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Molecular and Pathological Characterization of Velogenic Newcastle Disease Virus Causing Late Embryonic Death in Ostrich (*Struthio camelus*) in Egypt

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Abstract

In Egypt, ostrich farming has had a great increase during the last few years. The most common problems facing ostrich farmers are a high percentage of late embryonic mortality and low hatchability. This study was designed to survey the main causes of late in-shell ostrich embryonic death. A total of 100 freshly dead in-shell ostrich embryos, all of them were in the second half of the incubation period with different ages ranging from 25 to 39 days old, were received during the breeding seasons of the period from October 2018- October 2021, from different ostrich farms in Egypt, suffered from late-stage embryonic death during artificial incubation, respiratory signs and diarrhea in breeders, moreover, the surveyed ostrich farms were near to chicken farms suffered from Newcastle Disease outbreaks. Gross findings in all embryos were recorded as anasarca, severe general congestion, and hemorrhages. In this study, 66 dead shell ostrich embryos out of 100 (66%) were immunohistochemically positive for Newcastle Disease Virus. Four Velogenic Newcastle Disease Virus strains (genotype VIIb) were molecularly characterized in 4 immune-positive ND embryos (from 4 different ostrich farms in 4 different Egyptian governorates) by RT-PCR and F gene sequencing. In addition, all embryos were negative for bacterial isolation except for one vNDV positive embryo (1%) (25 days old) that was superinfected by *Citrobacter* spp. The main histopathological lesions of vNDV-positive ostrich embryos were general edema, extensive congested blood vessels, hemorrhages, necrosis, syncytial cells formation in the upper respiratory tract, and leukocytic cells infiltrations. To the best of our knowledge, this study is the first report of the Velogenic Newcastle Disease Virus that immunohistochemically and molecularly characterized in dead ostrich embryos in Egypt, therefore, Newcastle Disease Virus vaccination of ostrich breeders is strongly recommended.

KEYWORDS
Ostrich, vNDV, Late embryonic death, IHC, Histopathology, F gene sequencing, Vertical transmission.

INTRODUCTION

Ostrich (*Struthio camelus*), was described as the largest flightless bird that can run rapidly at a speed of 55 km/h. The females were found to lay the largest eggs over land (Huchzermeyer, 1996; Davies, 2003). In Egypt, ostrich farming has had a marked flourishment over the last few years. The most common problems facing ostrich farmers are low hatchability, the high embryonic mortality rate (over 30%) in the last 10-14 days of incubation, and the death of newly hatched chicks which resulted in high economic losses (Amer, 2012).

Newcastle disease (ND) was defined as a highly contagious viral disease caused by avian paramyxovirus serotype 1 (APMV-1) viruses, genus Avulavirus, subfamily Paramyxovirinae, family Paramyxoviridae (Rima *et al*., 1995). ND could cause high mortality of all ages in different poultry species besides young ostriches. It was reported to occur worldwide causing massive economic losses due to deaths, cost of vaccination, and laboratory investigations (Elboraay *et al*., 2013). NDV was first reported in ostriches in Egypt in 2010 as there were high deaths in affected birds that suffered from neck edema, facial hemorrhage, and abdominal enlargement. The internal examination revealed congestion in the respiratory tract with air sacculitis. Proventriculus and gizzard showed hemorrhagic spots. Kidneys showed severe enlargement and the ureters filled with urates (Elboraay *et al*., 2013). Moreover, hemorrhagic tracheitis, and spleen enlargement were reported by other authors (Abbas and Abbas, 2018). Microscopically, perivascular lymphocytic cuffing in the brain was noted (Huchzermeyer, 1998; Verwoerd, 2000; Ghiamirad *et al*., 2010; Abbas and Abbas, 2018).This study aimed to survey the main causes of late in shell ostrich embryonic death.

MATERIALS AND METHODS

Birds

A total of 100 freshly dead in shell ostrich embryos, all of them were in the second half of the incubation period with different ages ranging from 25 to 39 days old, were received during the breeding seasons during the period of October 2018-October 2021, from 4 different ostrich farms, in Ismailia, Damietta, Menofia and Al- Behera Governorates, which suffered from low egg hatchability due to late-stage embryonic death during artificial incubation period. All affected ostrich farms were near to chicken houses that suffered from ND outbreaks. The embryos were then aseptically transported to the Mansoura Laboratory

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of Animal Health Research Institute (AHRI) for further laboratory examination.

Sampling

Tissue specimens were taken after external and internal gross examinations of the freshly dead embryos. The tissue specimens were collected and fixed in 10% neutral buffered formalin for about one day before processing for histopathological and immunohistochemical examination. The collected formalin-fixed tissue specimens from the affected dead embryos were the larynx, trachea, lung, heart, liver, kidneys, proventriculus, gizzard, small intestine with the pancreas (duodenal loop), brain and leg, also frozen tissue specimens (brain, trachea, Lung, kidneys, small intestine) were taken for viral identification by RT-PCR. Additional liver samples were taken under complete aseptic condition from embryos for bacteriological examination.

Polymerase chain reaction (PCR) and agar gel electrophoresis for Citrobacter spp

The primer used for the detection of *Citrobacter* spp. was listed in Table 1 (Lin *et al*., 2007). All the PCR reactions were carried out in a final 25 µL reaction volume using 2xPCR Master Mix (Emerald Amp GT PCR Master Mix (Takara) Code No. RR310A). 12.5 µL, DNA template 6 µL, 20 pmol of primer 1 µL each, and PCR grade water 4.5 µL. Cycling profiles used for the *Citrobacter* spp-specific PCR consisted of primary denaturation at 94°C for 5 minutes, followed by second denaturation at 94°C for 30 seconds, annealing at 55°C for 1 minute, extension at 72°C for 1 minute, and a final extension at 72°C for 10 minutes. Electrophoresis of the PCR products was done using 2.0% (w/v) agarose gel. After electrophoresis, the DNA was stained for 10 minutes in Ethidium bromide (at 0.5 µg/mL) and visualized using an ultraviolet cabinet. The gel was photographed by a gel documentation system and the data was analyzed through computer software.

Reverse transcription Polymerase Chain Reaction (RT-PCR) of vNDV

In brief, extraction of RNA from samples was done using the QIAamp viral RNA Mini kit (Qiagen, Germany, GmbH) following the manufacturer's directions. In that way, 140 µl of the sample suspension was incubated with 5.6 µl of carrier RNA and 560 µl of AVL lysis buffer for 10 min at room temperature. After that, 560 µl of 100% ethanol was added to the mixture. Then, the sample was washed and centrifuged following the manufacturer's instructions. Lastly, the nucleic acid was eluted with 60 µl of the kit elution buffer.

