Research Article

Marek's Disease Virus in Egypt: Historical Overview and Current Research Based on the Major MDV-Encoded Oncogene Meq

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Abstract | An update survey of the MDV strains circulating in the Egyptian poultry flocks during 2015 -2016 was conducted. Twenty one vaccinated chicken flocks suffering from different form of lymphomas, representing different governorates in the Egyptian Delta, were screened for MDV infection. Pathological findings in the visceral tumours and presence of viral DNAs with variable mitotic figures were observed in the examined visceral organs. Eight samples were screened positive for the major MDV-encoded oncogene Meq which was subjected for alignment and phylogenetic analysis. The eight studied consensus amino acid sequences of Meq proteins possess several amino acid mutations associated with the MDV virulence and a unique distortion in the Proline repeats (Proline-to-Alanine) at position 176 in the Egyptian MDV strains. The Phylogenetic analysis grouped the eight analysed sequences with the previously investigated Meq from Egypt (2011-2013) together with the very virulent European and Chinese MDV isolates. The latter confirmed the geographical structuring of the Egyptian MDV strains together with the Eurasian origin viruses in an independent taxonomic unit distant from the North American strain.

Received | April 01, 2018; **Accepted** | May 07, 2018; **Published** | June 30, 2018
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DOI | http://dx.doi.org/10.17582/journal.hv/2018/5.3.35.43
Citation | Abdallah, F., O. Hassnain, E. Attar, H. Ali, M. Megahed and V. Nair. 2018. Marek's disease virus in Egypt: historical overview and current research based on the major mdv-encoded oncogene meq. *Hosts and Viruses*, 5(3): 35-43.
Keywords: Marek's, Meq, Egypt, Poultry, DNA

Introduction

Neoplastic diseases in chickens are comprised mainly of three etiologically distinct disease complexes. One of them is a member of the *Herpesviridae* family, Gallid herpesvirus II or usually known as Marek's disease virus (MDV) and the other two belong to *Retroviridae* family, avian leukosis (AL) and reticuloendotheliosis (RE) viruses. MDV causes economically important contagious neurolymphoproliferative and immunosuppressive diseases in the domestic chickens. Although, it consists of three serotypes, only MDV type 1 is able to cause disease in chickens. It is able to establish infection in a variety population of lymphocytic cells during distinct phases of infection as it causes early cytolytic infection in B and T lymphocyte as well as a latent infection followed by transformation of T lymphocytes followed by neoplastic transformation (Calnek, 2001). MDV has been associated with numerous clinical and pathologic syndromes

in chickens such as lymphoproliferative syndromes, lymphodegenerative syndromes, CNS syndromes and vascular syndromes (Witter and Schat, 2003).

For the last four decades, the Egyptian poultry flocks have been suffering from recurrent outbreaks of MD with increase in virus virulence. The existence of the classical clinical form of MDV in Egypt was firstly reported as early as 1953 (Soliman et al., 1954) as clinical cases of neurolymphomatosis. The investigated cases were categorized as a neural type of the avian leukosis complex. Thereafter, many seroepidemiological and pathological studies have documented the spread of MDV in the Egyptian commercial poultry flocks, with the authorization of MDV vaccine (HVT) from 1980. Those studies documented that MDV is the primary cause of the problems of chicken neoplasms in Egypt, even in the vaccinated flocks (Sheble et al., 1973; El-Saiad, 1977; Amin et al., 2001). The first proven isolation trial of virulent MDV from Egypt was performed at the beginning of this century by Awad (2002). Hence, he succeeded in the isolation of three highly virulent MDV from the buffy coat of 25 apparently healthy commercial broiler flocks slaughtered at poultry processing plants. The isolated viruses were confirmed to be very virulent by pathogenicity tests in specific pathogen free and commercial chicks with lesions of diffuse lymphocytic infiltration in visceral organs and nerves (Abd El-Hamid et al., 2002; Ahmed et al., 2002; Awad, 2002).

