Biological Characterization of Wild Birds-origin Avian Avulaviruses 1 and Efficacy of Currently Applied Vaccine against Potential Infection in Commercial Poultry

Rania F. El Naggar[a,b,c] , Mohammed A Rohaim[b,c,d], AbdelHamid H. Bazid[a], Kawkab A Ahmed[e,], Hussein A. Hussein[d], Muhammad Munir[b,c*]

[a]Department of Virology, Faculty of Veterinary Medicine, University of Sadat City, 32897 Egypt.
[b]The Pirbright Institute, Ash Road, Woking, Surrey, GU24 0NF, United Kingdom.
[c]Division of Biomedical and Life Science, Lancaster University, LA1 4TB, United Kingdom.
[d]Department of Virology, Faculty of Veterinary Medicine, Cairo University, 12211, Egypt.
[e]Department of Pathology, Faculty of Veterinary Medicine, Cairo University, 12211, Egypt.

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[*] **Corresponding Author:** Muhammad Munir, muhammad.munir@lancaster.ac.uk

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Abstract

Newcastle disease virus (NDV), a type-specie of Avian Avulaviruses 1 (AAvV-1, formerly known as avian paramyxovirus serotype 1), causes a highly contagious and economically important disease in myriad of avian species, around the globe. While extensive vaccination programs have been implemented in ND-endemic countries, the disease is continuously spreading in commercial, backyard and wild captive poultry birds. In order to assess the virus evolution and efficiency of currently applied vaccine regimens in commercial poultry, four wild birds-origin AAvV-1 strains were biologically characterized through mean death time and intracerebral pathogenicity index, and were genetically assessed on the basis of cleavage motif (^{112}RRQKRF^{117}) in the fusion (F) protein. Based on these features, all isolates were characterized as velogenic strains of AAvV-1. Phylogenetic analysis based on the complete genome sequences revealed clustering of these isolates within class II, genotype VII. This class of AAvV-1 remained the most predominant genotype in the Egyptian as well as many Asian and African poultry industries. To further delineate the potential of these wild-bird's origin AAvV-1 isolates in causing infection in domesticated poultry and the efficacy of currently available vaccines in protecting commercial poultry, an extensive animal challenge experiments was performed. Cumulative clinicopathological and immunological investigations in challenged-chickens indicate that these isolates can potentially transmit between chickens, cause systemic infections, and currently applied vaccines are unable to prevent clinical disease and virus shedding. Taken together, the data presents a comprehensive evaluation of Egyptian wild-birds origin AAvV-1 in causing infection in commercial poultry and highlights the need for a continuous and large-scale surveillance as well as revised vaccine approaches. These integrated and multifaceted strategies would be crucial in any efforts to globally control and eradicate the disease.

Keywords: Contagious; Epidemiology; Transmission; Shedding; Control.

Introduction

Newcastle disease (ND) is a highly contagious viral disease of both domesticated and wild bird species, throughout the world [26]. The ND is caused by avian avulaviruses 1 (AAvV-1), which belong to the genus *Avulavirus* [6, 21]. All AAvV-1 strains are enveloped and carrying a genome of approximately 15.0 kilobases, which is negative sense, non-segmented and single stranded RNA [19]. The genome encodes six linear transcripts for structural (3′-NP-P-M-F-HN-L-5′) proteins, and two additional non-structural transcriptional units (V and W) are transcribed by the RNA-editing mechanism in the phosphoprotein (P) gene [19].

Based on the fusion (F) gene sequence, two systems have been developed to classify AAvV-1 isolates into either lineages or classes. The first system classifies AAvV-1 isolates into at least 6 lineages and 13 sub-lineages [2, 7, 33] whereas the second system divides all AAvV-1 isolates into two distinct classes (I and II) with different genotypic entities. Class I strains are avirulent and mainly affect wild birds whereas Class II strains can be either virulent or avirulent and are primarily isolated from wild and domesticated birds [9, 18, 22]. Further analysis based on the full-length F gene sequences of the AAvV-1 from all lineages and classes revealed the presence of multiple genotypes in Class I and 18 genotypes in Class II [8, 11, 12, 34]. These genotyping systems provide convincing epidemiological association of emerging viruses; however, these merely represent any pathological or host association of AAvV-1. Alternatively, based upon pathogenicity and sequence signature at the protease cleavage site of the precursor F protein, AAvV-1 strains are commonly categorized into velogenic, mesogenic and lentogenic pathotypes [3, 32]. Specifically, the amino acid sequence in velogenic and mesogenic strains is ^{112}R/K-R-Q-R/K-R↓F^{117}, which is cleaved by a variety of cellular proteases in various organs and thus resulting in systemic infection (respiratory system, gastro-intestinal tract and nervous system). However, the sequence in less virulent (lentogenic) strains of AAvV-1 is ^{112}G/K/R-Q-G/ E-R↓L^{117} [3].

Since the expansion of the Egyptian commercial poultry sectors, the ND is causing devastating losses to the poultry and poses a major threat to the commercial poultry [17, 28]. In order to effectively control the diseases; mass vaccination programmes have been implemented in the commercial poultry with limited success [17, 28]. While appropriate vaccination and subsequent humoral immune responses are key to prevent the disease, this approach is limited in logistically immunization of both domesticated and wild birds. There are emerging evidences which dictate the failure of live and/or inactivated vaccines in protecting birds from current field isolates of AAvV-1 and thus warrant the establishment of comparative relationship among AAvV-1 isolates being used in vaccines and are reported from wild bird and commercial poultry [28]. These investigations will establish foundations to delineate the nature and dynamics of viruses that are causing clinical disease in vaccinated flocks as well as unvaccinated commercial, and backyard poultry birds.