Real-time PCR amplification of vNDV

Primers of vNDV were used in a 25 µl reaction containing 12.5 µl of Quantitect probe rt-PCR buffer (QIAgen, Gmbh), 1 µl of each primer of 20 pmol concentration, 0.25 µl of rt-enzyme 4.25 µl of water, and 6 µl of a template. The polymerase chain reactions were completed in a Biometra thermal cycler. The reverse transcription step was done at 50°C for 30 min, the principal denaturation step was completed at 95°C for 5 min, followed by 35 cycles of 94°C for 30 seconds, 50°C for 40 seconds, and 72°C for 45 seconds. The final extension step was finished at 72°C for 10 min. Oligonucleotide primer supplied from (Metabion Germany) was put in Table 1.

Analysis of the PCR Products

PCR products were separated by electrophoresis on 1.5% agarose gel (Applichem, Germany, GmbH) in 1x TBE buffer at room temperature using gradients of 5V/cm. For gel analysis, 15 µl of the PCR products were loaded in each gel slot. A gene ruler 100 bp DNA ladder (Fermentas, Germany) was used to detect different fragment sizes. The gel was photographed by a gel documentation system (Alpha Innotech, Biometra) and the data analysis was done through computer software (Automatic Image Capture Software, protein simple formly Cell Bioscience, USA).

Sequencing

Products of PCR were purified using a QIAquick PCR Product extraction kit. (Qiagen, Valencia). Bigdye Terminator V3.1 cycle sequencing kit (Perkinelmer) was utilized for the sequence reaction and then after, it was purified using Centrisep spin column. DNA sequences were obtained by Applied Biosystems 3130 genetic analyzer (HITACHI, Japan), and a BLAST® analysis (Basic Local Alignment Search Tool) (Altschul *et al*., 1990) was primarily performed to establish sequence identity to GenBank reference strains. The phylogenetic tree was formed by the Meg Align module of Laser gene DNA Star version 12.1 (Thompson *et al*., 1994) and Phylogenetic analyses were done using maximum likelihood, neighbor-joining and maximum parsimony in MEGA6 with bootstrap 500 phylogenetic tree (Tamura *et al*., 2013).

GenBank accession numbers

F gene nucleotide and the amino acid sequences of vNDV ostrich strains were deposited in the GenBank online database under the following accession numbers OM930810, OM930811, OM930812, and OM930813.

Ethical committee code: (Ph. D/2).

Bacteriological examination

Firstly, the enrichment of bacteria was done by inoculated swabs, taken from the deep tissues of affected livers after sterilizing their surfaces with hot scalpels, onto nutrient broth which was incubated at 37°C for 24 hours under aerobic conditions. Subculturing was done on 10 % defibrinated sheep blood agar, Mac-

Table 1. Primer sequences of vNDV F gene and amplicon size.

Virus	Gene	Primer/ probe sequence 5'-3'	Amplified Segment (bp)	Reference
vNDV	F	F+4839 TCCGGAGGATACAAGGGTCT F-4939 AGCTGTTGCAACCCCAAG F+4894 [FAM]AAGCGTTTCTGTCTCCTTCCTCCA[TAMRA]	101	Wise *et al*. (2004)
Citrobacter	*viaB*	TGTCGAGCAGATGGATGAGCAT ACGGCTGAAGGTTACGGACCGA	516	Lin *et al*. (2007)

Conkey's agar, Nutrient agar, and Eosin Methylene Blue (EMB) agar plates which were incubated at 37°C for 24 hours, then colonies were checked for their morphological characters (non- hemolytic, grayish whitish colonies on blood agar, white colonies on nutrient agar and Muller-Hinton agar, deep purple or pink colonies on EMB and pink colonies on MacConkey's agar). Biochemical identification of *Citrobacter* spp. was done using some biochemical tests (catalase, urease, citrate utilization, indole production, motility, sugar fermentation, ornithine decarboxylation, and lysine decarboxylation) as described by (Holt *et al*., 1994).

Histopathological examination

Tissues from internal organs like the trachea, larynx, lung, heart, liver, kidney, proventriculus, gizzard, intestine, brain, and edematous leg were collected in 10% neutral buffered formalin and processed by paraffin embedding technique for histopathological examination. 5µ thickness sections were stained with hematoxylin and eosin (H&E) as described by Kiernan (2015). H&E-stained sections were examined under the optical microscope, and lesions were documented.

Immunohistochemistry (IHC)

The laboratory method was performed at the Pathology Department, Faculty of Medicine, Mansoura University (Carrasco *et al*., 2015) protocol for Immunohistochemistry was applied. The primary NDV polyclonal anti-HN protein antibody, which was prepared in chickens at Animal Health Research Institute, Cairo, Egypt, was used at the optimal dilution (1:800). After being deparaffinized, hydrogen peroxide (3%) was added to the sections for 5 minutes to block endogenous peroxidase. Antigen retrieval was done using the wet method by incubating slides in a wet chamber for 2 hours. The slides were then immuno-stained with the primary NDV antibody at 25°C for 60 minutes, then, the secondary antibody, rabbit anti-chicken antibody with dilution (1:1000), was added to the sections and incubated for an hour. The IHC reactions were established using 3, 3-diaminobenzidine DAB (from DAKO Carpenteria, CA) for 5-10 minutes. The slides were counterstained with Mayer's hematoxylin, then they were examined under the light microscope. Control positive and negative samples were used.

RESULTS

History and clinical signs

Four different ostrich farms in four different governorates, Ismailia, Damietta, Menofia, and Al- Behera Governorates, had a history of smaller egg size than the previous breeding seasons, low egg hatchability (about 50%) due to late in-shell embryonic death during the artificial incubation period. There was no decrease in egg production. The eggs were rapidly collected after being laid and immediately were disinfected with diluted common approved disinfectants (Povidone-iodine or quaternary ammonium compounds), also the incubators and hatchers were cleaned and disinfected. All farms did not apply for any vaccination programs. There was a history of respiratory manifestations (lacrimation, discharges from nostrils, and beaks) and diarrhea with low feed intake appeared on a few breeders who were given different types of antibiotics. There were no deaths in the affected breeders.