The previously mentioned studies stimulated another work to evaluate the protective efficacy of HVT vaccine against challenge with a highly virulent locally isolated strain 11/Alex/99 (Ahmed et al., 2004). Infection with this virulent strain induced clinical signs of variable degree in severity in both vaccinated and unvaccinated groups. However, the clinical findings were relatively milder and less frequent in vaccinated birds compared with unvaccinated (Ahmed et al., 2004). In this study, MDV histopathological findings were in the form of variable degrees of lymphodegenerative and or cytolytic changes in thymus, bursa and spleens. Lymphoproliferative visceral lesions were observed in the liver and spleens of sacrificed birds as well as lymphoproliferative and inflammatory changes in the sciatic nerve (Ahmed et al., 2004; Awad et al., 2004). This work concluded that the cell free HVT vaccine gave unsatisfactory protection under controlled experimental conditions and recommended the necessity of introduction of Rispens strain CVI988 or R2/23 strain to be applied individually or as combine vaccines with serotype 2 or 3 in the Egyptian broiler industry. This finding was later confirmed by Hussein and colleagues who reported that the bivalent vaccination in the Egyptian broiler breeder flocks is recommended to induce protection from infection with the hypervirulent MDV strains (Hussein et al., 2004).

Since 2011, a few molecular surveys have been conducted to investigate the molecular situation of MDV in Egypt. The main focus of these screens was the sequence analysis of Meq protein, one of the major determinants of MDV virulence. In the period from 2011 to 2016, Meq protein from several MDV strains circulating in Egypt was shown to have high sequence identity to many hypervirulent European and Chinese MDV isolates (Hassanin et al., 2013; Lebdah et al., 2017). World Organization for Animal Health (OIE) has recently mapped Egypt and China as major endemic areas for MD with outbreaks reported on a yearly basis before and after 2009 (Boodhoo et al., 2016). Here, we investigate clinical, histopathological and molecular changes associated with MD outbreaks circulating in vaccinated poultry flocks in Egypt during 2015-2016.

Materials and Methods

Field sampling and histopathological examination

The samples were collected during 2015 -2016 from 21 vaccinated chicken flocks with either HVT or Rispens vaccine at one day old age, suffering from progressive tumours in different visceral organs, located in five distinct provinces in the Egyptian Delta: EL-Sharqyia (13 flocks), Kafr-Elsheikh (one flock), EL-Dakahlia (three flocks), Domiate (three flocks) and EL-Gharbia (one flock). Feather tip pulps and visceral organs with diffuse or focal tumours were collected from three birds per flock after sacrificed according to the slaughterhouse guidelines in Egypt. All feather tip pulp samples were preserved at -20 ˚C until genomic DNA extraction and PCR amplification of MDV. On the other hand, liver, spleen, bursa of Fabricius, ovary and proventriculus with focal and diffuse tumours were fixed immediately in 10 % buffered formalin for histopathological examination. Thin paraffin-embedded sections of the fixed tissues were stained with hematoxylin and eosin stain (Suvarna et al., 2013) for light microscopy. The present study was approved ahead by the Animal Ethical Committee of the Faculty of Veterinary Medicine, Zagazig University, Egypt.

Genomic DNA extraction

Viral DNA was extracted from Feather tip pulps using PathoGene-Spin™ Viral DNA RNA extraction kit (iNtRON Biotechnology, Korea) according to the manufacturer's instructions.

PCR amplification of full-length Meq gene

Meq-specific forward and reverse primers (Metabion International AG, Germany) were designed as described (Hassanin et al., 2013) and were used to amplify the full-length of the Meq gene. The amplification was performed using i-TaqTM 2x PCR Master Mix kit (iNtRON Biotechnology, Korea). The plasmid (pDONR^{207}) encoding the full length of the Meq gene was used as a positive control in the present study. Amplification was performed under the following conditions: 94 ˚C for 2 min followed by 40 cycles at 94 ˚C for 20 sec., 55 ˚C for 20 sec. and 72 ˚C for 1 min, with a final extension at 72 ˚C for 5 min. After amplification, 5 µl of PCR products were separated by electrophoresis on stained agarose gel (1%) with ethidium bromide against Sizer™1000 DNA Markers (iNtRON Biotechnology, Korea).