Here we completely characterized AAvV-1 strains collected from wild birds across the Egyptian provinces. Although several reports of disease outbreaks in Egypt [17, 27, 28] are recorded especially in commercial poultry, these findings mainly focus on partial sequencing of the F gene, which limits the assessments of evolutionary nature of the virus across the genome. Owing to recent reports of novel strains of AAvV-1 [6], it is of paramount importance to establish epidemiological links among adjacent countries and to underline the risks and challenged to commercial poultry for securing the future food security. To assess the evolutionary dynamics of these isolates in the Middle East, broad biological and genetic characterizations were performed, and possible transmission routes of wild bird-origin

AAvV-1 to the commercial poultry were drawn. Finally, we assessed the potential of currently applied vaccines against these AAvV-1 to further highlight the risk and preparedness in disease control in the event of viruses' jumps from wild birds to commercial poultry.

Materials and Methods

Sampling History, Virus Isolation and Biological Characterization

A total of one hundred-twelve (n=112) cloacal/faecal swabs were collected from different wild birds from NDV-endemic Egyptian governorates, which are rich in wild bird's population and are continuously and frequently affected during 2016 (Table 1 and Table 2). Capturing and sampling of birds were carried out in accordance with all relevant guidelines, regulations and animal ethics permits issued by the Faculty of Veterinary Medicine, University of Sadat City, Egypt and The Pirbright Institute, UK. Individual swabs were shipped in sterile phosphate-buffered saline (PBS, pH 7.4) supplemented with 50 U/mL Penicillin–Streptomycin mixture (PenStrep; Lonza). Swabs samples were cleared by centrifugation for 15 minutes at 2000 rpm at 10 °C and clear supernatants were collected and stored at −80 °C until further used.

Virus isolation was carried out in 10-day-old specific pathogen free (SPF) eggs *via* allantoic sac inoculation route as described previously [26]. Mean death time (MDT) and intracerebral pathogenicity indices (ICPI) were calculated according to the reference protocol [26] in Rhode Island Red SPF chicks. The egg lethal dose 50 (ELD_{50}) was calculated by Reed and Muench method [29]. Hemagglutination inhibition (HI) assay was performed as previously described [26, 37] using four hemagglutinating units for LaSota, NDV/Cattle egret/Egypt/SDU-1/2016 and NDV/Teal/Egypt/SDU-4/2016 and antisera collected from SPF chickens vaccinated with LaSota (Genotype II) live attenuated and commercial killed vaccine (Genotype VII).

Genome Amplification and Sequencing

Total RNA was extracted from the allantoic fluid using RNA extraction kit (QIAamp Viral RNA Mini Kit, Qiagen, USA) according to the manufacturer's instructions. Real-time RT-PCR assays targeting the matrix (M) and F genes of AAvV-1 were conducted as described previously [38]. Positive samples were subjected to conventional RT-PCR in order to amplify the complete genome of AAvV-1, as described earlier [24]. The amplified PCR products were purified using PCR Clean-Up System (Promega, Co., Madison, WI) according to the manufacturer's instructions and were directly sequenced using ABI PRISM BigDye Terminator version 3.1 (Applied Biosystems, Foster City, CA).

Phylogeny and Evolutionary Analysis

Sequences alignment, editing and analyses were performed using BioEdit version 7.0.9.0 [14]. The phylogenetic trees were constructed using the Neighbour-Joining method implemented in the *Kimura-two-parameter* model with 1000 bootstrap replicates in Molecular Evolutionary Genetics Analysis (MEGA) version 6 [35]. Well-known prototype strains of AAvV-1 were used as representative sequences of each genotype to establish reliable epidemiological association. Sequences performed in this study were deposited in the GenBank and are available under the accession numbers MG717683- MG717686. To estimate the recombination events, AAvV-1 sequence datasets were analysed using RDP V.3.44. The window size was adjusted to 30 with the highest *p value* of 0.05. The detection

of recombination events was applied between sequences sharing 0 and 100 % identity. The NetNGlyc 1.0 Server was used to predict the potential N-glycosylation sites in the surface F glycoprotein of isolated strains of AAvV-1 (http://www.cbs.dtu.dk/services/NetNGlyc/).

Transmission and Challenge experiments.

Pathobiological assessments of the AAvV-1 in immunized, challenged, mock-infected and contact birds were performed individually. In the animal experiment, a total of two representative isolates were chosen to study the pathobiology and possibility of horizontal transmission of wild bird origin- AAvV-1 to commercial poultry and evaluate the efficacy of currently used commercial vaccines against isolated AAvV-1. Sixty SPF Rhode Island Red chicks (kindly provided by the Egyptian SPF Production Farm, Egypt) were housed separately into two groups; vaccinated (n=20) and non-vaccinated (n=40). These experiments were conducted in BSL3-compatible isolators in accordance with all relevant guidelines animal ethics permits issued by the Faculty of Veterinary Medicine, University of Sadat City, Egypt and The Pirbright Institute, UK. Chicks in non-vaccinated group were divided into three sub-groups named challenged non-vaccinated (n=20, 10 for each isolate), contact (n=10, 5 for each isolate) and mock infected (n=10). However, chicks in vaccinated group (n=20) were kept in two groups of 10 for each isolate (Fig. 1). Following the vaccination commonly practised by the broiler industry in the country, chicks in vaccinated group were administered with LaSota vaccine (Genotype II) on day 7 and a subcutaneous injection of a widely used commercial inactivated vaccine (Genotype VII) on day 12. The lyophilized vaccine (LaSota, 1000 doses), used for primary immunization, was dissolved in vaccine-provided sterile buffer and administered via eye drops individually to each bird. The inactivated vaccine was administered with a dose of 0.5 ml per bird *via* the subcutaneous route around the neck region.

Each chick in the vaccinated-challenged and non-vaccinated challenged groups was inoculated with 100 ul of 10^6 EID_{50}/ml dose from each isolate through the oculonasal route on day 28. After 24 h post challenge, the contact birds were kept together with the vaccinated-challenged birds of each isolate to assess the horizontal transmission of both viruses (Fig. 1). The mock-infected group served as a negative control and was administered with a 100ul of sterile normal saline.

