific pathogen free (SPF) chicks was contaminated with allantoic fluid of NDV and kept at room temperature ranged from 20 to 25°c which is nearly the same as poultry farms on different coupon surfaces (metal, plastic and cooling pads). Daily examination of poultry manure for the persistence of NDV was conducted on the basis of virus isolation followed by haemagglutination assay (HA) which revealed that NDV survived in the manure up to 28 days. Likewise, monitoring for the manure pH and moisture % was carried out. Our explanation for the long-time course for NDV persistence due to increasing the moisture %. In this context, the findings of this study revealed that genotype VII viruses of class II NDV are circulating in Egypt. Furthermore, genotype II-based vaccines seem to be not efficient in stopping or controlling NDV infections among Egyptian poultry farms. On the other hand, the vaccination strategy depending on such vaccines may lead to the emergence of new variants and, consequently, the emergence of a different evolutionary pattern of NDV in Egypt. Therefore, it is necessary to test if genotype II-based vaccines apply unique selective pressures on NDV glycoproteins and their role in changing the NDV evolution in Egypt.

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***Correspondence** | Osama K. Zahran, Department of Animal Hygiene and Veterinary Management, Faculty of Veterinary Medicine, Cairo University, 12211, Giza, Egypt; **Email:** zahran771953@gmail.com
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Introduction

Newcastle disease (ND) is one of the most important diseases of poultry worldwide, negatively affecting trade and poultry production because of the high mortality and morbidity associated with it (Ganar et al., 2014). The disease was first observed in Java, Indonesia, in 1926, and

spread to England in the same year, where it was first recognized in Newcastle-upon- Tyne, hence the name (Doyle, 1927). ND is caused by avian avulavirus 1 (AAvV-1), which belongs to the genus Avulavirus, family *Paramyxoviridae* (Amarasinghe et al., 2017). Newcastle disease virus (NDV) can infect a wide range of avian species and can be particularly pathogenic in chickens. The disease has significant impacts throughout the world in areas of Central and South America, Asia, Middle East and Africa, where it is endemic (Miller and Koch, 2013). It is also a significant biosecurity risk in ND free countries where sporadic outbreaks can have great impacts on trade.

Biosecurity programs should address the control of the major routes for transmission of diseases and pathogens which include; i) direct transmission from other poultry, domesticated and wild animals and humans, ii) fomites, such as equipment, facilities and vehicles, iii) vectors (e.g. arthropods and rodents), iv) aerosols, v) water supply and vi) feed. Outcome based measurables include incidence of diseases, metabolic disorders and parasitic infestations, mortality, performance. Animal health management include preventive medicine and veterinary treatment which should be aware of the signs of ill-health or distress, such as a change in feed and water intake, reduced growth, changes in behavior, abnormal appearance of feathers, dropping, or other physical features.

Since the expansion of the Egyptian commercial poultry sector, ND has caused devastating losses to the poultry industry and possesses a major threat to commercial poultry (Hussein et al., 2014; Radwan et al., 2013). To effectively control disease; mass vaccination programs have been implemented in commercial poultry facilities with limited success (Hussein et al., 2014; Radwan et al., 2013). There is an emerging evidence of failure for live and/or inactivated vaccines in protecting birds from current field isolates of NDV, and therefore, it is necessary to investigate the relationships among the NDV isolates that are being used in vaccines and those found in wild birds and commercial poultry which in commonly defined as vaccine matching (El Naggar et al., 2018). These investigations will establish the foundation for understanding the nature and dynamics of viruses that cause clinical disease in vaccinated flocks and in unvaccinated commercial and backyard poultry. In addition, it has become important to develop new vaccines formulated of currently circulating NDV strains to control and prevent disease outbreaks.

The purpose of this study is to provide more applicable virus persistence information for the establishment of more accurate manure management protocols for both field (Genotype VII) and vaccinal strains (LaSota as model for Genotype II) shedding in simulating conditions like farm environment at consistent temperature range between 20-25°C and relative humidity 40-50% simulating empty poultry house after cleaning and before disinfection when proper cleaning is not done especially on different farm surfaces as; metal (feeder turfs), plastic (drinkers) and paper carton (cooling pads).