Gross pathology

About twenty (20%) out of one hundred unhatched ostrich embryonated eggs were smaller than normal. All dead in-shell ostrich embryos could not do internal piping. The internal examination of eggs revealed abnormally colored watery albumin and dead embryos of different ages (25 to 39 days old) suffered from general edema which was prominent in the face, neck, and legs, severe general congestion, and hemorrhages in all internal

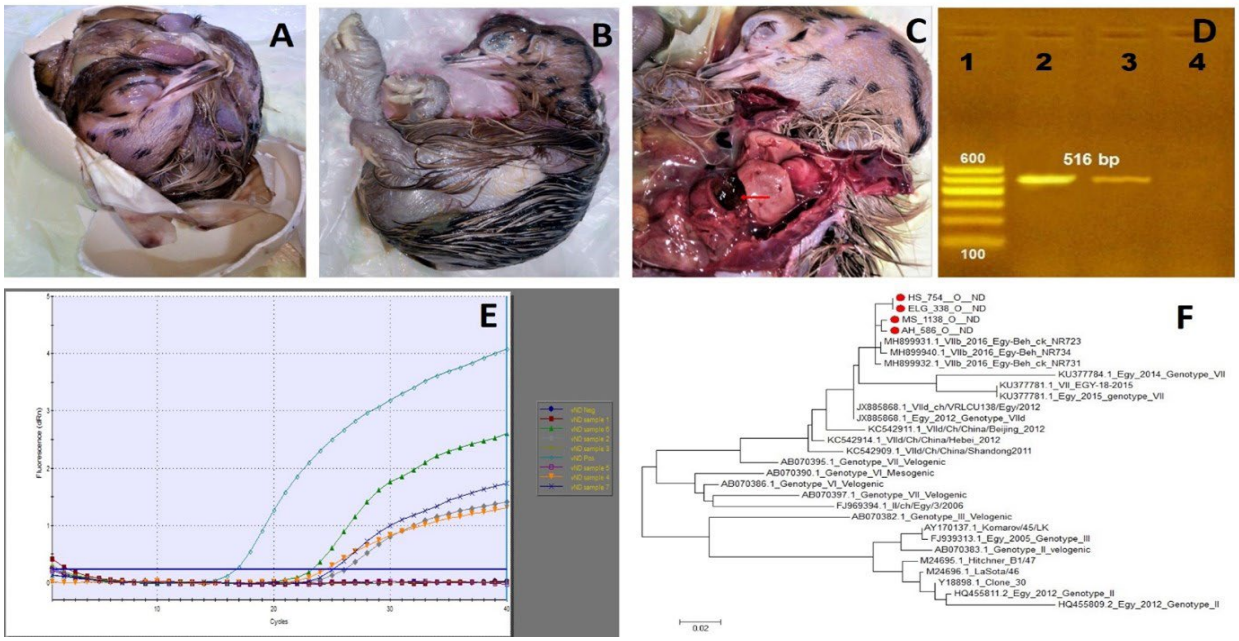

Fig. 1. A: A positively vNDV ostrich embryo (39d) shows edema in the face and submandibular region. B: A positively vNDV ostrich embryo (39d) shows edema in the neck and legs with ascites. C: A positively vNDV ostrich embryo (39d) shows congested and hemorrhagic GIT with bloody content in the gizzard (arrow), and edematous lacerated hemorrhagic liver. D: Agarose gel electrophoresis (1.4%) of cPCR (*viaB*) gene product of ostrich *Citrobacter* spp. isolate, lane 1: 100 bp DNA ladder, lane 2: control positive, lane 3: positive ostrich isolate for *Citrobacter* spp., lane 4: control negative. E: amplification curves of RT-PCR for vNDV F gene of ostrich samples indicate 4 positive ostrich isolates (no. 2, 4, 6, and 7) with CT values of [27(gray curve), 25 (orange curve), 24(green curve), 26 (deep blue curve)] respectively, also, control positive curve (light blue) is appearing. F: Phylogenetic tree analysis based on the nucleotide sequence of vNDV F gene of the current 4 study ostrich isolates (HS 754 _O_ND, ELG 338_O_ ND, MS 1138_O_ ND and AH 586_O_ ND) (indicted by compacted red circles) with other reference and vaccinal NDV strains on GenBank.

organs and yolk sacs (Fig. 1A-C).

Molecular identification of vND

Seven immune-positive ostrich embryos, from the surveyed 4 ostrich farms in the fore mentioned Egyptian governorates, were selected, and tested for the presence vNDV using RT-PCR to detect the F gene. Four embryos (one for each surveyed farm) out of seven (57.1%) were positive for vNDV with CT (threshold cycle) values 27, 25, 24, and 26 (Fig. 1E).

Sequence analysis of vNDV F gene in positive samples

The PCR products of the vND F gene of the 4 positive samples were purified, cloned, and sequenced. Nucleotide and deduced amino acid sequences of *F* genes in the positive samples were aligned and compared with the same gene of 25 reference strains on GenBank. The nucleotide and deduced amino acids sequences of F protein in the 4 present study ostrich strains (HS 754 _O_ ND, ELG 338_O_ ND, MS 1138_O_ ND, and AH 586_O_ ND) that had accession numbers in the GenBank online database (OM930810, OM930811, OM930812, OM930813) respectively, revealed that all ostrich strains had the velogenic motif 111 G-R-R-Q-K_R-F117 at the cleavage site of the F protein. Moreover, the ostrich strains were phylogenetically aligned and grouped in class II genotype VII, subgenotype VIIb (genotype VIIb subtype), which were found closely related to Egyptian chicken field reference strains isolated in 2016 with Nucleotide identity percent range (98.9-99.2%) and (78.6% -79.7%) identity percent for Hitchner, Clone, LaSota and Komarov vaccinal reference strains (Fig. 1F).

Biochemical and molecular identification of Citrobacter spp.

All embryos were negative for bacterial isolation except for one +vNDV embryo (25 days old) (1%) that was superinfected with *Citrobacter* spp. which was confirmed by successfully amplifying via B gene at 516 bp (Fig.1D).

Histopathological examination

Positively vNDV ostrich embryos showed various severe microscopic lesions in different organs. Embryonic upper respiratory tract showed lymphocytic laryngitis and tracheitis characterized by necrosis, ballooning, and loss of epithelium lining with the formation of syncytial cells by the viral fusion protein, moreover, Laryngeal muscles showed necrosis, calcification, marked inter muscular edema with Leukocytic cells infiltration (Fig. 2 A-C). Embryonic heart showed hemorrhagic myocarditis characterized by coagulative necrosis in the myocardium, leukocytic cells infiltrations mainly lymphocytes and heterophile with hemorrhage and hemosiderin pigment release. Myocadiac edema which caused thinning and compression in myocadiac muscle fibers. Moreover, hemorrhages were seen in coronary fat (Fig. 2 D-F). Embryonic liver showed severe congestion of hepatic blood vessels, also, aneurysms were seen in all positive vND embryos which were characterized by outward balloon-like bulge in a blood vessel due to severe chronic congestion along with weakness in the blood vessel wall (Fig. 3 A). Hepatocytes showed coagulative necrosis and degeneration as vacuolation, fatty change, and individualizations with the presence of lipofuscin pigment. (Fig. 3 B). The embryonic kidney showed interstitial nephritis, edema, and hemorrhage. renal tubules showed necrosis and degeneration, whereas glomeruli showed congestion in the tuft of capillaries and edema in Bowman's space (Fig. 3 C & D). Embryonic gizzard showed hemorrhagic gastritis which characterized by necrosis in gastric glands with loss of kaolin (cornified) layer, necrosis and fragmentation in the muscular layer with inter muscular edema and congested blood vessels, (Fig. 3 E & F). Embryonic Intestine showed catarrhal enteritis characterized by degeneration of intestinal epithelium and Goblet cell proliferation with accumulation of mucus, thickening in mucosa due to dense submucosal leukocytic cells infiltration and edema with fusion and stretching in intestinal villi beside congested serosal blood vessels (Fig. 4 A&B). Embryonic brain showed congested cerebral blood vessel, with diffuse cerebral edema which appeared as marked perivascular edema with degeneration, vacuolation and necrosis in the

