Research Article

Evaluation of Moisture percentage and pH Scale in Relation to Survivability of Vaccine Escape Mutant Newcastle Disease Virus Genotype VII in Poultry Manure

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Abstract | Newcastle disease virus (NDV) is still a havoc to the poultry flocks and outbreaks have occurred even in vaccinated flocks. Commercial Newcastle disease virus vaccines can provide various levels of protection against challenge with different NDV genotypes, raising the importance of the relationship between vaccines and field strains. The aim of the current work is to evaluate the effect of moisture percentage and pH on the persistence of velogenic NDV genotype VII in the poultry manure which considers a major threat to the Egyptian poultry industry since 2011 onwards. In the present study, manure of specific pathogen free (SPF) chicks was contaminated with allantoic fluid of 8 $\log^2$ NDV and kept at room temperature then daily examination of poultry manure for the survival of NDV on the basis of virus isolation and haemagglutination assay (HA). Our results revealed that pH of the manure from time of its mixing with the NDV infected allantoic fluid till day 4 was 7, then pH values changed progressively and recorded 8.5 on day 12. After that, pH increased towards the alkaline side and recorded pH = 9 till day 33 of the experiment. Regarding moisture %, after mixing the manure with allantoic fluid the moisture % of the mixture was 67%, and this wet manure remained moist (67%) till day 4; due to the humid environmental conditions applied at room temperature. Later, the moisture content of the treated manure decreased gradually and recorded 52.5%, 40% and 32% at the 8th, 28th and 33rd days of experiment; respectively. To recapitulate, both moisture % and pH of the poultry manure is greatly affect Newcastle disease virus persistence in the farm environment which is a critical point during cleaning and precleaning for the poultry farms to eliminate the viruses along with the use of effective disinfectants.

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Introduction

Newcastle disease virus (NDV) is a major threat to the poultry industry around the globe. The disease is endemic in many developing countries while the disease-free countries are prone to accidental outbreaks (Aldous and Alexander, 2001). Newcastle disease virus (NDV) belongs to family *Paramyxoviridae*

and is well characterized member among the avian paramyxovirus serotypes. NDV strains having varying degree of virulence and circulate among avian species. The topographical distribution of NDV is not well under-stood and regular sporadic cases are reported throughout the years from endemic areas (Aldous and Alexander, 2001). First confirmed outbreaks of NDV occurred in 1926 in Java, Indonesia and in Newcastle-Upon Tyne, UK (Aldous and Alexander, 2001). Poor manufacturing standards, lack of adequate storage facilities, application of expired vaccine batches, faulty application and vaccine handling during transportation (heat stress and water deprivation) also lead to production of steroids and thus consequently lead to vaccination failure and immunosuppression (Vui et al., 2002). Newcastle disease (NDV) was identified for the first time in Egypt in 1948 (Daubney and Mansy et al., 1948), since then, Egypt has been regarded as an endemic country by NDV.

Reports of vaccination failure from many countries and from our field observations on reduced the ability of classical vaccines to significantly decrease viral replication and shedding create an interest in developing vaccines formulated with genotypes homologous to the virulent field NDVs (Kapczynski et al., 2013). While intensive ND vaccination programs using LaSota like strains, vaccination failure against velogenic NDV genotype VII in Egypt was reported, as they were still circulating in the field causing significant economic losses (El Naggar et al., 2018). The genetic variation between the used vaccine strains and the circulating NDV viruses may explain the inability of the currently used vaccines to protect chicken against NDV genotype VII. Further studies are needed to screen the protection of the currently used vaccines against recently isolated ND strains (El Naggar et al., 2018). Previous studies reported that the homologous vaccine to the field virus reduced virus shedding significantly more than the heterologous vaccines (Dimitrov et al., 2017). Proper vaccination protects the birds from clinical disease, but it does not prevent virus replication and shedding, which results in infection (Cho et al., 2008).