Materials and Methods

Viruses

The suspected field pathogenic velogenic Newcastle disease virus was isolated from the litter of broiler breeder flock aged 32 weeks old showing high mortalities, sever decrease in egg production and deformity in eggs shape and color. The herd was vaccinated with inactivated LaSota (Genotype II) vaccine at 8, 65 and 133 days and live attenuated LaSota vaccine at 12, 35, 70, 103, 149, 185 and 210 days. The clinical symptoms started 10 days before sample collection. The purpose of using such locally isolated virus strain was to give the test the maximum simulation of Egyptian field conditions. LaSota strain (genotype II) was kindly provided by Prof Osama Zahran, Faculty of Veterinary Medicine, Cairo University, Egypt.

Virus isolation from infected poultry litter and antigenic characterization

Dilute the pooled litter/fresh feces with PBS, clarify the sample by centrifugation at 3000 rpm/30 minutes and finally collect the supernatant followed by addition of antimicrobial agent)Antibiotic-Antimycoplasma -Antimycotic) to the supernatant and allow standing for 1 hour at room temp. The virus was propagated by inoculation of 9-10-day old specific pathogen free embryonated chicken eggs (SPF ECEs) (white leghorn flock SPF Fayum, Kom Oshim, Fayoum, Egypt). Titration of the virus stock was completed by preparing 10-fold serial dilutions of the allantoic fluid with antibiotics; 0.2 ml of each dilution was inoculated into five SPF eggs. Virus titer was determined by calculation of the 50% egg infectious dose (EID_{50}) (Reed and Muench, 1938). The harvested allantoic fluid

was used in hemagglutination test. Positive samples were subjected to hemagglutination inhibition test (OIE, 2012) using NDV specific antisera to confirm the presence of NDV. A mixture of the diluted virus with 50 gm SPF dropping 1:1. The virus titer for the mixture titration to evaluate start titer of the field/vaccine virus used for contamination of coupons used to simulate the farm environment was 4 $\log^2$ and 5 $\log^2$ for both field and vaccine strain respectively.

Genetic characterization, sequencing, sequence analysis and phylogeny of Velogenic Newcastle disease virus

Total RNA was extracted from allantoic fluid with the use of the QIAamp Viral RNA Mini Kit (Qiagen, USA) and reverse transcribed with Superscript First Strand Synthesis RT-PCR kit (Invitrogen, USA) and random primers. The presence of NDV was confirmed by PCR targeting F gene (Mase et al., 2002) and virulence was determined by partial sequencing of F gene covering the cleavage site. Virus stock was titrated and virus EID_{50} was calculated using Reed and Muench (1938). The NDV F gene sequence was initially analyzed in Chromas PRO v1.7.3 (http://technelysium.com.au/) to confirm the quality of read data. After alignment, the sequence of the NDV isolates was subjected to blast analysis in NCBI BLAST tool (https://blast.ncbi.nlm.nih.gov/Blast.cgi). The phylogenetic analysis based on the partial nucleotide sequence of F gene in the highly variable region including cleavage site was performed in MEGA6 software by using the Maximum Likelihood method with default settings and 1000 bootstrap re-sampling (Tamura et al., 2013). NCBI BLAST was used for nucleotide and amino acid identity comparisons between representative isolates within identified clusters. The filed NDV virus reported in this study was submitted to GenBank under the accession number KF709445.

Invitro comparison for the persistence between NDV genotype II and genotype VII

SPF manure dropping was diluted by one part/one part of PBS resample feces that occurs after poultry infection. Virus contamination was carried out using field strain of genotype VII and the LaSota vaccine strain of genotype II which are usually shedded from chickens' droppings following infection and or vaccination, respectively. The virus was diluted using 50 ml PBS solution and subjected to HA test. A mixture of the diluted virus with 50 gm SPF dropping (1:1) was used. HA test was conducted to evaluate the virus start titer for contamination of coupons (metal, plastic and cooling pads) which was 4 and 5 $\log^2$ for both field and vaccine strain, respectively. Three types of surface coupons with dimensions (2x2x1cm^3) were used (Figure 1); simulating the poultry farm environment; metals like feeders and cages, plastic as drinker's bill or nipples and cooling pads like cellulose paper.