DNA sequencing

The obtained Meq gene PCR products were purified using MEGA quick-spin ™ Plus Fragment DNA Purification Kit (iNtRON biotechnology) following the manufacturer's instructions. The purified PCR products were sent for sequencing commercially using AB13730XL sequencer (ELim Biopharmaceuticals). Meq gene specific primers, spanning the entire length, were used for sequencing.

Sequence analysis of full-length Meq gene

Meq gene sequences of the positive samples were compared and aligned with the sequences of selected MDV reference strains accessible in the GenBank database, and their accession numbers from the GenBank database were listed (Table 2). Comparative alignment of the Meq protein amino acid sequences was performed using the Clustal W Multiple alignment of BioEdit Version 7.0 software (Hall, 1999). Sequence identities and divergences were computed utilizing MegAlign software (DNA STAR® Lasergene® version 7.2, USA). A phylogenetic tree was constructed using the neighbor-joining method employing the Kimura 2-parameter model in MEGA6.06 software (Tamura et al., 2013) with bootstrapping 1000.

Molecular screening of avian leukosis (ALV)

The positive Meq samples were screened for ALV infection using common primers for all ALV- subgroups (H5-5'-GGATGAGGTGACTAAGAAAG-3') and (AJ1-5'-ATGAACGGCCCATTCCCCTATTCC-3') (Venugopal, personal communication). The sequence of ALV primers were kindly A touchdown gradient PCR was carried out under the following conditions; 94 ˚C for 2 min followed by 20 cycles at 94 ˚C for 20 sec, 60-50 ˚C for 20 sec, and 72 ˚C for 2 min then 20 cycles at 94 ˚C for 20 sec, 55 ˚C for 20 sec, and 72 ˚C for 2 min with a final extension at 72 ˚C for 5 min. The amplification was performed using using i-TaqTM 2x PCR Master Mix kit (iNtRON Biotechnology, Korea), 1 µL of DMSO and 1.5 mM of MgCl2 were included in each reaction.

Results and Discussion

In spite of an intensive vaccination policy with either CVI988 alone or with a combined CVI988 and HVT vaccine at one day old age, Egypt was mapped as one of the most endemic areas for MD with outbreaks reported on a yearly basis (Boodhoo et al., 2016). Therefore, in the last few years, very virulent MDV strains in the Egyptian poultry flocks gained special attention and was investigated in many studies (Hassanin et al., 2013; Lebdah et al., 2017; Zanaty et al., 2017). For this reason, an update survey on the MDV strains circulating in the Egyptian poultry flocks during 2015 -2016 was conducted. Twenty one vaccinated chicken flocks suffering from different form lymphomas, representing different governorates in the Egyptian Delta, were screened for MDV histopathological findings in the visceral tumours and DNA presence in the feather follicles.

Gross pathological and Histopathological findings

Samples were obtained from chicken flocks between 70 and 360 days of age. In the 21 flocks examined, necropsy revealed visceral lymphomatosis in liver, spleen, proventriculus, Bursa of Fabricius and gonads in the form of diffuse or localized lymphomas. The nodules size ranged from pin point foci to large nodules. Microscopically, the tissues of the ovaries, proventriculus, spleen, liver and the bursa of Fabricius were obliterated by pleomorphic neoplastic lymphoid cells (Figure 1). The ovaries showed heavy infiltration with myelocytes with its characteristic eosinophilic cytoplasm (Figure 1a). Numerous mitotic figures were seen in the neoplastic cells infiltrating the ovarian tissues (Figure 1b)