Research priorities are toward the improved diagnostics and better vaccine development while there is a need to study the effect of environmental farm conditions and focusing on novel biosecurity measures. The overarching goal of biosecurity is to prevent, control and/or manage risks to life and health as appropriate to the biosecurity sector. In doing so, biosecurity is an essential element of sustainable agricultural development. The success of NDV vaccination program may depend on a minimum of 85% of the flock receiving a proper dose and responding to vaccination to achieve a high antibody titer after vaccination to ensure that no epidemic spread is possible in vaccinated populations. Previous studies indicated that a high fraction of birds (>85%) needs to have a high antibody titer (log^2 titer ≥3) after vaccination to ensure that no epidemic spread is possible (van Boven et al., 2008). The organic content of the fecal material, amount of moisture, exposure to the sunlight, residual disinfecting chemicals may all play a role in avian influenza virus survivability. Linear regression analysis of the virus infectivity in the embryonated eggs indicated the persistence of the virus did not deviate from the expected values for all the temperatures. The linear regression model indicated that the difference in the persistence of the virus in dry and wet feces at all temperatures was not very marked and there was a negative correlation between the virus survival and temperature while the virus was highly susceptible to the higher temperature (Kurmi et al., 2013). In the present study, isolation and genetic characterization for velogenic NDV from vaccinated flock with heterologous vaccine (LaSota) was carried out. Then, evaluation for the persistence of velogenic NDV in chicken manure with evaluation of moisture % and pH changes in manure conditions over a time period up to 33 days.

Materials and Methods

Sampling, virus isolation and propagation and antigenic characterization

Velogenic Newcastle disease virus (NDV) genotype VII was isolated from vaccinated commercial broiler flock of 40 days old from Giza governorate. The herd was vaccinated with inactivated LaSota (Genotype II) vaccine at 8 days and live attenuated LaSota vaccine at 12 and 35 days. 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, Egypt). Titration of the virus stock was completed by preparing 10-fold serial dilutions of the allantoic fluid in PBS with antibiotics; 200 mg gentamicin/ml, 2000 units penicillin/ml, and 4 mg amphotericin B/ml (Sigma Chemical Co., St. Louis, MO); 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}). The harvested allantoic fluid was used in hemagglutination test for the presence of a hemagglutinating virus. Positive samples were subjected to hemagglutination inhibition test (OIE, 2012) using NDV specific antisera to confirm the presence of NDV.

Molecular detection, sequencing and sequence analysis

For molecular identification of NDV, total RNA was extracted from the allantoic fluid which show positive HA using RNA extraction kit (QIAamp Viral RNA Mini Kit, QIAGEN, USA) according to the manufacturer's instructions. Conventional RT-PCR assays targeting the F gene of NDV were conducted as described earlier (Mase et al., 2002). The amplified PCR products were purified using a PCR Clean-Up System (Promega, Co., Madison, WI) according to the manufacturer's instructions and were sequenced directly using ABI PRISM Big Dye Terminator version 3.1 (Applied Biosystems, Foster City, CA). Sequence alignment, editing, and analysis were performed using Bioedit version 7.0.9.0 (Hall, 1999). Phylogenetic trees were constructed using the neighbor-joining method with the Kimura two-parameter model with 1000 bootstrap replicates in Molecular Evolutionary Genetics Analysis (MEGA) version 6 (Tamura et al., 2013). Well-known prototype strains of AAvV-1 were used as representative sequences of each genotype to establish reliable epidemiological associations. Sequence determined in this study was deposited to the GenBank database and is available under the accession number KF709445.

Contamination of chicken manure with the NDV isolate

Manure from specific pathogen free (SPF) chickens was kindly provided from kom oshime (SPF) egg production farm, Fayoum, Egypt. The manure was prepared as suspension and the filtered supernatant was firstly tested for HA activity which show negative result, then was inoculated into 9-11-days old SPF embryonated chicken eggs (ECEs) and incubated up to 3-7 days at 37◦C, then the harvest was tested with HA test to confirm that it is free from any HA viruses. This experiment contains two groups; tested and control at which each group consists of twenty-five grams of SPF chicken manure. The manure in the test group was contaminated with 5 ml of the allantoic fluid containing the vNDV isolate (8 $\log^2$). While, in the control group, the manure was mixed with 5 ml clean allantoic fluid. Both groups were left at room temperature in humid condition for up to 33 days according to similar studies conducted on avian flu (Horm et al., 2012).