Three coupons from each surface (steel, plastic and cooling pad) were sterilized by autoclaving and placed in sterile petri dish. Coupons were covered with 3 ml of 4 $\log^2$ and 5 $\log^2$ of chorioallantoic fluid of Genotype VII and LaSota, respectively. The virus-manure mixture was kept on coupons till end of experiment day after day. Each coupon was scraped with sterile pipette, and the fluid was aspirated from the petri dish and jetted back onto the coupon three times to dislodge virus from the coupon. The fluids from petri dish was pooled into a single tube, then three 10- fold serial dilutions were made in sterile PBS resulting in dilutions from 10^{-1} to 10^{-3}. Virus re-isolation attempts were made using each dilution by injecting 9-11-day old SPF chicken eggs via allantoic route, Eggs were candled daily for 3 days and the dead eggs were chilled for 24 hrs., then opened and the allantoic fluids were aspirated, examined for HA activity and EID_{50} was calculated via the method of Reed and Muench (1938).

Results and Discussion

Vaccination against NDV dates back more than 60 years (Dimitrov et al., 2017). However, to date, the virus continues to cause outbreaks in numerous poultry farms around the world. Vaccines prevent many millions of illnesses and save numerous lives every year (WHO, 2017). Conventional vaccine approaches, such as live attenuated, inactivated and subunit vaccines, provide durable protection against a variety of dangerous diseases (Plotkin, 2009). Despite this success, there remain major hurdles to vaccine development against a variety of infectious pathogens, especially those better able to evade the adaptive immune response. Moreover, for most emerging virus vaccines, the main obstacle is not the effectiveness of conventional approaches but the need for more rapid development and large-scale deployment. The decision to introduce and choose a vaccine is greatly influenced by several factors such as bio-burden, epidemiological aspects with

Figure 1: *Different coupon surfaces (metal, plastic and cooling pads) used in this study.*

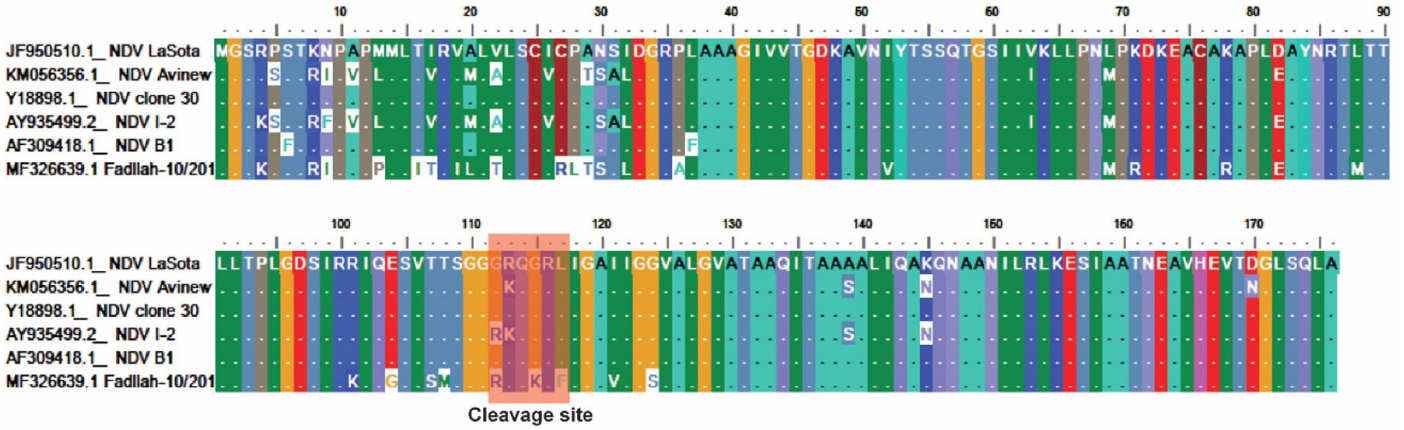

Figure 2: *Amino acids mutation trend analysis for F protein of field strain velogenic NDV genotype VII in compare to commonly used vaccine strain of Genotypes I and II.*

