Histopathological changes and sequence analysis of field strains of MDV associated with tumors in commercial layer farms in 2015 to 2019.

Mohamed A. Meligy[1], Ayman H. El Deeb[2], Laila A. Tantawey[1] and Hussein A. Hussein[2*]

[1] Poultry Disease and Research Department, Animal Health Research Institute, Agriculture Research Center, Giza, Egypt.
[2] Department of Virology, Faculty of Veterinary Medicine, Cairo University, Giza, Egypt.

* Corresponding author: Hussein A. Hussein, E-mail: husvirol@cu.edu.eg

1. Abstract

Marek's disease is considered one of the major economic problems in all poultry sectors especially commercial layers despite of intensive vaccination. This study reports the existence of MDV serotype1 very virulent plus (VV+) in commercial layer farms aged 16-40 weeks during 2015 to 2019 in eight Egyptian governorates. The study includes 31 affected layer farms demonstrating characteristic tumors with clear typical picture of Marek's disease (high mortality and paralysis). Tumor samples (Liver and spleen) were collected for detection of MDV viral genome by (PCR) using oligonucleotide primers to amplify 132 bp tandem repeat of specific DNA sequence to differentiate between the field strain (434 bp) or vaccine strain (multiple repeats of 132 bp) of MDV. Results revealed that 4 samples out of 31 were positive (two field and two vaccinal strains of MDV). The two field isolates of MDV were from Qalioubia flocks at 15 weeks old, and from Sharkia at 21weeks old, whereas the other two positive vaccinal strains were from Qalioubia at 22 weeks old and Gharbia at 16 weeks old. Sequencing of the amplified fragments of MDV genome was carried out and the obtained sequences were submitted to gene bank with accession numbers **(OK040950 MDV1/AHRI-1/Egypt and OK040951 MDV1/AHRI-2/Egypt)**. Sequence analysis of the 2 isolates showed that both viruses are identical. Indeed, the study reports the circulation of VV+ MDV in commercial layer farms. Whether the existence of field strains due to a vaccination failure or emerging of new strains of MDV need further investigation.

Keywords: Marek's disease virus (MDV), Polymerase Chain Reaction (PCR), Very Virulent Plus (VV+)

2. Introduction

MDV is first described in poultry in 1907 in Hungary causing 10-30% of mortality in rare cases and up to 60% mortalities with multiple economic drawbacks **[1]**. Every year, there are multiple economic problems reaching up to 1-2 billion dollars in poultry projects although MDV problems reported in sporadic cases **[2]**. After consistent usage of vaccination regimens for MDV problem, the spread of the MDV was dropped in last years **[3]**. MDV is highly attached to cells and classified as follow **[4]**:

Order *Herpesvirales*
Family *Herpesviridae*
Subfamily *Alphaherpesvirinae*
Genus *Mardivirus*

Replication of MDV is classified into three stages, the cytolytic, latent and T-cell transformation stage then after last stage MDV can transform multiple

Online ISSN: 2537-1045
Print ISSN: 1110-1423

CD4+T-cell and tumor produced **[5]**. The T-cell transformation stage is one of the important severity index of MDV to produce lymphoma **[6]**. The main clinical picture of MD (neural form) involved partial or complete paralysis of wing, limb and neck and the paralysis is made by lesions in vagus, brachial and sciatic nerves also from histopathological side, there are infiltration by multiple mononuclear cells noticed which is the prove of inflammation and in severe cases we can notice cloudy eyes and small nodules in visceral organs **[7].** In Egypt, three highly virulent MDV were isolated from the buffy coat of 25 apparently healthy commercial broiler flocks at slaughtering house, pathogenicity tests carried out on these viruses in specific pathogen free and commercial chicks induced lesions of diffuse lymphocytic infiltration in visceral organs and nerves **[8].** After 2011, molecular situation in Egypt was investigated by multiple molecular surveys. Sequence analysis of Meq protein was the main component of these surveillance, which is the strong 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 **[9,10].** 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 **[11].** The plan of the present study is to detect and molecular characterize MDV associated with tumors in commercial layers farms using PCR, sequence analysis and histopathology.