For the next 10 days, birds were monitored twice daily for clinical signs that include depression, anorexia, sneezing/coughing, facial swelling, respiratory sounds, ocular/nasal discharge, conjunctivitis, ruffled feathers, reluctance to move, dyspnoea, greenish diarrhoea, paralysis, tremors, opisthotonus, twisting of head and neck, circling, paresis and or necropsy lesions from dead chicks that include haemorrhages in proventriculus and caecal tonsils. Based upon typical necropsy lesions, tissue tropism and histopathology were performed. All remaining chicks were maintained to observe clinical signs until the end of study period. Euthanized/dead contact birds were collected every alternative day for necropsy lesions together with collection of oropharyngeal and cloacal swabs to monitor shedding of the challenged virus. Likewise, different internal organs were collected from the euthanized/dead chicks both control and challenged groups for examination of detailed necropsy.

Serology, Evaluation of Virus Shedding and Histopathology.

Sera samples were obtained pre- (day 27) and post-challenge (day 38) from vaccinated-challenged groups and assessed by HI assays. The HI assay was performed using inactivated NDV antigen according to standard procedures with 4 HAU virus/antigen in 0.025 ml [26]. Titers were calculated as the highest reciprocal serum dilution providing complete hemagglutination inhibition. Serum titers of 1:8 (2^3) or lower were considered negative for antibodies against NDV. The virus shedding was determined through previously described assays for identification of velogenic strains of AAvV-1 [38] through oropharyngeal and cloacal swabs.

Selected tissues such as whole brain, trachea, lung, liver, kidney, spleen and intestine were collected and fixed by immersion in 10% neutral buffered formalin at room temperature for 48 h followed by processing and embedding in paraffin wax. Tissue sections of 5 µm were stained with Haematoxylin and Eosin and examined for microscopic lesions under light microscope.

Statistical analysis

Comparison of treated groups was analysed using one-way analysis of variance (ANOVA) and significant differences among means were tested using a student's *t*-test. Kaplan-Meier analysis was performed to calculate the survival percent. All statistical tests were conducted in the GraphPad Prism 7 (GraphPad Software, La Jolla, CA, USA). For all assays and comparisons, the difference was considered significant at a p value of <0.05.

Results

Prevalence of AAvV-1 in different Egyptian provinces, Biological characterization and Genetic Diversity of Egyptian Wild bird-origin AAvV-1 strains

A total of 112 samples were individually screened by RT-qPCR targeting the M and F genes, followed by complete genome amplifications for positive samples. From this screen, 3.6 percentage positivity (4 out of 112) was detected among all tested tracheal and faecal/cloacal samples. We classified all species that were included in the analysis into four different families that reflected both their taxonomy and their ecology. These families were *Passeridae, Phasianidae, Anatidae and Ardeidae.* The *Anatidae* family was the most frequently AAvV-1-infected family followed by *Phasianidae* and *Ardeidae*, whereas *Passeridae* was found negative (Table 1). Furthermore, there were notable variations in the proportion of positive samples between provinces; Sharqia, Kafr El Sheikh, Gharbia, Qalubia, Menofia, Giza and Fayoum. The lowest prevalence rates were observed in Kafr El Sheikh and Giza whereas Fayoum and Sharqia had the highest rates while no positive samples were detected in Gharbia, Qalubia and Menofia as shown in Table 2. Four positive AAvV-1 strains reported in this study were subjected to pathogenicity index using SPF chicks, which were 1.6 for NDV/Cattle egret/Egypt/SDU-1/2016, 1.79 for NDV/Quail/Egypt/SDU-2/2016, 1.83 for NDV/Teal/Egypt/SDU-3/2016 and 1.72 for NDV/Teal/Egypt/SDU-4/2016 per eight days observation period. Mean death time (MDT) was 63.2h for NDV/Cattle egret/Egypt/SDU-1/2016, 64.1h for NDV/Quail/Egypt/SDU-2/2016, 65.0h for NDV/Teal/Egypt/SDU-3/2016 and 63.8h for NDV/Teal/Egypt/SDU-4/2016 indicating the velogenic nature for these isolates.

The genome lengths of the reported isolates in this study followed the 'rule-of-six', which is considered the essential feature for efficient replication of paramyxoviruses [19]. Annotation of the genome indicate that the entire length of isolated viruses encodes transcriptional units for 3'-N-P-M-F-HN-L-5' as expected for AAvV-1. The percentage nucleotide identity between the studied isolates ranged between 97.5% and 99%, and these isolates revealed a

varying degree of genetic diversity with other representative genotypes of AAvV-1. Four isolates showed multiple basic amino acid residues at the cleavage site (F_0) of the F protein that are characteristic features for mesogenic/velogenic strains of AAvV-1 [19]. The consistent residues ^{112}R-R-Q-K-R^{116} with phenylalanine at position 117 ($_{116}$↓F$_{117}$) were found at the F_2 protein and the NH_2-terminus of the F_1 protein. Previous studies have reported six potential glycosylation sites in the F protein, which are highly conserved in most AAvV-1 genotypes. The glycosylation motif of Asn-X-Ser/Thr [(N-X-S/T, where X could be any residue except proline (P) and aspartic acid (D)] was identified in the studied isolates. These sites include ^{85}N-R-T^{87}, ^{191}N-N-T^{193}, ^{366}N-T-S^{368}, ^{471}N-N-S^{473} and ^{541}N-N-T^{543}.

The transmembrane region (aa from 501-521) in the F protein of four isolates, characterized in this study, showed several substitutions (summarized in the Table 3). Interestingly, four isolates carried three substitutions in the fusion peptide. However, amino acid substitutions in the fusion peptide and heptad repeat region (HR) region (Table 3) can affect the fusogenic activity of the F protein [5, 9, 30]. Similarly, differences in amino acid length of the HN protein have been reported previously from different strains of AAvV-1 [30]. However, isolates in the current study carried an ORF (1716 bp) encoding 571 residues; a feature common to most of the virulent AAvV-1 [25, 30]. In the transmembrane domain of the HN protein (25-45 aa), there were seven amino acid substitutions which were prominent within NDV/Cattle egret/Egypt/SDU-1/2016 isolate, nine substitutions within NDV/Quail/Egypt/SDU-2/2016 isolate and six substitutions within NDV/Teal/Egypt/SDU-3/2016 and NDV/Teal/Egypt/SDU-4/2016 (Table 3). Analysis of the neutralizing epitopes in the HN protein identified a total of four amino acid substitutions, which are important in the formation of antigenic epitope and could result in neutralizing escape variants [16]. AAvV-1 isolates, reported in this study, carried five potential N-glycosylation sites, key residues for receptor binding and crucial amino acids in the hydrophobic core of the stalk 4HB [16, 40] (Table 3).