special reference to transmission, vaccine factors that include safety, efficacy and availability, feasibility of introduction, financial implications and projected or expected benefits in terms of morbidity, mortality and cost effectiveness. Thus, a vaccine that is ideal for use would be one that is highly efficacious, economical, safe and protects against disease. Therefore, in designing effective vaccines certain key elements are essential such as; 1) An antigen that can stimulate good immune response, 2) presentation of antigens to augment the immune response and 3) presentation of antigens in native form. The purpose of this study was to provide more applicable virus persistence information for the establishment of more accurate manure management protocols for both field (Genotype VII) and vaccine (LaSota) strains for the conditions simulating the farm environment from temperature and relative humidity especially on different farm equipment as; metal (feeder turfs), plastic (drinkers) and cooling pads (paper carton).

As mentioned earlier, antigenic differences between the vaccine strains belonging to genotypes I and II, and the virulent NDV strains of genotypes V, VI and VII causing ND outbreaks may contribute to the increased virus shedding and maintenance of the virus in vaccinated flocks or the environment (Miller et al., 2013). Virulent strains from new genotypes continue to be isolated from chickens suggesting that vaccination using conventional strategies has not been, nor will be the complete solution to control the disease (Miller et al., 2013). The challenge is ever increasing, due to the diversity of viruses that appear every year; thus, it is expected that sooner or later adjustments to increase antigenic similarity between the vaccines and field viruses will be needed. As depopulation is often not a viable economical alternative and biosecurity is limited to highly advanced farms, it has been suggested that other strategies need to be developed to enable better control of ND. These strategies should not only aim