Survivability of NDV in chicken manure and evaluation of moisture % and pH changes in manure conditions overtime

The infected and control groups were monitored and tested daily during the first week of experiment, then day after day till the 33rd day; for detection of HA activity. One gm from each manure group was added to 5 ml sterile PBS with antibiotics (Penicillin G $2x10^6$ IU, Streptomycin, 200 mg, Mycostatin $0.5x10^6$ IU, Gentamycin 250 mg) to make 20% suspension. The suspension was mixed thoroughly, centrifuged at 3000 rpm for 10 min, and filtrated by syringe filter (0.45 µm filter) prior inoculated in SPF ECEs (OIE, 2012). ten gm of thoroughly mixed litter were weighed, then put in hot air oven at 105 °C for 24 hours, cooled in a desiccator before being weighed again. The difference in weight represented the moisture content (Tucker, 1967). The variations in pH were measured (1:10 w/v manure–water extract) using pH electrode from day 0 till day 33.

Statistical analysis

Data analyses were performed using Microsoft Excel 2016 to examine frequency distribution. Correlation between changes in manure condition and NDV titers were determined by calculating Pearson's coefficient (r). The effect of change in pH and moisture % of manure on the NDV titer was presented through linear regression plot and coefficient of determination (R2).

Results and Discussion

Commercial NDV vaccines can provide various levels of protection against challenge with different NDV genotypes, raising the importance of the relationship between vaccines and NDV field strains. Evaluation of the vaccination program showed that vaccinated commercial broilers with genotype II vaccines (LaSota and Hitchner B1) on the day of hatching and 14 days of age were susceptible to velogenic NDV challenge which confirmed that heterologous vaccines cannot face of the virus filed infection with velogenic genotypes (e.g. genotype VII) (Hu et al., 2009). Vaccination might significantly decrease the amount of cloacal and oropharyngeal virus shedding compared to non-vaccinated birds which depend on the host

immunity, the host species, the amount and virulence of the infected virus, the dose and type of ND vaccine and the time between vaccination and infection (Miller et al., 2009). The virus shedding can be controlled by choosing vaccines that are more genetically like the infected virus (homologous vaccine) and which may be a useful strategy to limit the spread of the disease. For vaccine evaluation of their ability to provide protection against NDV, it may be useful to include their ability to decrease the amount of virus shedding after challenge, which potentially would decrease the spread of NDV. Likewise, the amount of NDV shed into the environment from vaccinated birds has been arisen as a potential indicator of vaccine efficacy (Miller et al., 2009).

The general approaches to biosecurity and control Newcastle disease are depend mainly on hygienic measures and vaccination especially in semi-intensive systems where birds are confined within a fenced yard or house. Hygienic measures include cleaning, disinfection, limiting access of wild birds, and personal hygiene for the farm staff. Vaccination in combination with appropriate hygiene measures, this remains the most effective way to control NDV (Moerad et al., 1987). Problems with NDV outbreaks may have to be expected unless the vaccination practice is improved substantially. The wider range of NDV in birds may be due to natural infection which is responsible to produce higher antibody titers than vaccination titers (Luc et al., 1992). Biosecurity measures include bird-proof houses, feed and water supplies, minimizing travel on and off the facility, disinfecting vehicles and equipment's that enter the farm (Abdisa and Tagesu, 2017). Pests such as insects and mice should also be controlled. If possible, employees should shower and change into dedicated clothing prior entry into the poultry farm (Abdisa and Tagesu, 2017).

The Egyptian poultry industry has experienced enormous economic losses due to continual outbreaks of velogenic ND in different sectors of the commercial poultry industry (El Naggar et al., 2018). It has been proposed that increased genetic divergence between field strains and vaccines may be responsible for disease outbreaks in vaccinated flocks due to insufficient immune protection induced by be the vaccine strains (Munir et al., 2012). Due to these problems, it is overbearing to investigate the evolutionary dynamics of field NDV strains together with their potential pathobiology in vaccinated mismatched poultry flocks. Genotype VII is the most prevalent group of NDV in the Middle East, and most NDV strains isolated from poultry dwelling this genotype (El Naggar et al., 2018). In this study, NDV isolate from broiler poultry farm was classified genetically into velogenic genotype VII.