To determine the epidemiological clustering of isolates in the current study with the AAvV-1, all available complete genomes from National Centre for Biotechnology Information (NCBI) databases were downloaded and used for the phylogenetics and comparative genomics. To further enrich the datasets with Egyptian isolates, all available Egyptian AAvV-1 sequences in the NCBI databases were considered for epidemiological assessment of isolates. The Bayesian consensus phylogenetic analysis, verified by the Neighbour-Joining method, clearly divided the AAvV-1 strains into six lineages, and the four reported isolates in this study clustered with isolates of Lineage 5 (Genotype VII) in association with strains reported previously from Egypt and China (Fig 2a). The clustering pattern of selected isolates within Lineage 5/Genotype VII at a higher resolution (Fig 2b), showed their close association within their sub-genotypes/sub-lineages. Based on sequence comparison, four isolates showed higher than 15% nucleotide difference compared to routinely used vaccines in the country [LaSota (Genotype II) and Avinew (Genotype I)].

Horizontal transmission, virus-host relationships, and Efficacy of the commercially available vaccines (LaSota and Genotype VII inactivated).

The protective efficacy of commonly used commercial vaccines (LaSota and inactivated vaccines) were assessed against wild bird originated AAvV-1, as outlined in Fig. 1. Moreover, it is important to investigate the infectious nature of isolates that are originated from non-chicken hosts but cluster within the group of AAvV-1 that are reported from domesticated chickens. Moreover, HI assay was carried out for both Cattle egret/Egypt/SDU-

1/2016 and Teal/Egypt/SDU-4/2016 isolates using sera collected from SPF chicks immunised with commercial LaSota and an inactivated vaccine. However, as was expected, a lower cross-reactivity was observed between the sera collected from LaSota and inactivated vaccine immunized chicks in relation to both isolates (Fig 3a).

Shedding of virus was detected using RT-qPCR in contact groups challenged with Cattle egret/Egypt/SDU-1/2016 and Teal/Egypt/SDU-4/2016 isolates from day 6 to 10 in comparison with mock-inoculated chicks that revealed no virus detection throughout the experiment. Cloacal and oropharyngeal swabs were collected at every alternate day (on 2, 4, 6, 8 and 10 days) until the end of the experiment. Viral shedding in cloacal swabs was identified in both isolates, on day 4 post-challenge in vaccinated-challenged group with Cattle egret/Egypt/SDU-1/2016 while it was detected on day 6 post-challenge in vaccinated-challenged group with Teal/Egypt/SDU-4/2016. However, virus shedding was detectable in contact chicks from day 6–10 in Cattle egret isolate while in Teal-isolate from day 8-10. Nevertheless, we found lack of shedding through cloaca route in contact birds challenged with Teal-isolate on day 6.

Likewise, observed clinical signs for AAvV-1 started to appear in both non-vaccinated challenged groups on the 4th day post-challenge/virus-inoculation that include depression, anorexia, mild respiratory sounds and oculonasal discharge. Interestingly, all chicks in both non-vaccinated challenged groups died on day 7 and 8 post infection for Cattle egret and Teal isolates, respectively due to severe neurological disease (Fig 3b and 3c). Kaplan-Meier analysis has been performed which revealed that the protective effect of the vaccine on chick's survival was statistically significant with *P*- value < 0.0001 that indicate partial cross protection to the challenged viruses at which the survival percentage was 40% for vaccinated challenged group with Cattle egret/Egypt/SDU-1/2016 and 60% for vaccinated challenged group with Teal/Egypt/SDU-4/2016. The nervous signs were also prominent in the vaccinated challenged groups before death, potentially indicating lack of complete protection induced by vaccination. Although severity of clinical signs such as twisting of the head and neck, circling movement and opisthotonus was more often observed in chicks challenged with the virulent Cattle egret isolate, the disease outcome coupled with the post-mortem AAvV-1 pathognomonic lesions that were observed on day 7 post-challenge. Pathogenicity of isolates showed typical gross necropsy lesions of AAvV-1. Various tissues were collected from dead chicks that revealed typical necropsy lesions compared to mock-infected negative control group.

Sera samples were collected from survived birds in both challenged-vaccinated groups on day 38 (day 10 post-infection) and HI assay was conducted. The antibody titres to immunizing LaSota strain, killed vaccine and the respective challenge virus were compared as pre-challenge (day 27) versus post-challenge (day 38) immune response (Fig 3d). We observed an increased immune titre, which was statically non-significant with *p*-value= 0.6 (based on Student's *t*-test) between two challenged vaccinated groups. Birds challenged with Cattle egret/Egypt/SDU-1/2016 isolate showed GMT 10.7, while it was 9.3 in birds challenged with the Teal-isolate (Fig 3d). This increase in post-challenge antibody titres may be due to the replication of the challenged viruses in both groups and partial cross-reactivity to vaccine strains.

The microscopic changes were relatively less profound in chicks challenged with Teal/Egypt/SDU-4/2016 isolate compared to Cattle egret/Egypt/SDU-1/2016 isolate. Both strains caused detectable histopathological lesions including congestion of blood vessels in

the lamina propria associated with moderate oedema in the lamina propria/submucoas layer (Fig 4). Severe histopathological alterations were noticed in the lung of chickens infected with Cattle egret/Egypt/SDU-1/2016 isolate, congestion of blood vessels, focal pulmonary haemorrhage, pneumonia described by infiltration of the air capillaries with inflammatory exudate (mainly mononuclear inflammatory cells) as well as focal pulmonary emphysema. Likewise, marked fibrinoid necrosis, lymphocytic necrosis and depletion were noticed in the spleen. Caecal tonsils revealed massive heterophils infiltrating the lamina propria with lymphocytic necrosis and depletion. Examined liver revealed Kupffer's cells activation, dissociation of hepatic plates, cytoplasmic vacuolization of hepatocytes as well as focal hepatic necrosis and apoptosis with inflammatory cells infiltration and hyperplasia of biliary epithelium and fibroplasia in the portal triad. Lesions in brain revealed congestion of the cerebral blood vessels, neuronal oedema, perivascular oedema, necrosis of neurons and neuronophagia of necrotic neurons (Fig 4).