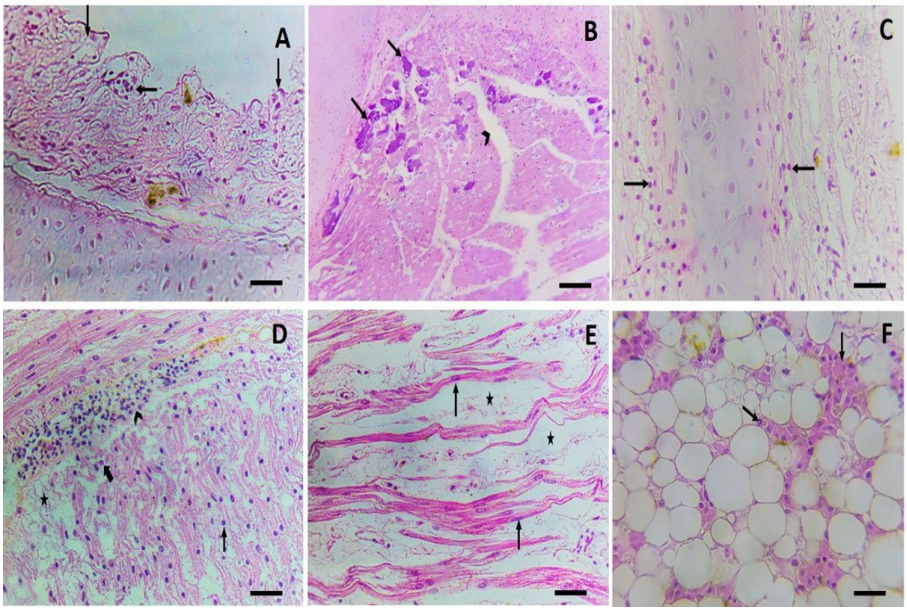

Fig. 2. A&B: Ostrich embryonic Larynx shows (A): necrosis and ballooning in epithelium lining, with the formation of syncytial cells by the virus (arrows) (H&E. Bar, 50µm). (B): laryngeal muscles show necrosis and calcification (arrows), with intermuscular edema (arrowhead) and Leukocytic cell infiltration, (H&E. Bar, 100µm). (C): Ostrich embryonic trachea shows lymphocytic tracheitis characterized by diffuse lymphocytic infiltration in a tracheal wall (arrows), (H&E. Bar, 50µm). D-F: Ostrich embryonic heart shows (D): hemorrhagic myocarditis characterized by myocardium hemorrhage with hemosiderin (arrowhead), inter myocardial edema (asterisk) with leukocytic cells infiltrations mainly lymphocytes (thin arrow) and few heterophile (thick arrow) (H&E. Bar, 50µm). (E): Marked intramyocardial edema (astrix), with Zinker's necrosis, thinning, and compression in the myocardium (arrows) (H&E. Bar, 50µm). (F): Coronary fat shows hemorrhage (thin arrows) (H&E. Bar, 50µm).

surrounded cerebral tissue (Fig. 4 C), moreover, focal gliosis was seen. Embryonic leg showed marked diffuse edema in the skin and leg muscles represented by hydropic degeneration of stratum spinosa cells, cutaneous and subcutaneous edema, along with marked intermuscular edema. Moreover, myositis was noted represented by leukocytic cells infiltration in muscle fibers and the surrounding connective tissue capsule mainly lymphocytes and a few heterophiles (Fig. 4 D-F).

Immunohistochemistry (IHC) for NDV

Sixty-six dead in-shell ostrich embryos (66%) out of one hundred showed positive brown immunolabelling for NDV in different organs with variable degrees seen either intracellularly or extracellularly in necrotic tissues as bright brown fine or dense granules. The embryonic upper respiratory tract (trachea) showed moderate positive brown immunolabelling reaction for

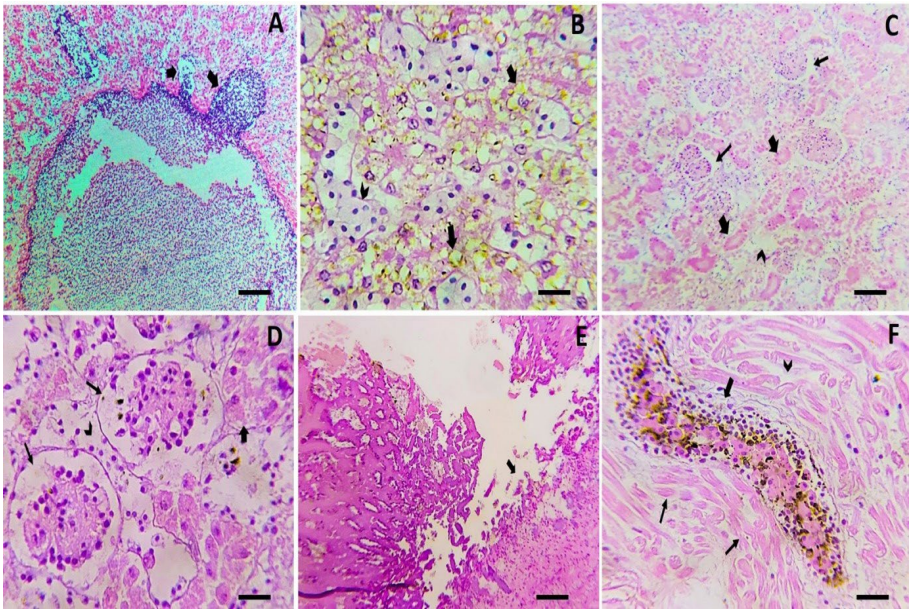

Fig. 3. A&B: Ostrich embryonic liver shows (A): Hepatic aneurysms in some congested angiopathic blood vessels (arrows) which are characterized by an outward balloon-like bulge in the blood vessel due to severe chronic congestion and weakness in its wall (H&E. Bar, 100µm). (B): Hepatocytes show Lipofuscin pigment (thick arrows), coagulative necrosis, individualizations, and fatty change with marked congested sinusoids (arrowhead) (H&E. Bar, 50µm). C& D: Ostrich embryonic kidney shows (C): interstitial edema (arrowhead), necrosis in renal tubules (thick arrows), with congested glomeruli and edema in Bowman's space (thin arrows) (H&E. Bar, 100µm). (D): high power to show edema in Bowman's space (thin arrows) and congestion in glomeruli, interstitial nephritis which is characterized by interstitial edema and leukocytic cells infiltration (arrowhead), with necrosis in renal tubules (thick arrows) (H&E. Bar, 50µm). E&F: Ostrich embryonic gizzard shows (E): necrosis in gizzard glands with loss of kaolin layer (arrow) (H&E. Bar, 100µm). (F): necrosis and fragmentation in the ventriculus muscles (thin arrows) with intermuscular edema (arrowhead) and congested blood vessel (thick arrow) (H&E. Bar, 50µm).