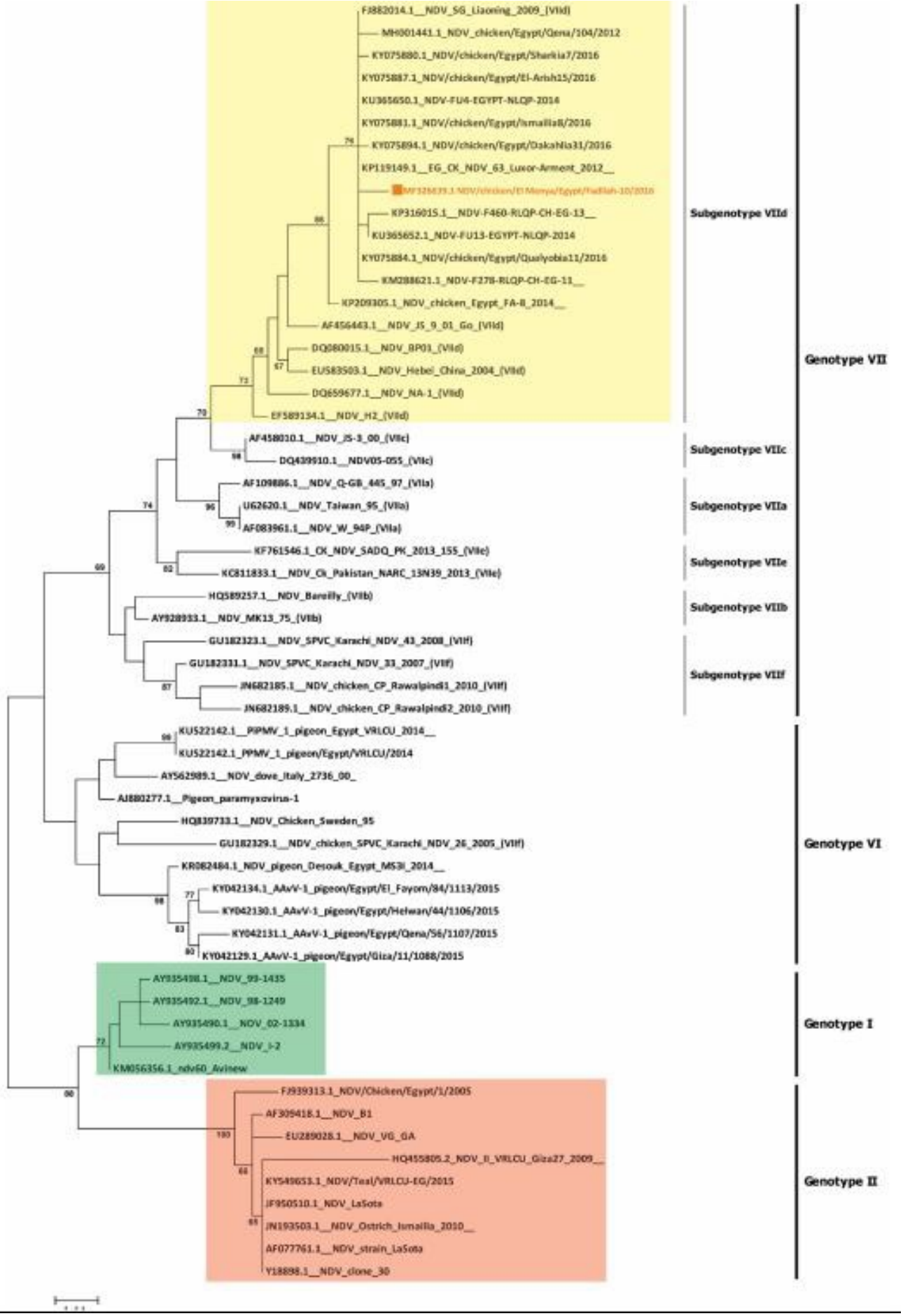

Figure 3: *Phylogenetic analysis of the studied NDV isolate genotype VII and their clustering patterns with representative AAvV-1 isolates of each genotype. Reported isolates clustered within genotype VII of class II. The position of clustering is indicated by a yellow box.*

to prevent mortality but also to reduce the virus shedding, thus reducing the persistence of the virus in the flock, with both parameters being indicators of vaccine efficacy (Miller et al., 2010). For that purpose, one strategy proposed in recent years has been the development of antigenically matched vaccines; i.e., vaccines formulated based on a vaccine viral seed that belongs to the same circulating genotype as the challenge virus. This strategy has shown to be effective for both inactivated and live vaccines developed from homologous genotypes of the challenge virus, to increase efficacy against virulent challenge strains circulating in the field, and above all, on reducing the virus shedding (Miller et al., 2007; 2009a; Hu et al., 2009; Absalón et al., 2012b; Dimitrov et al., 2017a, b). Antigenically matched low virulence vaccine seeds are normally created through reverse genetics following established procedures (Cardenas-Garcia and Afonso, 2017). Antigenically matched NDV vaccines have demonstrated to be efficient for preventing mortality while significantly reducing viral shedding (Hu et al., 2009, 2011; Absalón et al., 2012). It has been reported that immunization with recombinant NDV vaccines of genotype VII conferred protection against mortality with the absence of clinical signs when challenged with virulent NDV strains of genotype VII like those circulating in Asia (Ji et al., 2018).

In Egypt, ND has been endemic since its first identification in 1948 (Daubney and Mansy, 1948) and despite routine massive vaccination programs, severe outbreaks occur frequently, and the source of the virulent variants is still unclear. To control NDV infections in Egyptian poultry, avirulent and attenuated vaccines, whose strains belonged to genotype II NDV (e.g. LaSota and Hitchner) have been used. Therefore, most studies on the phylogeny and evolution of ND viruses are relying on the characterization of partial F gene sequence including the F0 cleavage site. However, there are several regions within F protein (e.g. FP and HR a, b and c) involved in the protein conformational changes, which are crucial for viral infectivity and pathogenicity (Ayllon et al., 2010). Additionally, a relatively new system for classifying ND viruses showed the importance of the full-length F protein for the accurate phylogenetic analysis of NDV isolates (Diel et al., 2012). In this study, the entire F gene of NDV isolate obtained from El Menya province in Egypt during 2016 was sequenced. Further analysis of its deduced amino acid sequence was performed to test for the presence of substitutions within the other critical regions of the F protein beside the cleavage site motif (Figure 2). By phylogenetic analysis, the studied isolate showed close genetic relatedness and were clustered within genotype VII (Figure 3). Thus, the failure of preventing and controlling infections caused by strains of such genotype (VII) since its detection from 8 years ago in Egypt (Radwan et al., 2013) is considered a major enigma requiring more investigations. The studied isolate had an identical polybasic motif ^{112}RRQKRF^{117} at the F protein cleavage site (Figure 2), indicating their high virulence (de Leeuw et al., 2003, 2005). Genotype VII matched chimeric vaccines showed a high protection level against infection by NDV genotype VII viruses (Kim et al., 2013). Therefore, the production of genotype VII-matched chimeric vaccines on the basis of field strains could be a promising choice for controlling recent ND outbreaks in Egypt.