Discussion

The Egyptian poultry industry has experienced enormous economic losses due to continuous outbreaks of velogenic ND in different sectors of commercial poultry industry. It has been proposed that increased genetic divergence between field strains and vaccine may be responsible for disease outbreaks in vaccinated flocks due to non/partial-protective immune induction be the vaccine strains [23]. Since wild birds are known to be natural reservoir of AAvV-1, viruses of low virulence could emerge as velogenic AAvV-1 that carry mutation in the F_0 cleavage site [2, 4, 10]. Moreover, phylogenetically related AAvV-1 of class II, possibility causing disease outbreaks, have been isolated from wild birds [22]. Due to these complications, it is imperative to investigate the evolutionary dynamic of field AAvV-1 strains together with their potential pathobiology in vaccinated and non-vaccinated poultry birds. The genotype VII represents the most prevalent group of AAvV-1 in the Middle East countries and all AAvV-1 isolated from wild birds mostly belong to genotype VII [18]. In this study, four AAvV-1 isolates from different wild birds were classified into genotype VII/lineage 5. Analysis of the deduced residues at the cleavage site of characterized isolates in the current study and previously reported isolates from Egypt [17, 27, 28] indicated a high prevalence, and circulation of genotype VII of AAvV-1 in the country.

While assumptions can be drawn based on the cleavage site and biological characterization, pathobiology of these isolates has not been studied in animal challenge experiments. Biological characterization revealed mean death time (ranged from 63.2 to 65 h), whereas genetic characterization ($_{112}$RRQKRF$_{117}$) confirmed the virulent nature of these isolates. Furthermore, comparative analysis of functional domains of F and HN proteins of studied isolates highlight evolutionary constrains on the F protein compared to other representative velogenic genotypes and vaccine strain. Since the neutralization antibodies raised against the F protein define the protection status of the host, these modifications in the F protein explain the potential of immune escape and lack of sterile immunity caused by currently available vaccines. Moreover, the substitutions particularly in the fusion peptide, hydrophobic regions and transmembrane region of the F protein and neutralizing epitopes of the HN protein could result in altered fusion activity and neutralizing escape variants [14, 15, 37]. Taken together, nucleotide and subsequent amino acid substitutions explained the evolving nature of RNA viruses including the AAvV-1 however warrant future investigation to delineate the importance of these mutations in the pathobiology of AAvV-1 [16, 20, 36].

Phylogenetic analysis of the complete genome and hypervariable region of the F gene of wild bird-originated AAvV-1 revealed evolutionary relationships to lineage 5/genotype VII, which is predominantly among the Egyptian poultry sectors. The findings of this study provide genetical and clinicopathological characterization of wild bird-originated AAvV-1 in Egypt. Four studied isolates, NDV/Cattle egret/Egypt/SDU-1/2016, NDV/Quail/Egypt/SDU-2/2016, NDV/Teal/Egypt/SDU-3/2016 and NDV/Teal/Egypt/SDU-4/2016, clustered within lineage 5/genotype VII and were found to be closely related to isolates previously reported from Egyptian domesticated chickens. Lineage 5/genotype VII is thought to have originated from the Far East with the first isolation from Taiwan in the 1980s [39]. Since then, there have been detections of this genotype in various parts of the world [2]. A number of velogenic AAvV-1 strains, with variable genetic diversity at the sub-lineage level, have been reported from several Middle East countries [13]. Results presented here demonstrate the predominance of lineage 5/genotype VII, which highlight the risk of genotype VII transmission among different avian hosts.

Since there is only one reported serotype for all AAvV-1, lentogenic strains (e.g. LaSota or B1) are being used as live-attenuated vaccine to protect birds from velogenic NDVs. These classic vaccines are known to prevent disease but not infection; replication and shedding of the virus occur even in vaccinated animals. Due to the increasing wave of ND outbreaks since 2010, killed vaccines from genotype VII have been included into the vaccination schedule in ND-endemic countries including Egypt [17, 27, 28]. Owing to the fact that the vaccine strains belong to genotype II, which 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 antigen differences and may result in lack of complete protection against field viruses and shedding of virulent AAvVs in the environment.

In the assessment of the clinicopathological impact of two representative isolates, we observed sudden deaths in challenged non-vaccinated chicks, which was expected, as death with no apparent clinical indications is considered the most noteworthy evidence of velogenic NDVs. Similar observations have been reported by Samuel et al. [31] in immunologically naive birds challenged with virulent isolates of African origin. Although severity of observed neurological clinical signs was relatively less for Teal/Egypt/SDU-4/2016 isolate than Cattle egret/Egypt/SDU-1/2016 isolate, the morbidity, mortality and virus shedding were comparable between these groups. Interestingly, the nervous signs were prominent in the vaccinated group, potentially indicating lack of complete protection induced by the vaccine. Viral shedding together with increase in antibody titre suggests that the commonly practised vaccine schedule and vaccine types (LaSota and killed vaccine of genotype VII) give partial protection from disease, and are unable to protect from infection and virus replication, at least in laboratory experiments. Moreover, a lower HI was observed between sera collected from LaSota and inactivated vaccine-immunized SPF chicks against Cattle egret/Egypt/SDU-1/2016 and Teal/Egypt/SDU-4/2016 isolates. These findings further highly a lack of complete neutralization potential of vaccines strains against the field strains.