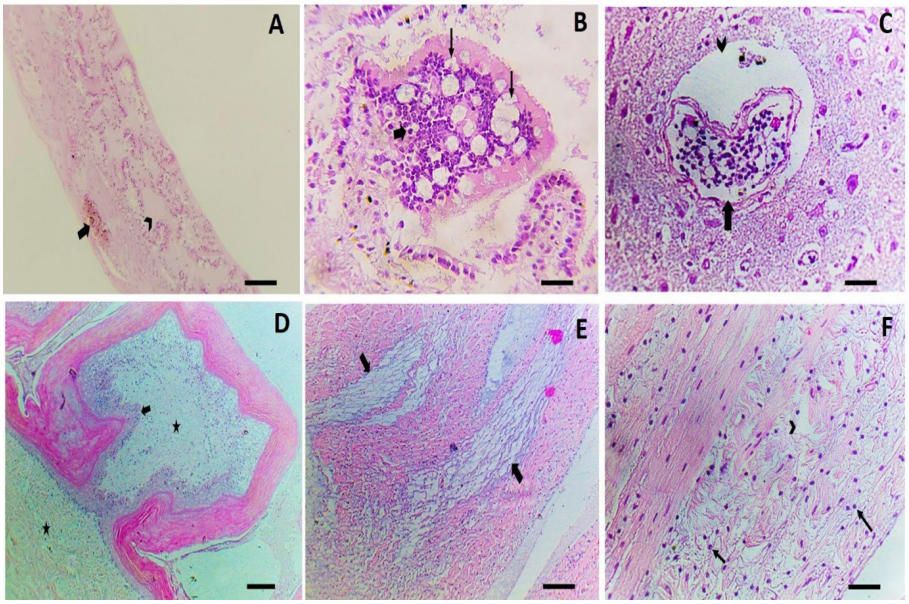

Fig. 4. A&B: Ostrich embryonic Intestine shows (A): Congested serosal blood vessel (arrow), mucosal and submucosal edema (arrowhead) (H&E. Bar, 100µm). (B): Catarrhal enteritis which is characterized by thickening in the mucosa due to dense focal leukocytic infiltration (thick arrow), degeneration of intestinal epithelium (goblet cell proliferation), with an accumulation of mucus secretion (thin arrows) (H&E. Bar, 50µm). (C): Ostrich embryonic brain shows congested cerebral blood vessel (arrow), with cerebral edema which appeared as marked perivascular edema (arrowhead) with degeneration, vacuolation, and necrosis in the surrounded cerebral tissue (H&E. Bar, 50µm). D-F: Ostrich embryonic leg shows (D): skin of leg shows hydropic degeneration in cells of stratum spinosa (arrow), cutaneous and subcutaneous edema (asterixis) (H&E. Bar, 100µm). (E): leg muscles show myositis which is characterized by intermuscular edema with leukocytic cells infiltration (arrows) (H&E. Bar, 100µm). (F): high power of the previous figure to show myositis which is characterized by intermuscular edema with leukocytic cells infiltration in muscle fibers and connective tissue capsules mainly lymphocytes and few heterophiles (arrows) (H&E. Bar, 50µm).

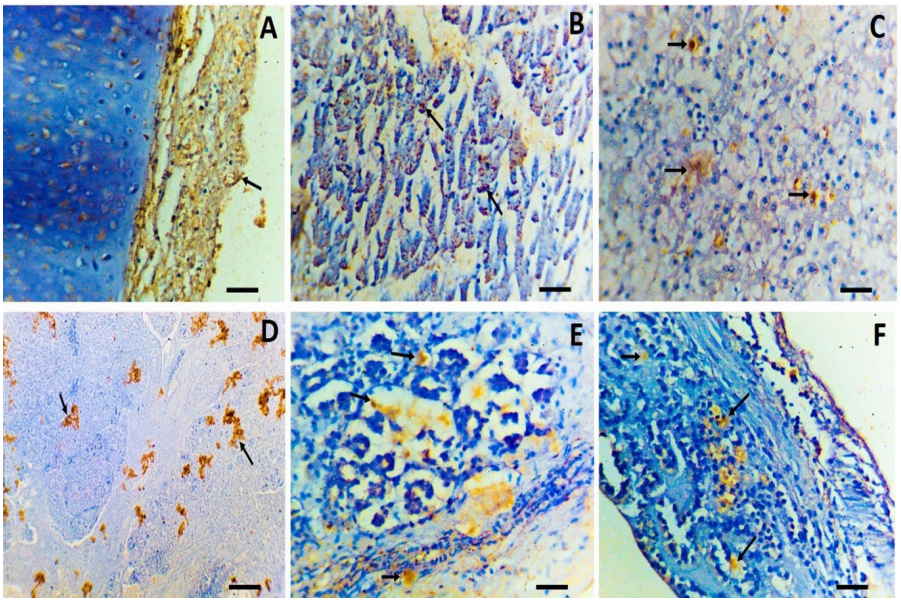

Fig. 5. A: Ostrich embryonic trachea shows moderate positive brown immunolabelling for NDV in the tracheal cartilage and mucosa (arrow), (Immunoperoxidase stain Bar, 50µm). B: Ostrich embryonic heart shows moderate positive brown immunolabelling reaction for NDV in myocardium (arrow), (Immunoperoxidase stain. Bar, 50µm). C: Ostrich embryonic liver shows moderate positive brown immunolabelling reactions for NDV in the hepatic parenchyma and inside hepatocytes (arrows), (Immunoperoxidase stain. Bar, 50µm). D: Ostrich embryonic kidney shows strong positive brown immunolabelling reactions for NDV in renal parenchyma (arrows), (Immunoperoxidase stain. Bar, 500µm). E: Ostrich embryonic proventriculus shows moderate positive brown immunolabelling reaction for NDV in lining glandular epithelium (arrows), (Immunoperoxidase stain. Bar, 50µm). F: Ostrich embryonic small intestine shows moderate positive brown immunolabelling reactions for NDV in mucosal epithelium and submucosa (arrows), (Immunoperoxidase stain. Bar, 50µm).