In this study, NDV virus when mixed with SPF dropping on different surfaces (metal, plastic and cooling pads) of poultry farm, it remained stable and was able to be infective for 9-11 days old embryonated SPF eggs resulting in embryos death. The harvested allantoic fluid was subjected for centrifugation at 10000 rpm for 10 minutes for clarification then it was examined by HA test which was positive for up to three weeks before the titer dropped below Mean Infectious Dose (MID) value which couldn't be infective. Comparing the persistence time of NDV outside poultry (host) either genotype II or genotype VII on different surfaces, on the galvanized steel (metal) like feeder troughs or cages, the virus survived and be infective till day 22 in both genotype II and genotype VII (Figure 4A). While in case of plastic surfaces like drinking instruments, the virus was able to survive till day 19 in case of genotype II, however for genotype VII, it survived for 22 days (Figure 4B). On the other hand, NDV was still infective on cooling pads surface ill day 22 for both genotype II and genotype VII (Figure 4C). The main parameters that were considered, was the persistence of the NDV vaccine virus (genotype II) and field strain (genotype VII) when coated with SPF dropping and exposed to room temperature on different surfaces.

Our field epidemiological findings for ND outbreaks in poultry farms gave an impression that mortality due to ND in spite of vaccination with Genotype II

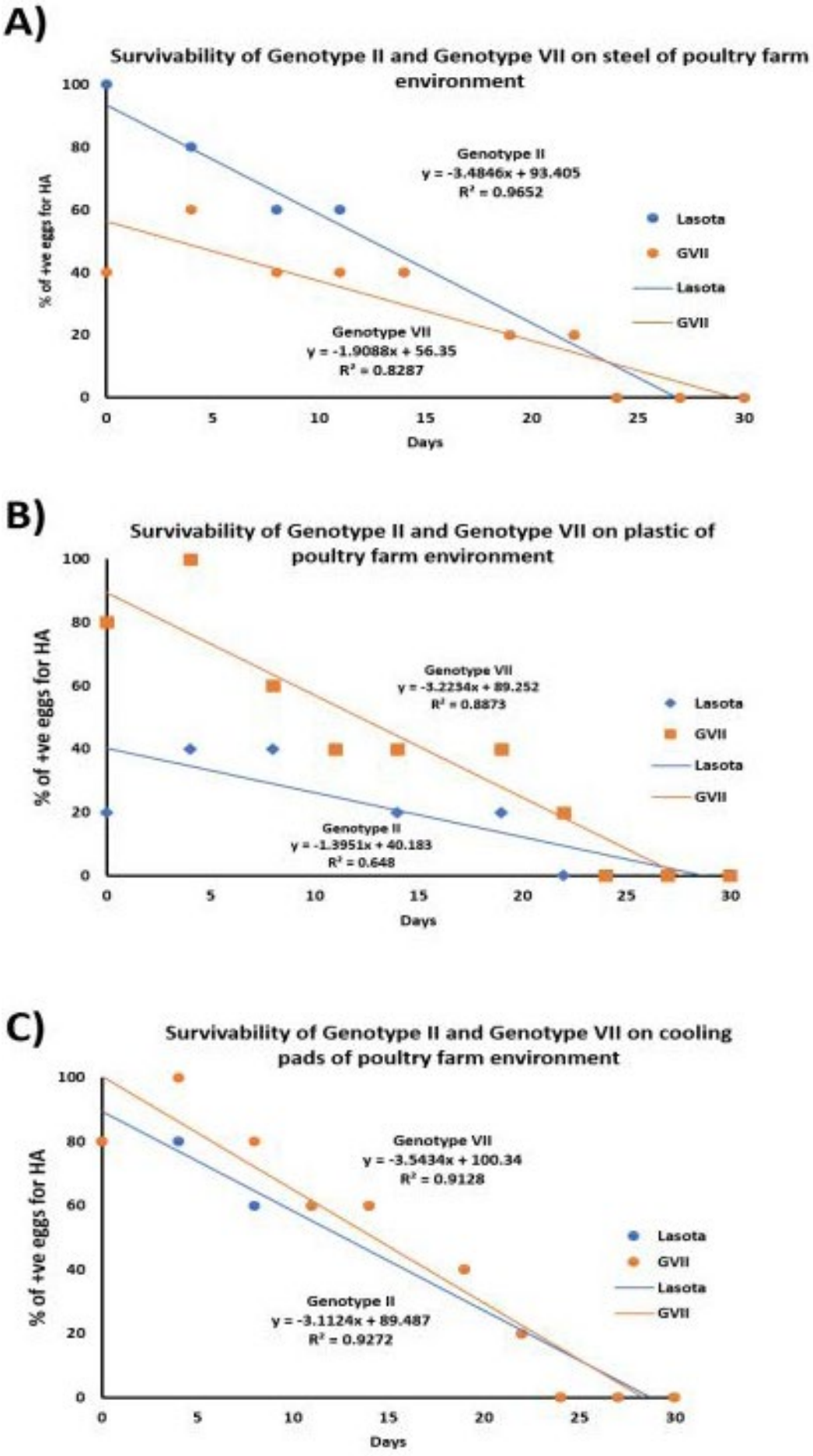

Figure 4: *Comparison of persistence of Genotypes II and VII on different surfaces A) metal, B) plastic and C) cooling pads of poultry farm environment.*

virus strain reached above 50% among broiler poultry flocks or sever egg drop and deformity in eggs in either layer or breeders farms. Virus shedding from live birds either through vaccine and or field strain are considered a source of reinfection in poultry farms. Coated virus droppings and the virus contaminated poultry surfaces could survive and caused reinfection to the same bird flocks beside poor sanitation and disinfection procedures