Furthermore, comparative analysis for functional domains of the F protein and analysis of the deduced amino acid sequence at the cleavage site in the isolate characterized in the current study suggests stronger evolutionary constrains on its F proteins compared to those of other representative vaccine strains (Figure 1). These modifications in the F protein might explain the potential of this isolate for immune escape and the lack of sterilizing immunity provided by LaSota vaccines could result in altered fusion activity and neutralizing escape variants (Hu et al., 2009; Wang et al., 2015). Phylogenetic tree based on the nucleotide sequence for the isolate characterized in the current study indicates the circulation of genotype VII of NDV in the country as shown in Figure 2. Because the vaccine strains belong to genotype II and were isolated approximately 60–70 years ago and that most of the circulating Egyptian strains are clustered within genotype VII, it is likely that the genetic distances reflect antigenic differences that could result in a lack of complete protection against field viruses and shedding of virulent NDV strains in the environment. We could loudly speak that these old vaccine stains from the biosecurity point of view acts as continuous challenge of earlier virus infection leading to increase the load of virus in poultry flock environments. So, concurrent disinfection of poultry houses during the production and or rearing is essential which might reduce the virus load shedding in the poultry environment. These results may be useful for revising and/or updating the vaccine and biosecurity schedules currently being followed in Egypt. We could announce through this work homologues vaccination better and efficient than heterologous vaccine and even no vaccination is better than heterologous vaccination schedules

The NDV infected allantoic fluid that was mixed with the manure had an initial HA titer of 8 $\log^2$. The supernatant of this mixture recorded HA titer 4 $\log^2$ just after mixing the manure with the allantoic fluid. The diluted manure-virus mixture remained recording 4 $\log^2$ titer till the 6th day of the experiment (Table 1).

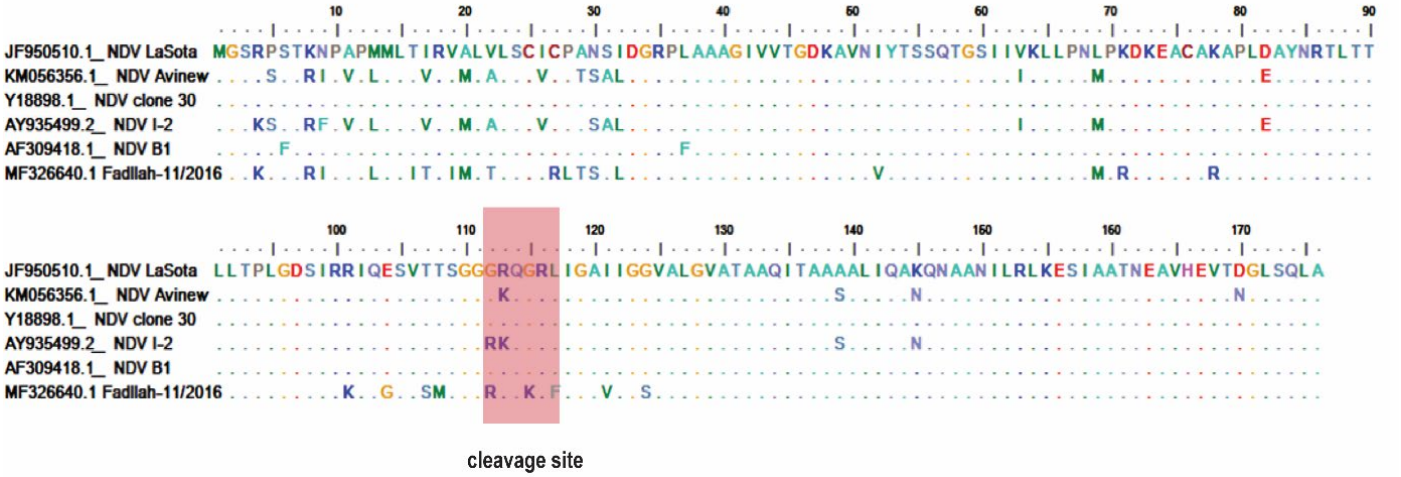

Figure 1: *Amino acids mutation trend analysis for F protein of field strain velogenic NDV genotype VII in compare to commonly used vaccine strain of Genotypes I and II.*