NDV especially in the lining mucosa, serosa, and cartilage, (Fig. 5 A). Embryonic heart showed moderate positive brown immunolabelling reaction for NDV in the myocardium and endocardium (Fig. 5 B). Embryonic liver showed a moderate positive brown immunolabelling reaction in the cytoplasm of some hepatocytes and hepatic parenchyma (Fig. 5 C). Embryonic kidneys showed a strong positive brown immunolabelling reaction inside renal parenchyma (Fig. 5 D). The embryonic digestive tract (proventriculus and small intestine) showed a moderate positive brown immunolabelling reaction in the glandular epithelium, mucosal lining epithelium, and submucosa (Fig. 5 E & F).

DISCUSSION

Embryonic mortality was described as one of the most common problems facing ostrich farming causing great economic losses (Deeming, 1996). Many microorganisms are related to ostrich egg contaminations, which could result in embryonic death (Jahantigh, 2010).

The present study surely suggested the vertical transmission of vNDV from infected ostrich breeders to their progeny, as vNDV was immunohistochemically and molecularly characterized in dead in-shell ostrich embryos, this was supported by Alexander (1997); Davies (2003); Landry and Hsiung (2000); Chen and Wang (2002); Roy and Venugopalan (2005) and Sá e Silva *et al.* (2016). The mechanism of NDV vertical transmission from infected ostrich breeders to their embryos might need further studies and follow-up.

In this study, it was noted that some vNDV-positive eggs (20%) were smaller than normal with watery albumin, this nearly agreed with Alexander *et al.* (2004) and OIE (2009). All collected ostrich embryos died in the last few days before hatching (36-39 days old), this agreed with Deeming (1995) and Brown *et al.* (1996). About (1%) of vNDV-positive embryos died earlier (25 days old) due to superinfection with a virulent strain of *Citrobacter* spp.

Clinical signs that appeared on ND-infected non-vaccinated ostrich breeders were respiratory manifestations and diarrhea without deaths or a decrease in eggs. These recorded clinical signs partially agreed with Elboraay *et al.* (2013), and Abbas and Abbas (2018), as they reported a drop of egg production.

The main gross pathological lesions in positively vNDV ostrich embryos were severe general edema, congestion, and hemorrhage, these findings nearly came in contact with those of Samberg *et al.* (1989); Huchzermeyer (1998); Verwoerd (2000); Elboraay *et al.* (2013), and Abbas and Abbas (2018), also Khorajiya *et al.* (2015) reported hemorrhage in artificially infected chicken embryos. On the opposite, the present study gross lesions disagreed with former authors (Huchzermeyer, 1996; Allwright (1996), who reported only head edema as the most constant lesion and no pathognomonic lesions were seen in positively vNDV ostrich chicks respectively.

To the best of the authors' knowledge, the present study is the first report that molecularly characterized vNDV infection as the main cause of late-stage embryonic death in ostrich in Egypt by RT-PCR and F gene sequencing. Four dead in-shell ostrich embryos were molecularly positive with vNDV which revealed velogenic motif 111 G-R-R-Q-K_R-F117 at the cleavage site of the F protein. Also, the sequences of the current study ostrich strains were closely related to Egyptian chicken reference strains on GenBank (Genotype VIIb) in 2016 with identity percent 98.9-99.2% which confirmed that ostrich caught up vNDV from the near infected chicken houses, this agreed with Samberg *et al.* (1989); Verwoerd (1995) and Ghiamirad *et al.* (2010), also Alexander (2000) proved that ND poultry vaccines were found to be protective in ostriches. On the other hand, the current findings disagreed with Elboraay *et al.* (2013), who supposed the source of vNDV infection in ostrich was unknown, moreover, they isolated the NDV strain from ostrich with lentogenic motif 111G-R-N-Q-G-R-L117 at the cleavage site of a fusion protein.

Publications about ostrich pathology are rare, but pathological studies on vNDV in other avian species were found to support the present study findings. Histopathological examination of positively vNDV ostrich embryos showed different severe lesions all over the organs as necrosis, general edema, and inflammation ranging from catarrhal to hemorrhagic and congestion, similar findings were reported in experimentally infected chicken embryos with vNDV by Wicaksana *et al.* (2019), who proved that vNDV (genotype VII) could cause a similar pathological lesion in different avian hosts. On the other hand, the present study's

histopathological findings disagreed with Allwright (1996), and Huchzermeyer (1996). The upper respiratory tract showed lymphocytic inflammation with necrosis and syncytial cells formation (cytopathic effect (CPE) in the respiratory mucosa, according to Ravindra (2009), who documented syncytia formation as vNDV cytopathic effect in cell culture. Foci of calcification were recorded in necrotic laryngeal muscles which indicated the earlier involvement and destruction of the respiratory tract during incubation perhaps due to high viral tropism and affinity to the respiratory tissues that still not functioned yet so death of the embryos delayed to the end of incubation. Bello *et al.* (2017), also documented mild mineralization and calcification in kidneys and proventricular mucosa of dead in-shell ostrich embryo. Khorajiya *et al.* (2015), reported severe involvement of respiratory tissues as edema and dense mononuclear cells infiltration with desquamation of tracheal epithelium lining, whereas, Susta *et al.* (2015) documented mild respiratory lesions in chickens artificially infected with vNDV (genotypes XIV and XVII). Hemorrhagic myocarditis with mononuclear cells and heterophilic infiltration with myocadiac edema were seen, former authors reported similar lesions in vNDV artificially inoculated chicken embryos by Wicaksana *et al.* (2019), and vNDV artificially inoculated chickens and quails by Anis *et al.* (2013); Brown *et al.* (1999) and Abd El Aziem *et al.* (2020). Embryonic liver showed severe lesions represented by marked congestion of blood vessels and sinusoids, diffuse coagulative necrosis, degeneration, and individualizations of hepatocytes. Wicaksana *et al.* (2019) reported similar findings in vNDV experimentally infected chicken embryos, whereas, Abd El Aziem *et al.* (2020), recorded mild lesions in the liver of vNDV (genotype VIId) experimentally infected quail, also, hepatic aneurysms were seen due to weakness of the vessel wall along with severe congestion of the vessels. Bello *et al.* (2017) documented aortic aneurysm in an adult ostrich female. Lipofuscin pigment was seen might be due to an increase cell death rate and hemorrhage which perhaps indicated a long course of infection. Embryonic kidneys showed interstitial nephritis, edema, and necrosis in renal tubules. Anis *et al.* (2013), reported mild tubulointerstitial nephritis in chickens and ducks. Embryonic gizzard showed hemorrhagic gastritis with loss of cornified layer and necrosis with intermuscular edema. Susta *et al.* (2015) and Ali and Abd EL-Dayem (2019) documented similar lesions in artificially and naturally infected chickens respectively. Embryonic intestine showed catarrhal enteritis and submucosal edema with fusion and stretching in intestinal villi. Etriwati *et al.* (2017); Ali and Abd EL-Dayem (2019) and Abd El Aziem *et al.* (2020), also reported catarrhal enteritis in naturally and experimentally infected chickens and quails. The embryonic brain showed diffuse cerebral edema, marked congestion in cerebral blood vessels and focal gliosis, the similar finding recorded by Brown *et al.* (1999); Khorajiya *et al.* (2015); Ali and Abd EL-Dayem (2019) and Abd El Aziem *et al.* (2020), in naturally and experimentally infected chickens and quals. Embryonic leg showed myositis with marked edema on skin and muscles. Wiercinska and Szczerbinska (2005) and Abbas and Abbas (2018) reported grossly subcutaneous edema in the head, neck, and legs.