3. Materials and Methods

3.1. Samples collection

Detailed history of thirty one affected layer flocks under study between (November 2015 to October 2019) as in table (1)

3.2. Histopathology

Heart, kidney, sciatic nerve and liver from the suspected flocks were collected and fixed on 10% formalin saline and subjected for histopathological examination. Dehydration of these samples was carried out by using ascending grades of alcohol. For cleaning, tissues placed in Xylol. Impregnation was conducted by transferring the specimens in three changes of methyl paraffin wax. Finally, samples were block in hard paraffin cut into sections of 5-micron thickness and prepared for staining by H&E. section covered with slides and examined by light microscope [**12**].

3.3. PCR for identification of MDV.

Using Q1AmpDNA minikit with oligonucleotide primers for BamH1-H 132 tandem repeats. The sequences of primers are:
M1-F (5- TACTTCCTATATAGATTGAGACGT)
M2-R (5- MGAGATCCTCGTAAGGTGTAATATA).
These primers used to detect specific DNA sequence (**434bp**) in positive field MDV results and with only one product in case of Serotype 1 while multiple copies of 132 bp repeats where observed in positive vaccinal strains **[13].**

Online ISSN: 2537-1045
Print ISSN: 1110-1423

3.4. Sequence and Phylogenetic analysis

The Two PCR products were purified from agarose gel using PCR purification kit (Qiagen), for sequencing, a purified PCR product was sequenced in the forward and reverse directions on an Applied Biosystems 3130 automated DNA Sequencer ***(*ABI, 3130, USA*).*** Using a **ready reaction Bigdye Terminator V3.1 cycle sequencing kit. (Perkin-Elmer/Applied Biosystems, Foster City, CA)**, with Cat. No. 4336817. Then sequences sent to the gene bank.

A comparative analysis of sequences was performed using the CLUSTAL W multiple sequence alignment program, version 1.83 of MegAlign module of Lasergene DNA Star software Pairwise, which was designed by **[14, 15, and 16].** Sequence analysis using bioedit file analysis of positive field sequences showed that 2 sequences isolated were identical to each other. Phylogenetic analysis when comparison of the nucleic acid sequences of tandem repeat genes of positive field results with other MDV strains on Gene Bank.

4. Results

4.1. Gross pathology:

Nerves showed loss of cross-striations, gray or yellow discoloration. Swollen and enlargement of liver, spleen, kidney and heart with whitish foci on their surfaces.

4.2. Histopathological examination:

The nerves showed edema and demyelination with infiltration with few small lymphocytes. The liver exhibited focal and diffuse infiltration with pleomorphic lymphocytes, few lymphoblasts and plasma cells. Spleen showed focal pleomorphic lymphocytes infiltration. Kidney infiltrated interstitially with pleomorphic lymphocytes with degenerative changes of renal tubules. Moderate to massive infiltration with lymphocytes in myocardium **Fig. (1).**

4.3. PCR (molecular identification) results:

Out of 31 farms there were 2 samples +ve for field isolate with specific single band (434 bp) **[12]** and 2 positive vaccinal strain with 132 tandem repeats whereas 27 samples were negative for MDV by PCR although these flocks showed typical picture of MDV and positive for histopathology. **Fig. (2).**

4.4. Sequencing:

A purified PCR product was sequenced in the forward and reverse directions then the obtained sequences submitted to the Gene bank with accession numbers of **(OK040950 MDV1/AHRI-1/Egypt and OK040951 MDV1/AHRI-2/Egypt).**

4.5. Sequence and phylogenetic analysis:

Sequence analysis of positive field sequences showed that 2 sequences isolated were identical to each other. Phylogenetic analysis when comparison of the nucleic acid sequences of tandem