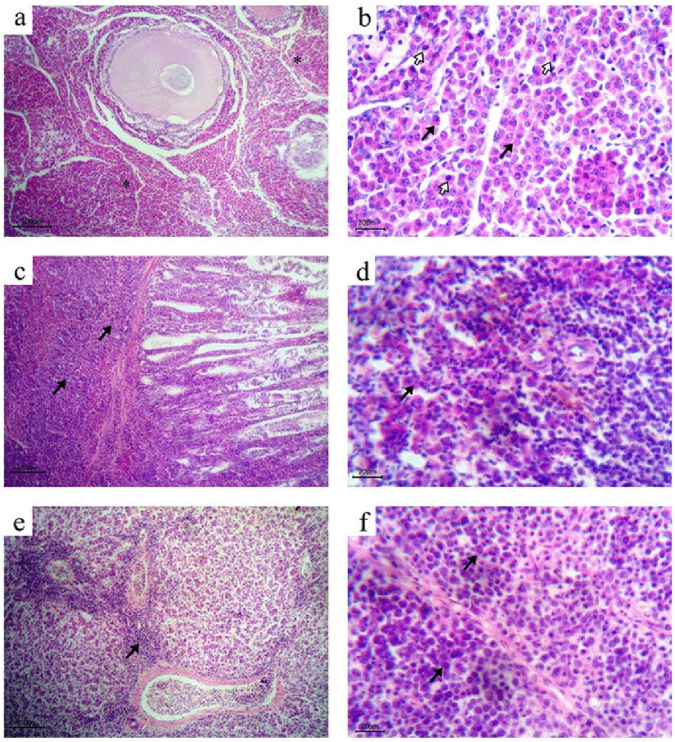

Figure 1: *Histopathology of MDV naturally infected birds (HandE stain).* **a):** *Ovary. Massive diffuse infiltration with myelocytes (asterisks) with its characteristic eosinophilic cytoplasm;* **b):** *Ovary. Myeloid cells proliferation (black arrows) with numerous mitotic figures (white arrows);* **c):** *Proventriculus. Diffuse infiltration with neoplastic lymphoid cells both in the lamina propria (arrows) and the proventricular glands;* **d):** *Spleen. Mixed population of lymphoid tumour cells and myeloid cells (arrow) adjacent to the central arterioles;* **e):** *Liver. Periportal infiltrations with neoplastic lymphoid cells (arrow);* **f):** *Bursa of Fabricius. Diffuse infiltrations with pleomorphic neoplastic lymphoid cells (arrows).*

which was frequently reported in the adult birds (Payne and Venugopal, 2000). Neoplastic lymphoid cells were seen in the lamina propria and the glandular epithelium of the proventriculus (Figure 1c). Similar findings were reported in a recent ALV-MDV co-infection in egg laying poultry flocks in Japan (Wen et al., 2018); however, histopathologically it was not clear whether MDV or ALV-J infection is incriminated in these findings. On contrary, in the present work we can confirm that MDV is responsible for this pathological lesion in different tissues including the Proventriculus, as the MDV-positive flocks were screened negative for ALV. Splenic tissues showed a mixture of lymphoid tumour cells and myeloid cells adjacent to the central arterioles (Figure 1d). In the liver, the lesion varied from few lymphoid nodules to massive infiltration and replacement of the hepatic tissues with neoplastic lymphoid cells (Figure 1e). This consistent micro-structural damage and pleomorphic lymphoid infiltration in different visceral organs are characteristic for MDV tumours and was described earlier by many reports (Payne and Venugopal, 2000; Zhuang et al., 2015). The bursa of Fabricius revealed necrotic lymphoid follicles and infiltrations with pleomorphic neoplastic lymphoid cells (Figure 1f) as was previously described by (Yavuz and Erer, 2017).

Detection of Meq oncogene by PCR

Meq, exists as two isoforms, a short form with 1017 bp in the oncogenic strain and a longer form with a 180-bp sequence insertion (L-Meq) in the non-oncogenic vaccine strains (Jones et al., 1992; Lee et al., 2000). Out of 21 flocks investigated, eight flocks were screened positive for the amplification of the entire full length of the Meq gene (1017bp). PCR products of the Meq-positive samples were further sequenced and submitted to GenBank under accession numbers (Table 1). It is worth mentioning that all MDV-positive samples were screened negative for all ALV- subgroups.