Taken together, genetic differences observed in the functional domains and neutralization epitopes of the F and HN proteins of wild bird origin isolates in compare to the vaccine strains could be attributed to increased virulence and vaccine escape mutants [1]. These results may be useful in revising and/or updating the vaccine schedule being currently practised in Egypt and other ND-endemic countries. Furthermore, it ascertains the need to establish and maintain active and passive surveillance for AAvV-1 in wild birds and to expect

increased disease emergence in case close interaction occurs between AAvV-1-positive wild birds and domestic poultry.

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Compliance with ethical standards

Conflict of interest

We declare that we have no significant competing financial, professional or personal interests that might have influenced the performance or presentation of the work described in this manuscript.

Ethical approval:

All animal studies and procedures were carried out under strict accordance with the guidance and regulations of animal welfare and health. As part of this process, the work approved by the Ethics Committee at the Faculty of Veterinary Medicine, University of Sadat City, Egypt, and The Pirbright Institute, UK.

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Figures captions

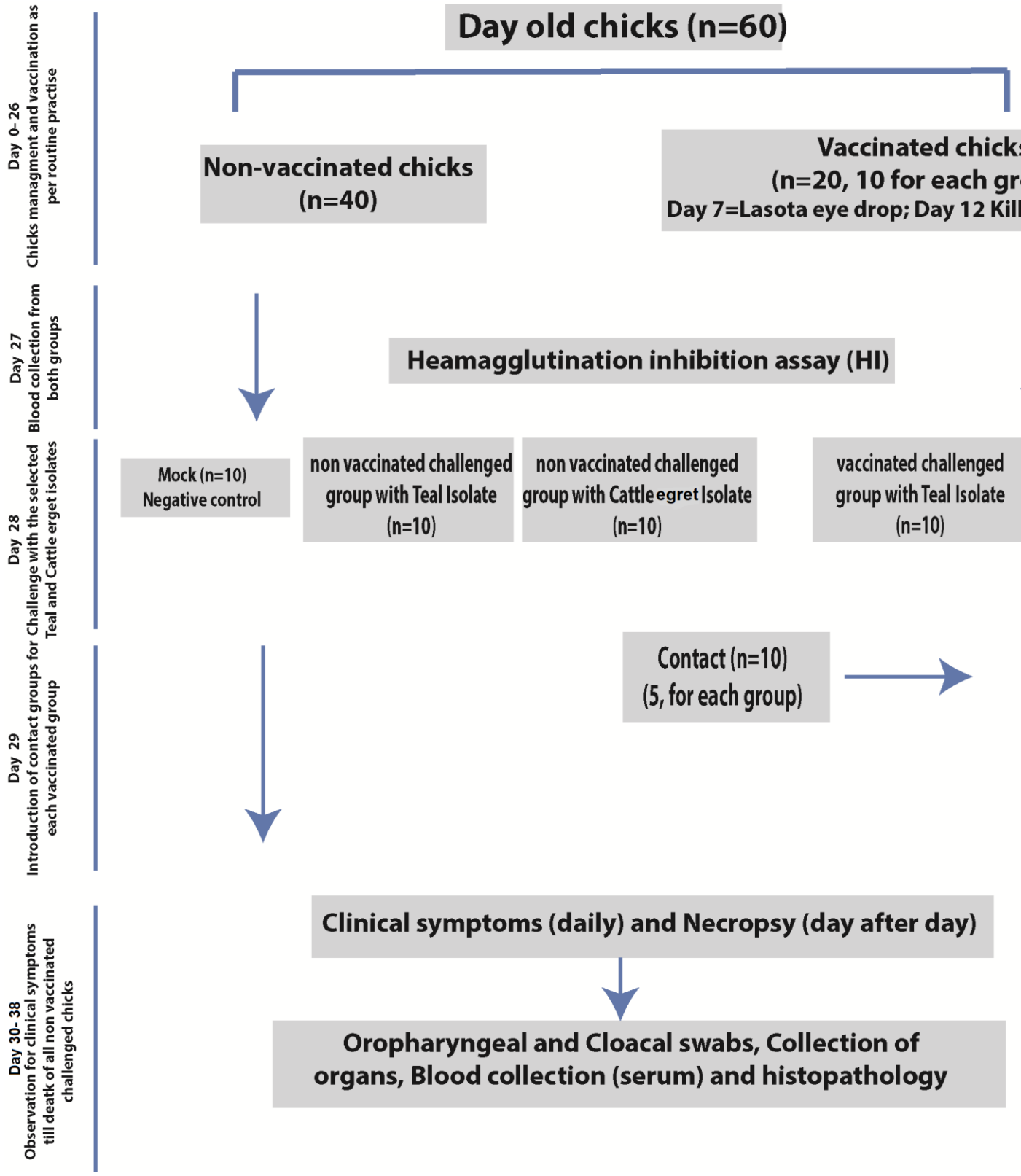
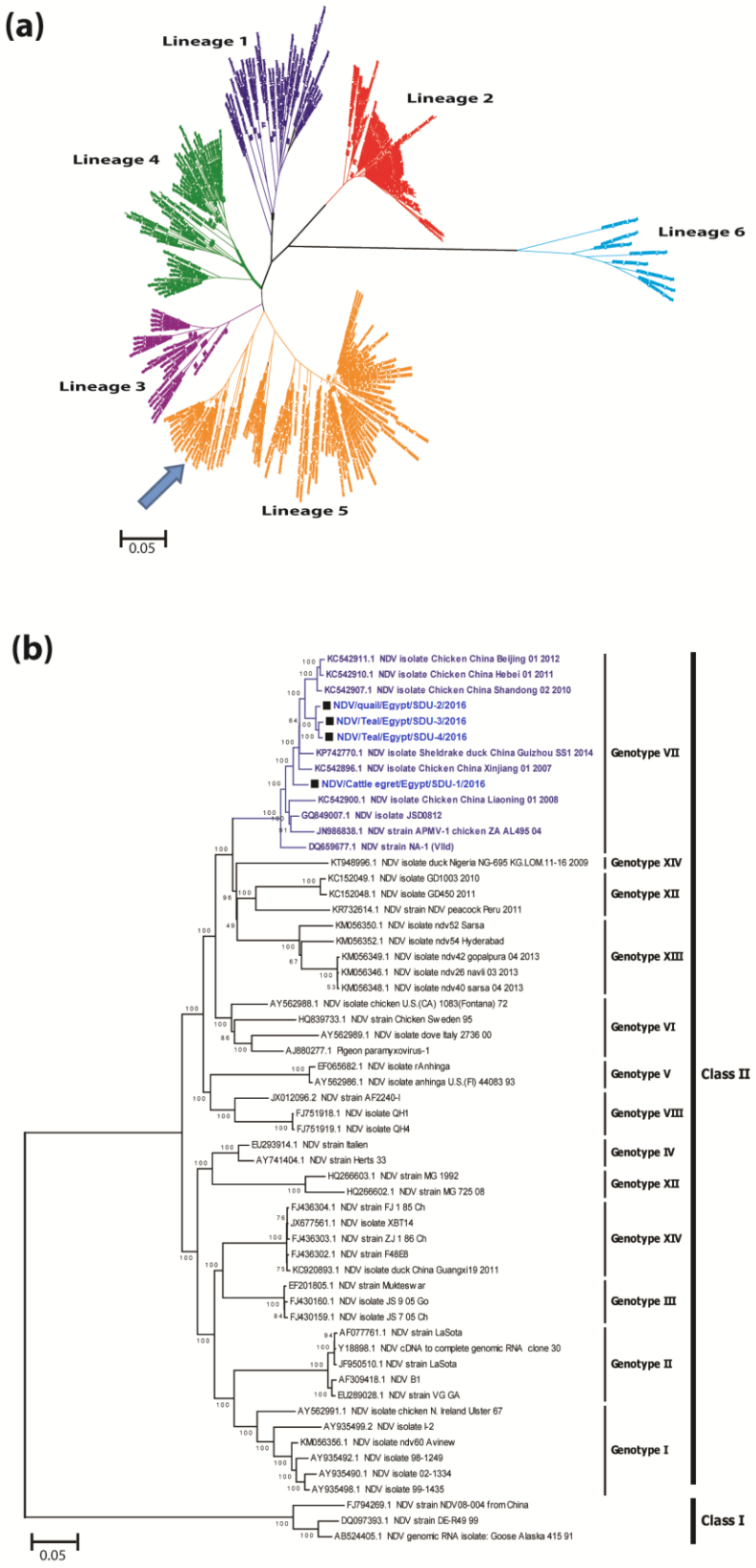