Some of the above-mentioned microscopic lesions indicated a long course of vNDV in ostrich embryos causing slow death, as hemosiderin and lipofuscin pigments in the heart and liver respectively, hepatic aneurysms and calcification in laryngeal muscles. Verwoerd (1995), mentioned NDV duration in ostrich about 3-16 days, unlike other avian species as chicken embryos which died within 3days post artificial infection as recorded by Khorajiya *et al.* (2015) and Wicaksana *et al.* (2019). Moreover, the severe generalized edema in all positive ostrich embryos perhaps related to hepato-renal and circulatory failure (diffuse necrosis, degeneration, and hemorrhage). Bello *et al.* (2017) supposed the main cause of late-stage ostrich embryonic death was circulatory failure associated with generalized subcutaneous edema.

Based on the available research studies, the present study is the first to detect NDV immunolabelling antigen in different organs (trachea, heart, kidneys, liver, proventriculus, and intestine) of freshly dead ostrich embryos by IHC in Egypt. It was clear out that the presence of viral labeled antigen in different embryonic tissues indicated pantropic features and high pathogenicity of vNDV ostrich strains, agreed with Cattoli *et al.* (2011). Similar previous immunohistochemical findings in chickens affected with NDV supported the current study findings (Anis *et al.*, 2013; Susta *et al.*, 2015; Etriwati *et al.*, 2017; Ali and Abd EL-Dayem, 2019). The kidneys of vNDV ostrich embryos showed the heaviest immunolabelling reaction for NDV antigen, this also indicates intensive replication in renal tissue. Anis *et al.* (2013) recorded immunolabelling of NDV in the chicken kidney. Additionally, Elboraay *et al.* (2013), documented the renal gross lesion was the most severe one.

CONCLUSION

This study the first that characterized vNDV (genotype VIIb) molecularly and immunohistochemically in late-stage dead in-shell ostrich embryos, causing low hatchability and great economic losses in Egypt. Moreover, the current study suggested that vertical transmission of vNDV (genotype VIIb) from infected ostrich breeders to their embryos through eggs causing embryonic death at the late stage of incubation. Ostrich perhaps caught up vNDV from the beside-infected chickens, as there was a high nucleotide identity percent between ostrich strains and chicken reference field strains in Egypt on GenBank. The main pathological lesions in vNDV positively ostrich embryos are severe general edema, hemorrhages, and leukocytic infiltration with the exhibition of immuno-positive labeling reactions for NDV in different organs. To the best of our knowledge, this study is the first report of vNDV detection (molecularly and immunohistochemically) in dead in-shell ostrich embryos in Egypt, therefore, ND vaccination of ostrich breeders is strongly recommended.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

REFERENCES

Abbas, G.a., Abbas, S.W., 2018. Health and Hygiene Guidlines for Ostriches. International Journal of Animal Husbandry and Veterinary Science 3, 2455-8567.

Abd El Aziem, A., Abd-Ellatieff, H., Elbestawy, A., Belih, S., El-Hamid, A. and Abou-Rawash, A.R., 2020. Susceptibility of Japanese quail and chickens to infection with newcastle disease virus genotype VIId. Damanhour Journal of Veterinary Sciences 3, 27-31.

Alexander, D., 1997. Newcastle Disease and other paramyxoviruses infection, in diseases of Poultry. Edited by: calnek BW, Barnes HJ, Beard CW, McDoughal LR, Saif YM, Ames LA. Iowa state University Press.

Alexander, D., 2000. Newcastle disease in ostriches (*Struthio camelus*)—a review. Avian Pathology 29, 95-100.

Alexander, D.J., Bell, J.G., Alders, R.G., 2004. A technology review: Newcastle disease, with special emphasis on its effect on village chickens. The Food and Agriculture Organization of the United Nations FAO TECA.

Ali, H.S.a., Abd EL -Dayem, G.A., 2019. Molecular characterization for Newcastle virus from some farms in Dakhalia Governorate years 2017-2018. Second International Conference of Animal Health Research Institute. Animal Health Research Journal 7 312-330.

Allwright, D., 1996. Viruses encountered in intensively reared ostriches in southern Africa. Proceeding of Improving our Understanding of Ratites in a Farming Environment, Oxford, UK, pp. 27-33.

Altschul, S.F., Gish, W., Miller, W., Myers, E.W., Lipman, D.J., 1990. Basic local alignment search tool. Journal of Molecular Biology 215,