Online ISSN: 2537-1045
Print ISSN: 1110-1423

repeat genes of positive field results with other MDV strains on Gene Bank. The results in this study revealed virulent MDV strains similar to the very virulent MDV reported in India, China, America and Europe **Fig.(3).**

5. Discussion

Marek's disease is one of the most commercial neoplastic illness of poultry. MD constituted a serious economic threat to the poultry industry causing up to 60 % layers mortality and 10 % broilers condemnations and sporadic losses still occur **[13]**. In the last few years in Egypt, very virulent MD strains in poultry flocks acquired high attention and was investigated in many studies **[17, 18 and 19].** In the present study, four out of thirty one farms exhibited gross tumor lesions and were positive by histopathology and PCR for MDV. Histopathology can be very useful and decisive to diagnose MD lymphoma and to evaluate the morphology and distribution of tumor cells **[20].** In this study, the pathological lesions of MD were observed in nearly all examined cases, the nerves showed demyelination and edema with lymphocytes infiltration between nerve fibers, massive focal and diffuse pleomorphic lymphocytes, few lymphoblasts, plasma cells infiltration were observed in spleen, liver, kidney, and heart. This infiltration could be attributed to the entrance of the virus via inhalation, then infection of B lymphocytes and macrophages in the lungs **[21]**, thus spreading towards the main lymphoid organs (bursa of Fabricius, thymus and spleen). After replicating in B lymphocytes, MDV infects activated T lymphocytes. Few T lymphocytes undergo transformation and are considered the origin of the T lymphoma. This lymphoma is mostly localized in visceral organs (kidneys, spleen, liver, gonads, and proventriculus), peripheral nerves, skin, and muscles. Sequencing of positive field isolate (**434 bp**) revealed forward and reverse sequences for MDV. Two of the obtained sequences were submitted to GenBank with accession numbers **(OK040950 MDV1/AHRI-1/Egypt and OK040951 MDV1/AHRI-2/Egypt).**

Phylogenetic analysis showed that both field samples are identical to each other with high similarities to very virulent isolates previously reported in India, Europe, Egypt, and USA. The existence and circulation of reported field virus in commercial layer farms may be due to either vaccination failure or emergence of new strains of MDV **[11]**. Phylogenetic analysis of studied sequences revealed high similarities to recently emerged MDV strains in Egypt during (2015-2016) which were previously grouped and investigated with circulating strains during (2011-2013) together with European and Asian strains in clade EUA **[17,18,19]**. Also, recent reports from China showed the emergence of virulent MDV strains that were causing mortalities from 15-60% in HVT-vaccinated chickens which supporting the occurrence and emergence of VV+ MDV strains in HVT-vaccinated flocks **[22]**. On the other hand, several previous studies noted multiple factors affecting efficacy of MDV vaccines, including problems in the preparation, storage handling and administration of vaccine. These pointed out factors referred to host genetics (MHC haplotype), strain or dose of challenge virus, vaccine challenge intervals, and maternal antibody status of the vaccinated chicks **[23]**. Studies on host genetics were carried out in five chicken

Online ISSN: 2537-1045
Print ISSN: 1110-1423

lines comprising four different MHC B-haplotypes. At a challenge dose above 8000 plaque-forming units, differences in protection were noted between the two very virulent strains examined (648A and 686). The interval between vaccination and challenge indicated a protective efficacy from 0 to 2 days varied greatly (12%-82%) after challenge with vv+686, the most virulent virus. Less variation and significant protection began at 3 days post vaccination and reached a maximum at 5 days post vaccination with about 80%-100% protection. Indeed, vaccination failure is not ruled out in the studied cases. The present study reports the circulation of vvMDV strains. Continuous characterization of strains of MDV in the field is needed to maintain and evaluate the efficacy of the utilized MDV vaccination strategy.

6. Conclusion

This study reports the existence of circulating vvMDV associated with tumors in commercial layer farms. These field viruses are similar to those in China, India, Europe and America.

Conflict of interest

Authors declared no conflict of interests exist.