Table 1: *Evaluation of Persistence of NDV Genotype VII in poultry manure.*

Days after inoculation of SPF manure with NDV	Manure pH	Manure moisture %	HA activity (log 2) of manure supernatant before egg inoculation	Percentage of HA positive eggs after inoculation	Virus recovery	Mean HA titer (log 2) ± SD
1	7.00	67%	4	100%	+	4.33±1.53
2	7.00	67%	4	66.67%	+	2.67±3.06
4	7.00	67%	4	66.67%	+	1.67±1.53
5	8.25	60%	4	66.67%	+	3.33±3.06
6	8.50	58%	4	66.67%	+	2.33±2.52
7	8.50	54%	3	66.67%	+	2.33±2.08
8	8.50	52.5%	3	66.67%	+	3.33±3.06
11	8.50	48%	3	66.67%	+	2.00±1.73
12	8.50	46%	3	66.67%	+	5.33±0.58
14	9.00	46%	2	33.33%	+	0.67±1.15
19	9.00	46%	2	33.33%	+	0.67±1.15
21	9.00	44%	2	33.33%	+	5.00±2.00
23	9.00	44%	1	33.33%	+	1.67±1.53
26	9.00	44%	1	33.33%	+	2.00±1.73
28	9.00	40%	1	33.33%	+	2.00±3.46
30	9.00	36%	0	0%	-	0.00±0.00
31	9.00	34%	0	0%	-	0.00±0.00
32	9.00	32%	0	0%	-	0.00±0.00
33	9.00	32%	0	0%	-	0.00±0.00

The diluted mixture revealed persisted HA activity till day 28 with sustained egg infectivity, however, the titers were gradually fading (Table 1). The HA activity completely disappeared from day 30 onwards, as shown in Table 1. The NDV field isolate remained viable and infectious to SPF-ECEs for up to 28 days with Mean HA titers ranged from 1.67 to 5.33 $\log^2$ (Figure 3 and Table 1).

Manure of SPF chickens was successfully infected with NDV virus isolate (Genotype VII) to study how long it will survive. Daily variations in manure pH and moisture % were recorded. At constant room temperature range 20°C to 25°C. Supernatants of infected manure were tested for HA activity over the study period beside moisture % and pH ranges throughout the experiment (Figure 3 and Table 1). The pH of manure from time of mixing with the NDV infected allantoic fluid till the 4th day was 7, then pH values changed progressively and recorded pH = 9