Alignment and phylogenetic analysis of Meq gene

Analysis of the Meq gene was performed between the studied sequences (Table 1) and 28 reference MDV strains (Table 2). The homology analysis revealed 100% deduced amino acid sequence identity between MDV-chicken-Egypt- EL-Sharqyia 1-2015, MDV-chicken-Egypt- EL-Sharqyia 2-2015, MDV-Chicken-Egypt-Kafr-Elsheikh1-2015, MDV-Chicken-Egypt-EL-Dakahlia1-2016, MDV-Chicken-Egypt-Domiate 1-2015 and MDV-Chicken-Egypt- EL-Gharbia 1-2016. Those six sequences showed 99.7% amino acid sequence identity to MDV-chicken-Egypt- EL-Sharqyia 3-2015 and MDV-Chicken-Egypt-EL-Dakahlia 2-2016. The eight deduced amino acid sequences showed high homology (≥ 98%) to the previously reported Egypt1, 2, 3 and _4 and 96% to Egypt_5 (Hassanin et al., 2013). The studied isolates have amino acid sequence homology of approximately ≥97% to the very virulent Chinese strains LMS (Cheng et al., 2012), WS03 (Tian et al., 2011) and HL-1111 (Zhang et al., 2016), the very virulent Indian strain GADVASU-M5, the Hungarian very virulent strain ATE, the UK very virulent strain pC12-130-10 (Spatz et al., 2011) and the virulent American strain 617A (Shamblin et al., 2004). On the other hand, the sequences showed lower amino acid sequence homology to the vaccine strain CVI988 (≤73%).

Recent data has suggested that the mutations in the Meq may be associated with virulence of MDV strains (Trimpert et al., 2017) and some of the mutations such

Table 1: *Descriptive data of MDV infected flocks investigated in this study.*

Strain	Province	Breeding	Age (day)	MDV vaccination history	Clinical examination	GenBank Accession number (Meq)
MDV-chicken-Egypt-EL-Sharqyia 1-2015	EL -Sharqyia	Layer	85	HVT	Emaciation	MF773445
MDV-chicken-Egypt-EL-Sharqyia 2-2015	EL -Sharqyia	Layer	210	HVT	Visceral tumors	MF773446
MDV-chicken-Egypt-EL-Sharqyia 3-2015	EL -Sharqyia	Layer	360	HVT	Visceral tumors	MF773447
MDV-Chicken-Egypt-Kafr-Elsheikh 1-2015	Kafr-Elsheikh	Layer	220	Rispens	Visceral/Neural tumors (enlargement sciatic nerve)	MG913293
MDV-Chicken-Egypt-EL-Dakahlia 1-2016	EL-Dakahlia	Layer	170	HVT	Visceral tumors	MG913294
MDV-Chicken-Egypt-EL-Dakahlia 2-2016	EL-Dakahlia	Layer	345	Rispens	Visceral tumors	MG913295
MDV-Chicken-Egypt-Domiate 1-2015	Domiate	Layer	110	HVT	Visceral tumors	MG913296
MDV-Chicken-Egypt-EL-Gharbia 1-2016	EL-Gharbia	Layer	113	HVT	Visceral tumors	MG913297

Table 2: *List of MDV reference strains used in the phylogenetic analysis study.*

Strains	Virulence	Number of PPPPs repeats	Country of origin	Accession number
LMS	Very virulent	3	China	JQ314003.1
WS03	Very virulent	3	China	HQ638152.1
ATE	Very virulent	5	Hungary	AY571784.1
pC12-130-10	Very virulent	5	USA	FJ436096.1
tn-n2	Very Virulent	5	India	HM749325.1
RB1B	Very Virulent	5	USA	HM488349.1
GA	Virulent	5	USA	AF147806.1
648A	Very virulent plus	2	USA	AY362725.1
584a	Very virulent plus	2	China	DQ534532.1
X	Very virulent plus	2	USA	AY362724.1
L	Very virulent plus	2	USA	AY362717.1
U	Very virulent plus	2	USA	AY362722.1
Md5	Very virulent	4	USA	AF243438.1
CU-2	Mild	7	USA	AY362708.1
CVI988	Attenuated	7	Commercial vaccine	DQ530348.1
BC-1	Virulent	7	USA	AY362707.1
617A	Virulent	4	USA	AY362712.1
Egypt1	n/a	3	Egypt	JX467678.1
Egypt2	n/a	3	Egypt	JX467679.1
Egypt3	n/a	3	Egypt	JX467680.1
Egypt_4	n/a	3	Egypt	KC161220.1
Egypt_5	n/a	4	Egypt	KC161221.1
GADVASU-M5	n/a	5	India	KY651235.1
HL-1111	n/a	5	China	KP888821.1
MPF57	virulent	5	Australia	EF523774.1
MDV-1-SA-2013	n/a	4	Saudi Arabia	KJ949617.1
New	Very virulent plus	2	USA	AY362719.1
HNGS201	n/a	7	China	HF546085.1