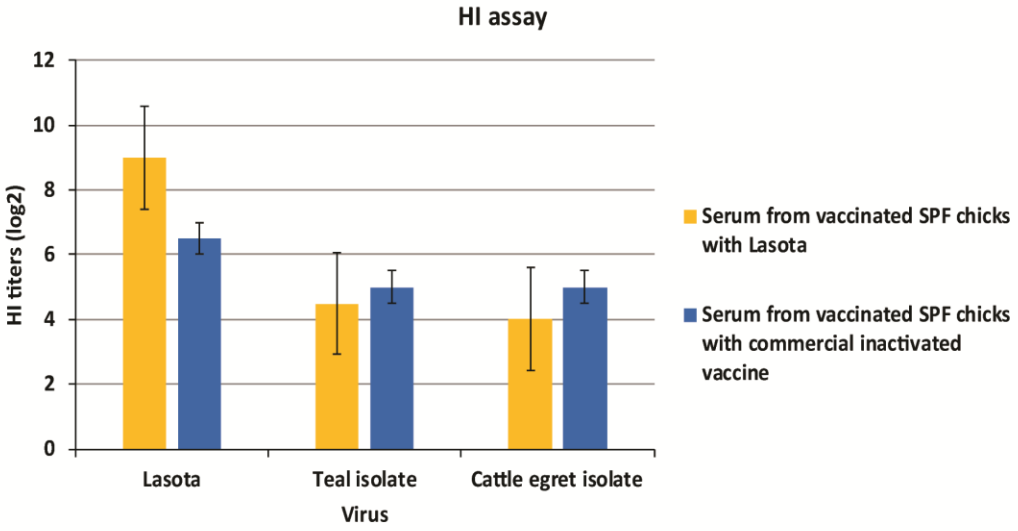
Fig. 1. Experimental plan for the assessment of virus infectivity and vaccine effectiveness in chickens.



719 Fig. 2.
720 Phylogenetic analysis of studied isolates and their clustering patterns with representative

AAvVs-1. (a) Complete genome-based phylogenetic analysis of four wild bird origin AAvV isolates with the representative strains of each lineage/genotype in class I and class II reported elsewhere in the world. (b) Full length F gene based phylogenetic analysis of four wild bird origin AAvV isolates with the representative strains of each genotypes. Reported isolates clustered in the lineage 5/genotype VII of class II. Position of clustering is marked with blue arrow.

(a) (b)



(c) (d)