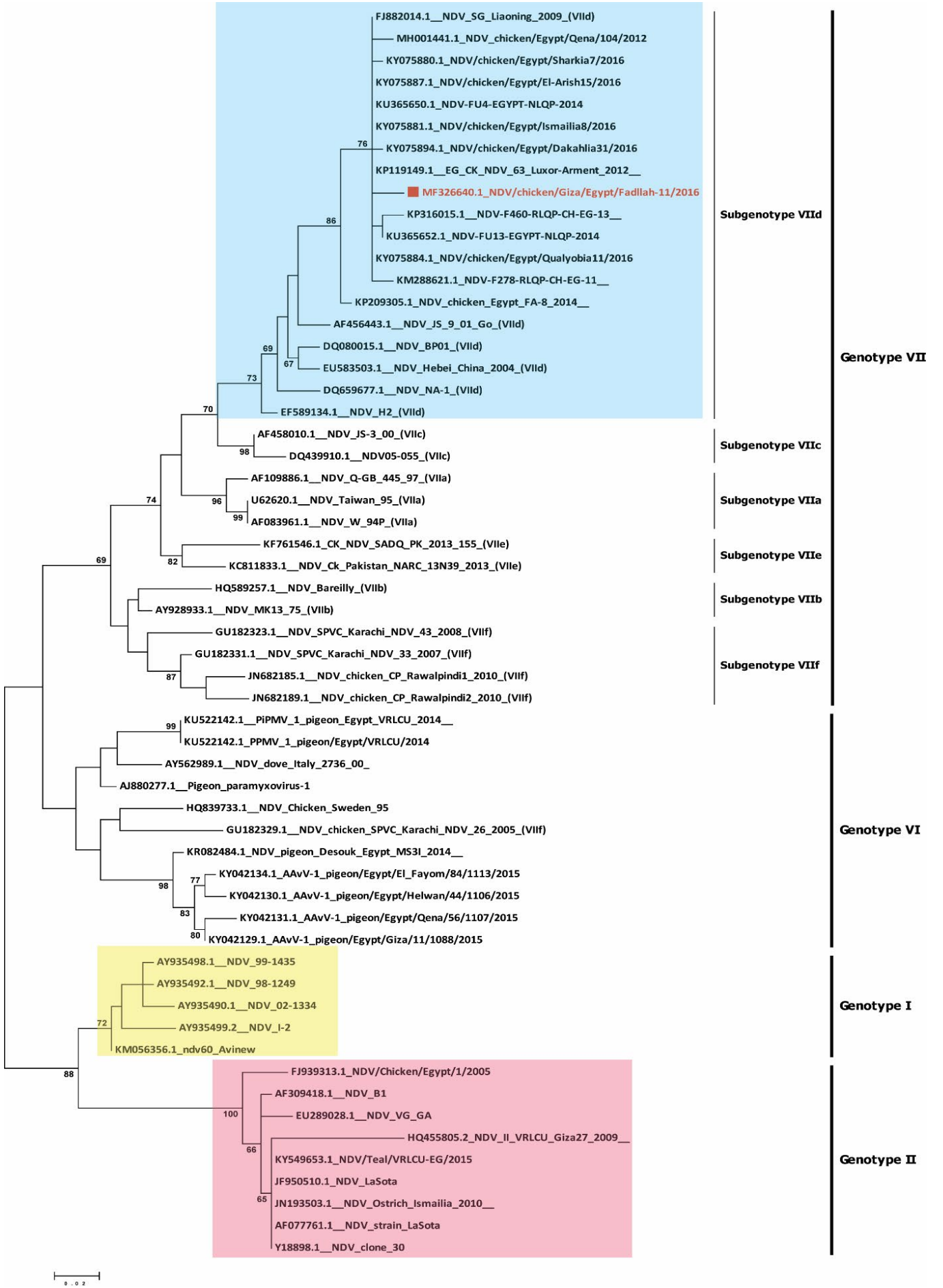

Figure 2: *Phylogenetic analysis of the studied NDV isolate genotype VII and their clustering patterns with representative AAvV-1 isolates of each genotype. Reported isolates clustered within genotype VII of class II. The position of clustering is indicated by a blue box.*