n/a: *not available.*

as 71 (Alanine), 77 (Lysine), 80 (Tyrosine), 115 (Alanine), 139 (Alanine), 176 (Arginine), 217 (Alanine) were used as genetic markers for the level of virus virulence. In our study, several amino acids mutations associated with the MDV virulence were observed in the eight sequences of Meq gene including 71 (Alanine), 80 (Tyrosine), 139 (Alanine), 176 (Alanine) and 217 (Alanine) (Table 3). Neither of mutations at 77 (Lysine) and 115 (Alanine) were detected in any of the eight sequences. However, these two mutations were not consistent in all the virulent MDV strains. For example, 77 (Lysine) was not observed in the previously studied virulent Egyptian and Chinese strains as well as in the American very virulent strains TK, L, X and U (Hassanin et al., 2013; Shamblin et al., 2004; Tian et al., 2011). Similarly, 115 (Alanine) was not seen in the European very virulent isolates ATE and pC12-130-10 or in most of the American virulent and very virulent strains. These two mutations are not correlated with the MDV virulence and not reliable genetic markers for the level of virus virulence.

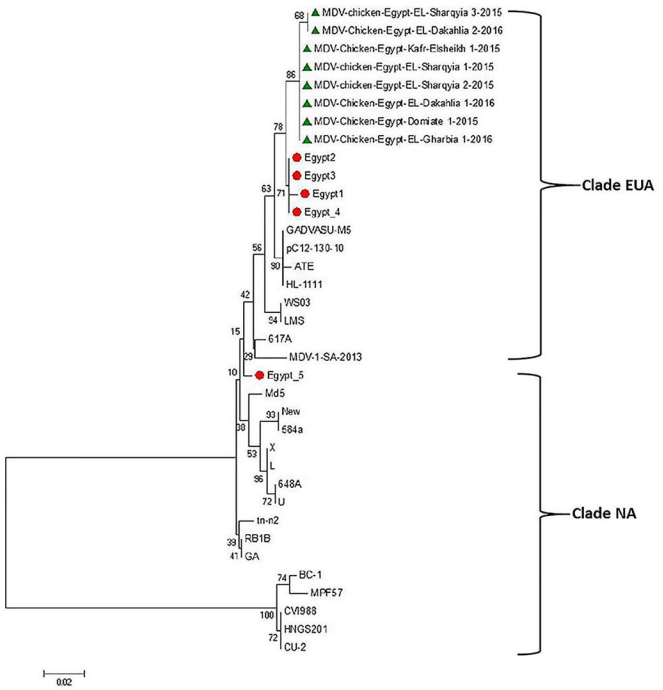

Figure 2: *Phylogenetic analysis of MDV based on Meq protein amino acid sequences. phylogenetic tree was constructed via multiple alignments of amino acid sequence of Meq protein of 28 reference strains and the eight Egyptian sequences using a distance-based neighbour joining method with bootstrapping (1000) with MEGA6.06 software. Egyptian MDV Sequences of this study and earlier one was indicated using green triangle and red circle, respectively. Clades EUA and NA are abbreviation for Eurasian and North American.*