between poultry cycles. The present study proved that, there was a great affinity for NDV not only able to survive but also to induce infection while residing outside live chicken. There wasn't a huge difference in persistence time between different NDV. The maximum duration for ND to be infectious outside the poultry body is 22 days while the minimum duration is only 19 days.

In conclusion, our findings revealed that genotype VII viruses of class II NDV are circulating in Egypt. The analysis of the entire F protein, not only the cleavage site motif, improves our understanding of NDV evolution. Furthermore, genotype II-based vaccines seem to be not efficient in stopping or controlling NDV infections among Egyptian poultry farms. On the other hand, the vaccination strategy depending on such vaccines may lead to the emergence of new variants and, consequently, the emergence of a different evolutionary pattern of NDV in Egypt. Therefore, it is necessary to test if genotype II-based vaccines apply unique selective pressures on NDV glycoproteins and their role in changing the NDV evolution in Egypt. Likewise, it is recommended to thoroughly and speedy remove of debris of litter as soon as removing the live flock from the farm as these debris are considered to be a main source of contamination and reinfection of different microbes. It is recommended to decrease the viral load in the farm through allowing adequate time between two flocks about three weeks including sanitation time.

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Authors Contribution

Conceptualization: F.S, R.F.E, M.M.H, A.M.G, S.A.E.N, E.I. and O.K.Z. Data curation: F.S, M.M.Z, S.E.L, S.A.E.N, E.I. and O.K.Z. Formal analysis: F.S, R.F.E, M.M.Z, M.M.H. and O.K.Z. Investigation: F.S, R.F.E, M.M.Z, A.M.G, S.E.L, E.I. and O.K.Z. Methodology: F.S, A.M.G, S.E.L, S.A.E.N. and O.K.Z. Supervision: E.I. and O.K.Z. Validation: S.E.L, S.A.E.N, E.I. and O.K.Z.. Writing: original draft: F.S, R.F.E, A.M.G, S.E.L, and O.K.Z. Writing review & editing: R.F.E, M.M.H, M.M.Z, and O.K.Z.

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