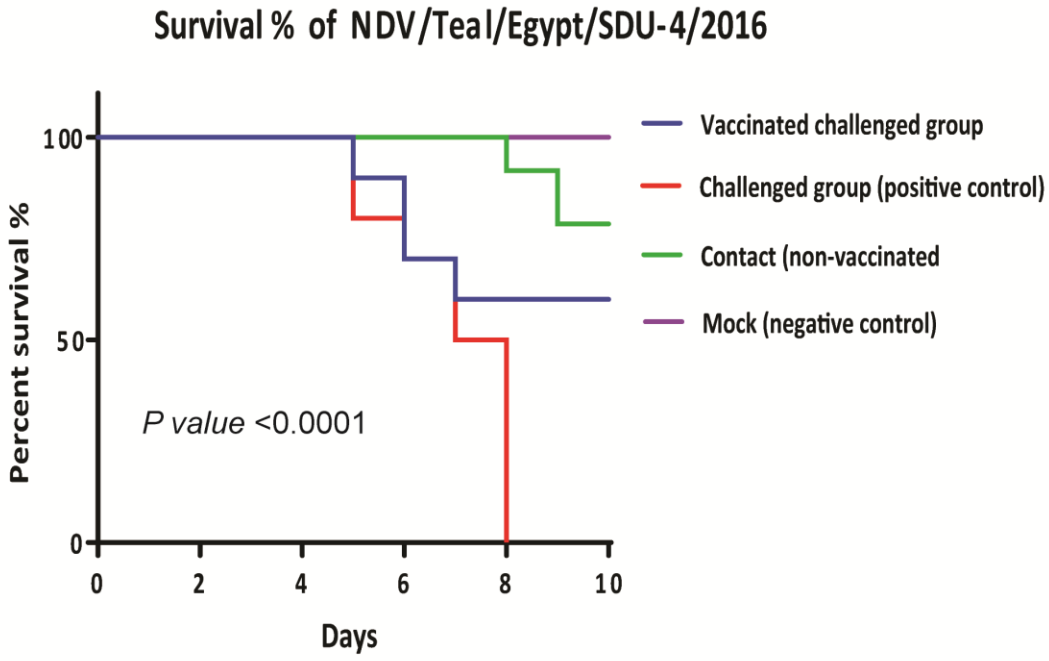


Fig. 3. Serological monitoring and survival curves for studied isolates. (a) Haemagglutination inhibition assay based cross reactivity between wild bird's origin AAvVs-1 in relation to antibodies from vaccinated SPF chicks with LaSota and commercial inactivated vaccine (genotype VII). Results indicate that low HI of wild bird origin AAvVs-1 (Cattle egret/Egypt/SDU-1/2016 and Teal/Egypt/SDU-4/2016 isolates) with commercial vaccine

(Lasota and Inactivated) in compare to Lasota antigen. (b) Percentage survival rates of vaccinated-challenged group (40%), non-vaccinated challenged (positive control) with Cattle egret isolate, contact (non-vaccinated and immunologically naive) (70%) and negative control groups. using one-way analysis of variance (ANOVA), $P<0.0001$. (c) Percentage survival rates of vaccinated challenged group (60%), non-vaccinated challenged (positive control) with Teal/Egypt/SDU-4/2016 isolate, contact (80%) and negative control groups. using one-way analysis of variance (ANOVA), $P<0.0001$. (d) Comparison of pre- and post-challenge sero-conversion of sera (GMT) samples in chicks that were immunized with LaSota and killed vaccine. Sera were taken prior to challenge on day 27 and post-challenge on day 38. Significant differences among means were tested using a student's *t*-test whereas $P=0.6$

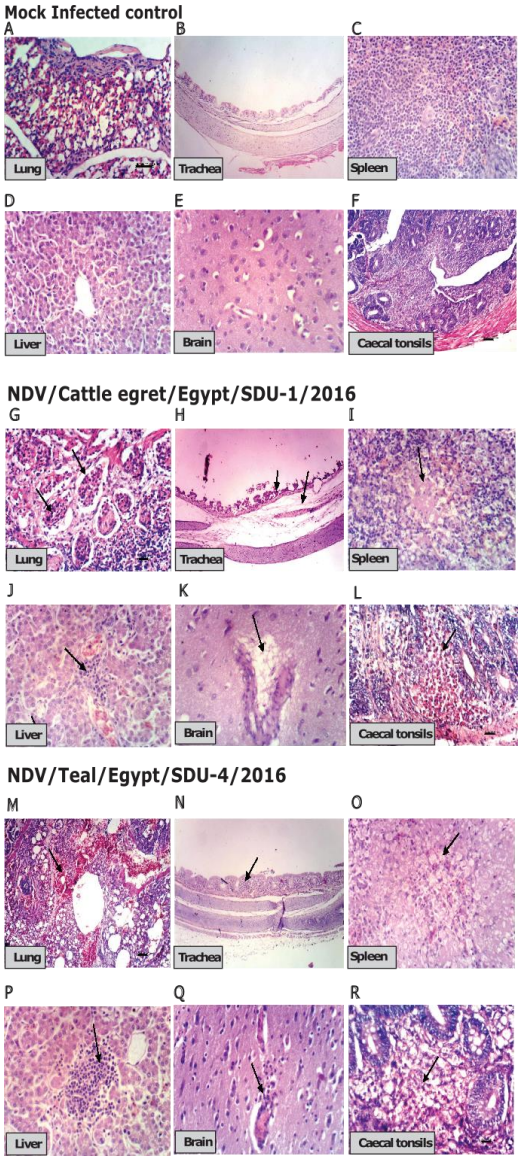


Fig. 4. Microscopic examination of different tissues collected from chicken infected with Cattle egret/Egypt/SDU-1/2016 and Teal/Egypt/SDU-4/2016 isolates. Arrows indicate lesions in the affected tissues. (G,H,I,J,K and L) histopathological lesions in different tissues collected from chicks which were infected with Cattle egret isolate. (G) Congestion of blood vessels, focal pulmonary hemorrhage, pneumonia described by infiltration of the air capillaries with inflammatory exudate (mainly mononuclear inflammatory cells). (H) Focal necrosis of the mucosa (small arrow) and edema in the propria/submucosal layer. (I)

Lymphocytic necrosis lymphocytic depletion and marked fibrinoid necrosis. (J) Kupffer cells activation (small arrow), and portal infiltration with few inflammatory cells (large arrow). (K) Perivascular edema. (L) Massive heterophils infiltrating lamina propria. (M,N,O,P,Q and R) Histopathological lesions in chicken tissues infected with Teal/Sharkia/Egypt/VRLCU/2016. (M) Pulmonary haemorrhage. (N) Inflammatory cells infiltration in thepropria/submucosal layer. (O) Lymphocytic necrosis, lymphocytic depletion and marked fibrinoid necrosis. (P) Focal hepatic necrosis associated with mononuclear cells infiltration. (Q) Perivascular cuffing with mononuclear cells. (R) Lymphocytic necrosis and depletion. (A,B,C,D,E and F) Histology of normal tissues: liver, kidney, caecal tonsil, trachea, lung and small intestine collected from the mock-infected group.