403-410.
Amer, N., 2012. Studies on improving ostrich egg hatch ability and its relation with some factors affecting embryonic development during artificial incubation. PhD. Thesis. Department of Animal Production Faculty of Agriculture, Cairo, Al-Azhar University, Egypt.
Anis, Z., Morita, T., Azuma, K., Ito, H., Ito, T., Shimada, A., 2013. Comparative study on the pathogenesis of the generated 9a5b Newcastle disease virus mutant isolate between chickens and waterfowl. Veterinary Pathology 50, 638-647.
Bello, A., Frei, S., Peters, M., Balkema-Buschmann, A., Baumgärtner, W., Wohlsein, P., 2017. Spontaneous diseases in captive ratites (Struthioniformes) in northwestern Germany: A retrospective study. PLoS One 12, e0173873.
Brown, C., King, D., Seal, B., 1999. Pathogenesis of Newcastle disease in chickens experimentally infected with viruses of different virulence. Veterinary Pathology 36, 125-132.
Brown, C., Peinke, D., Loveridge, A., 1996. Mortality in near-term ostrich embryos during artificial incubation. British Poultry Science 37, 73-85.
Carrasco, A.d.O.T., Seki, M.C., Mineo, T.W.P., Peres, J.A., Knychala, L.M., Alves, M.H., Benevenute, J.L., Ikeda, P., Pinto, A.A., 2015. Use of immunohistochemistry (IHC) in the detection of Newcastle disease virus (NDV) in experimentally and naturally infected birds. African Journal of Microbiology Research 9, 2225-2231.
Cattoli, G., Susta, L., Terregino, C., Brown, C., 2011. Newcastle disease: a review of field recognition and current methods of laboratory detection. Journal of Veterinary Diagnostic Investigation 23, 637-656.
Chen, J.-P., Wang, C.-H., 2002. Clinical epidemiologic and experimental evidence for the transmission of Newcastle disease virus through eggs. Avian diseases 46, 461-465.
Davies, S., 2003. Birds I tinamous and ratites to hoatzins. Grzimek's animal life encyclopedia 2, 99e101.
Deeming, D., 1995. Possible effect of microbial infection on yolk utilisation in ostrich chicks. Veterinary Record 136, 270-271.
Deeming, D., 1996. Production, fertility and hatchability of ostrich (*Struthio camelus*) eggs on a farm in the United Kingdom. Animal Science 63, 329-336.
Elboraay, I.M., Elshorbagy, M.A., Hassan, M.K. and El-sayed, H. S.,, 2013. Isolation and characterization of Newcastle disease virus from ostriches (*Struthio camelus*) in Egypt. Benha Veterinary Medical Journal 24, 27-33. .
Etriwati, R.D., Handharyani, E., Setiyaningsih, S., 2017. Pathology and immunohistochemistry study of Newcastle disease field case in chicken in Indonesia. Veterinary World 10, 1066.
Ghiamirad, M., Pourbakhsh, A., Keyvanfar, H., Momayaz, R., Charkhkar, S., Ashtari, A., 2010. Isolation and characterization of Newcastle disease virus from ostriches in Iran. African Journal of Microbiology Research 4, 2492-2497.
Holt, J.G., Krieg, N.R., Sneath, P.H., Staley, J.T., Williams, S.T., 1994. Bergey's Manual of determinate bacteriology. Published by LWW; Ninth edition.
Huchzermeyer, F., 1996. Velogenic Newcastle disease in ostriches in South Africa. Proceedings of Improving our Understanding of Ratite in a Farming Environment, p. 44.
Huchzermeyer, F.W., 1998. Diseases of ostriches and other ratites. Onderstepoort Veterinary Institute, Private Bag X05.
Jahantigh, M., 2010. Bacteriological study of dead-in-shell embryos of ostrich. Iran J Vet Res 11, 88-90.
Khorajiya, J., Pandey, S., Ghodasara, P.D., Joshi, B., Prajapati, K., Ghodasara, D., Mathakiya, R., 2015. Patho-epidemiological study on Genotype-XIII Newcastle disease virus infection in commercial vaccinated layer farms. Veterinary World 8, 372.
Kiernan, J., 2015. Histological and histochemical methods. Scion Publishing Ltd.
Landry, M.L., Hsiung, G.D., 2000. Primary isolation of viruses. In: Clinical virology manual. 3rd ed. S. Specter, R. L. Hodinka, and S. A. Young, eds. ASM Press, Washington, DC., 27-42.
Lin, C.L., Chiu, C.H., Chu, C., Huang, Y.C., Lin, T.Y., Ou, J.T., 2007. A multiplex polymerase chain reaction method for rapid identification of *Citrobacter* freundii and *Salmonella* species, including *Salmonella* Typhi. Journal of Microbiology, Immunology, and Infection= Wei Mian yu gan ran za zhi 40, 222-226.
OIE, 2009. Newcastle Disease: Aetiology, Epidemiology, Diagnosis, Prevention and Control References. OIE Technical Disease Cards.
Ravindra, P.V., Tiwari, A.K., Ratta, B., Chaturvedi, U., Palia, S.K., Chauhan, R.S. , 2009. Newcastle disease virus-induced cytopathic effect in infected cells is caused by apoptosis. Virus research 141, 13-20.
Rima, B., Alexander, D., Billeter, M., Collins, P., Kingsbury, D., Lipkind, M., Nagai, Y., Orvell, C., Pringle, C., Ter Meulen, V., 1995. Virus taxonomy. Sixth Report of the International Committee on Taxonomy of Viruses. Arch Virol Suppl 10, 268.
Roy, P., Venugopalan, A., 2005. Unexpected Newcastle disease virus in day old commercial chicks and breeder hen. Comparative Immunology, Microbiology and Infectious Diseases 28, 277-285.
Sá e Silva, M., Susta, L., Moresco, K., Swayne, D.E., 2016. Vaccination of chickens decreased Newcastle disease virus contamination in eggs. Avian Pathology 45, 38-45.
Samberg, Y., Hadash, D., Perelman, B., Meroz, M., 1989. Newcastle disease in ostriches (*Struthio camelus*): field case and experimental infection. Avian Pathology 18, 221-226.
Susta, L., Jones, M., Cattoli, G., Cardenas-Garcia, S., Miller, P., Brown, C., Afonso, C., 2015. Pathologic characterization of genotypes XIV and XVII Newcastle disease viruses and efficacy of classical vaccination on specific pathogen-free birds. Veterinary Pathology 52, 120-131.
Tamura, K., Stecher, G., Peterson, D., Filipski, A., Kumar, S., 2013. MEGA6: molecular evolutionary genetics analysis version 6.0. Molecular Biology and Evolution 30, 2725-2729.
Thompson, J.D., Higgins, D.G., Gibson, T.J., 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Research 22, 4673-4680.
Verwoerd, D., 1995. Velogenic Newcastle disease epidemic in South Africa II. Ostriches, waterfowl, exotic bird collections and wild birds. S. Afr. Vet. Med. 8, 44-49.
Verwoerd, D., 2000. Ostrich diseases. Revue Scientifique et Technique-Office International des Epizooties 19, 638-652.
Wicaksana, I.G.H.P., Adi, A.A.A.M., Kardena, I.M., 2019. Pathological Lesions in Chicken Embryo Caused by Newly Virulent Isolate of Newcastle Disease Virus. Jurnal Veteriner Jurnal Veteriner September 20, 337-344.
Wiercinska, M., Szczerbinska, D., 2005. The ostrich and emu egg hatchability with reference to dead embryo analysis. Electronic Journal of Polish Agricultural Universities. Series Biology 8.
Wise, M.G., Suarez, D.L., Seal, B.S., Pedersen, J.C., Senne, D.A., King, D.J., Kapczynski, D.R., Spackman, E., 2004. Development of a real-time reverse-transcription PCR for detection of Newcastle disease virus RNA in clinical samples. Journal of Clinical Microbiology 42, 329-338.