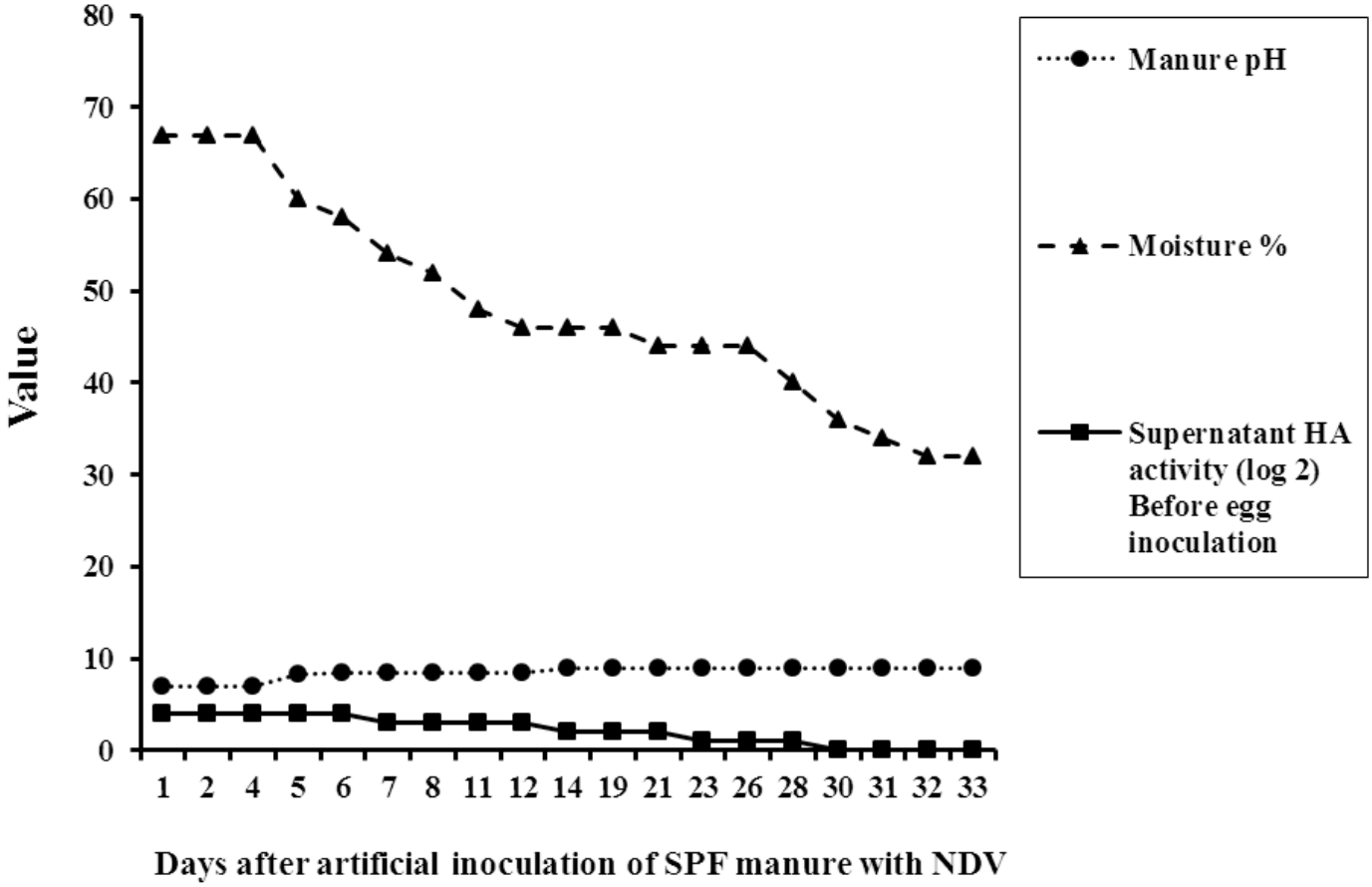

Figure 3: *Days interval for evaluation of moisture % and pH and their effect on velogenic NDV genotype VII survivability.*