Renz and his colleagues suggested that the number of the four Proline repeats is strongly associated with the level of MDV virulence (Renz et al., 2012). Hence, the overall range of the four Proline repeats in all MDV strains ranged from 2-8 and negative correlation is existed between their numbers and the virulence. In the present study, only three Proline rich repeats (PPPPs) sequences (152-155), (191-194) and (232-235) were observed. The distortion in the Proline repeats was seen because of a Proline-to-Alanine substitution at positions 176 and 217. A Proline substitution to Leucine/Serine/Arginine at position 176 have been detected in the recently isolated very virulent MDV-1 strains in China, Japan and the USA (Shamblin et al., 2004; Zhang et al., 2011; Murata et al., 2013). Previously sequenced Egyptian strains also possess a Proline substitution to Arginine at position 176 (Hassanin et al., 2013). Therefore, this newly detected Alanine substitution (176) in all currently the studied strains is considered a novel unique point mutation characteristic for the isolates associated with this outbreak.

For the Phylogenetic analysis of the studied sequences, the amino acid sequences of Meq protein of 28 reference strains and the eight sequences from this study were carried out using a distance-based neighbour joining method with bootstrapping (1000) Mega software. The amino acid tree of MDV based on the Meq protein revealed that the eight sequences circulating in Egypt (2015-2016) were grouped with the previously investigated Meq protein from Egypt (2011-2013) together with the European very virulent ATE and pC12-130-10 strains and the Asian very virulent MDV strains LMS, WS03, HL-1111 and GADVASU-M5 in clade EUA (Figure 2). On the other hand, the virulent, very virulent and very virulent plus MDV strains isolated from the USA and the vaccine strain CVI988, CU-2, BC-1and MPF57 were grouped together in clade NA (Figure 2). The nearest neighbours for the Egyptian MDV strains were the Asian and European very virulent strains, suggesting the geographical structuring of the Egyptian MDV strains with Eurasian origin viruses in an independent taxonomic unit distant from the North American strains (Trimpert et al., 2017). The latter observation suggested that MDV strains evolve separately and the evolution is driven by various factors including the strategy used to prevent MDV infection in each country.

In conclusion, the virulent MDV strains circulating in Egypt during 2015-2016 were in the same clade of viruses of Eurasian origin since 2011 with minor changes in the sequence composition of the Meq pro-

tein including the unique substitution at position 176 of the Proline rich repeats. It cannot be confirmed

Table 3: *Amino acid substitutions in the sequence of the Meq protein of the studied strains.*

Domains / Strains	Basic Region			Leucine Zipper					Transactivation domain (Proline-rich repeats)			
	A71	E77	Y80	T88	R93	V115	C119	A139	P153 PPPP(P)	A176 PPPP(A)	A180	A217 PPPP(A)
Egypt 1, 2, 3, _4										R	T	
ATE,pC12-130-10,HL-1111, GAD-VASU-M5										P	T	P
WS03, LMS				A	Q	A				R	T	
MDV/1/SA/2013				A	Q			T		P	T	P
617A				A	Q		R	T		P	T	
New, 584a		K	D	A	Q		R	T	Q		T	
L, X, U, 648A		K	D	A	Q		R	T	Q	R		
Egypt _5		K	D	A				T		P		P
Md5		K	D	A	Q			T		P	T	
RB1B, GA,Tn-n2		K	D	A	Q			T		P	T	P
CU-2, CVI988, HNGS201	S		D	A	Q			T		P	T	P
BC-1	S	A	D	A	Q	A		T		P	T	P
MPF57	S	A	A	A	Q	A		T		P	T	

wither the recurrent MDV outbreaks hit the Egyptian poultry industry are correlated to the vaccination failure from the vaccine itself or the strategy used in the vaccine application in the field. Further works are mandatory to address this raised question.

Authors Contributions

Mohamed Megahd provided the clinical samples in the present study. Ola Hassnain and Fatma Abdallah designed the study, performed the methodology and evaluated the results. ELsayed Attar and Haytham Ali performed histopathological examinations. Ola Hassnain and Fatma Abdallah wrote the original draft. Venugopal Nair involved in supervision of the work and revising the manuscript. All author approved the final version of the manuscript for submission.

Conflicts of interest

The authors whose names are listed have no affiliations with or involvement in any organization or entity with any financial interest or non-financial interest in the subject matter or materials discussed in this manuscript.

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