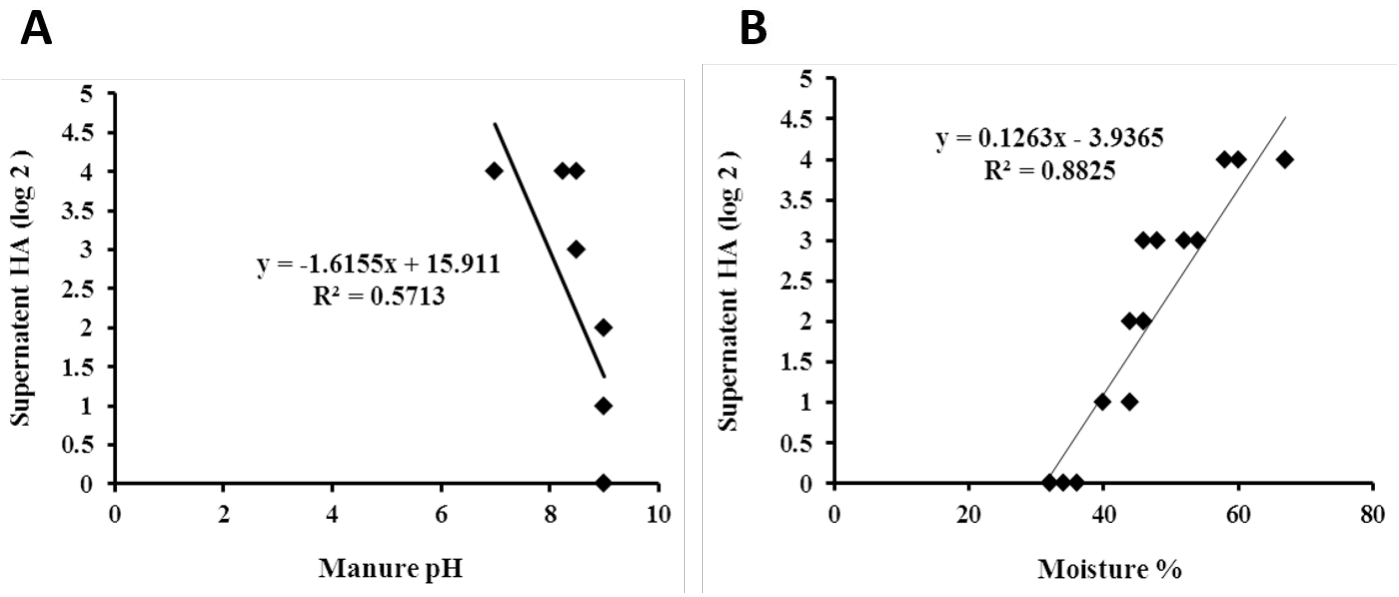

Figure 4: *Evaluation of A) pH and B) moisture % on the survival of velogenic NDV genotype VII in poultry manure.*

till day 33 of the experiment (Figure 3, Figure 4A and Table 1). Regarding moisture %, after mixing the manure with allantoic fluid the moisture % was 67%, and this wet manure remained moist (67%) till the 4th day; due to the humid environmental conditions applied at room temperature (Figure 3, Figure 4B and Table 1). Later, the moisture content of the treated manure decreased gradually and recorded 52.5%, 40% and 32% at the 8th, 28th and 33rd days of experiment; respectively (Figure 3, Figure 4B and Table 1). 8.5 till day 12 (Figure 3 and Table 1). After that, pH increased towards the alkaline side. In conclusion, the data regarding the hygienic disposal of manure from NDV infected farms in Egypt is limited. In this study, we investigated how long the NDV will survive in virus contaminated manure. At the first day, the

pH of the virus contaminated manure was 7. After incubation of the mixture in humid environmental conditions and 24 ºC air temperature, pH values changed progressively towards the alkaline side and reported pH= 9 from day 14 till the end of the experiment. At room temperature, NDV survived in SPF manure for 28 days. From day 14 onward, the pH of virus contaminated manure turned into 9 with decrease in its HA titer as in Table 1. Results of manure's pH during the 33 days showed moderate negative correlation with NDV HA activity in manure supernatant (r = - 0.76); which indicates that the virus titer in the manure decreased when pH increased; however, there was relatively low association (R2 = 0.57); which means that the increase of manure's pH could be a reasonable factor in decreasing NDV titer as shown in Figure 4A which shows that ND virus is relatively liable to the increased manure pH in accordance with previous studies reported by Lu et al., (2003). While manure's moisture % showed strong positive correlation with NDV HA activity in manure supernatant (r = 0.94), which indicates that the titer of NDV in manure decrease when moisture% decrease; and there was strong association (R2 = 0.88); which means that the increase of moisture% could be a main factor in maintaining NDV titer (Figure 4B and Table 1). Overall, our field investigations proposed that biosecurity alone is not sufficient to prevent virus outbreaks however, development of antigenically matched vaccines; i.e., vaccines formulated based on a vaccine viral seed that belongs to the same circulating genotype (homologous genotypes) has shown to be effective for both inactivated and live vaccines, to increase efficacy against virulent challenge strains circulating in the field, and above all, on reducing the number of excreted viral particles.

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Authors Contribution

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

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