Acknowledgments

Authors would like to acknowledge Dr. Ahmed Maher, Serum and Vaccines Institute and staff members of Animal Health Research Institute, P.O. Box 264-Dokki, Giza12618, Egypt as follow, Dr. Mervet Hamdy, Dr. Aalaa Samir Saad, Dr. Heba Ali and Dr. Ahmed Erfan for their support.

7. References

1. Kennedy, D.A.; Cairns, C.; Jones, M.J.; Bell, A.S.; Salathé, R.M.; Baigent, S.J.; Nair, V.K.; Dunn, P.A.; Read, A.F. Industry-Wide Surveillance of Marek's Disease Virus on Commercial Poultry Farms. Avian Dis. 2017. 61. 153–164.
2. Schat, K.A.; Nair, V. Neoplastic Diseases. In Diseases of Poultry; Swayne, D.E., Ed.; John Wiley & Sons, Inc.: Hoboken, NJ, USA, 2017. 513–673
3. Gimeno, I.M. Marek's disease vaccines: A solution for today but a worry for tomorrow? Vaccine 2008. 26. C31–C41.
4. Davison, A.J. Herpesvirus systematics. Vet. Microbiol. 2010. 143. 52–69.
5. Osterrieder, N.; Kamil, J.P.; Schumacher, D.; Tischer, B.K.; Trapp, S. Marek's disease virus: From miasma to model. Nat. Rev. Microbiol. 2006. 4. 283–294.
6. McPherson, M.C.; Delany, M.E. Virus and host genomic, molecular, and cellular interactions during Marek's disease pathogenesis and oncogenesis. Poult. Sci. 2016. 95. 412–429.
7. Najem, H.A. Clinical, Pathological and Molecular Study of Marek's Disease in the Gallus gllusDomesticus and Brahma Chickens of Basra Province, Iraq. Egypt. J. Vet. Sci. 2021. 52. 63–71.
8. Abd El-Hamid, S.H., Awad, A.M., El-Mankhly, S. and Ahmed, A.A.S. Studies on Marek's disease in commercial broiler chickens. 2. Pathogenicity of Marek's disease virus isolates recently recovered from commercial broilers. In: 10th Scientific Congress, Faculty of Veterinary Medicine, Assuit University. Egypt. 2002. 492-518
9. Hassanin O, Abdallah F, El-Araby IE. Molecular characterization and phylogenetic analysis of Marek's disease virus from clinical cases of Marek's disease in Egypt. Avian diseases. 2013. 57 (2s1). 555-61.

Online ISSN: 2537-1045
Print ISSN: 1110-1423

10. Lebdah MA, Nassif SA, Shahein AM, El-Basrey YF. Isolation and Identification of Very Virulent Strains of Marek's Disease Virus from MDVVaccinated Flocks in Egypt. Zagazig Veterinary Journal. 2017. 45 (3). 197-205.
11. Boodhoo N, Gurung A, Sharif S, Behboudi S. Marek's disease in chickens: a review with focus on immunology. Veterinary research. 2016. 47 (1). 1-9.
12. Suvarna KS, Layton C, Bancroft JD, editors. Bancroft's theory and practice of histological techniques E-Book. Elsevier health sciences; 2018. 40-52
13. Mitra N, Verma R, Singh A. Early detection of avian oncogenic viruses from blood of apparently healthy chickens. Proceedings of the National Academy of Sciences, India Section B: Biological Sciences. 2013. 83. 53-8.
14. Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ. Basic local alignment search tool. Journal of molecular biology. 1990. 215 (3). 403-10.
15. Tamura K, Stecher G, Peterson D, Filipski A, Kumar S. MEGA6: molecular evolutionary genetics analysis version 6.0. Molecular biology and evolution. 2013. 30 (12). 2725-9.
16. Thompson JD, Higgins DG, Gibson TJ. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic acids research. 1994. 22 (22). 4673-80.
17. Hassanin O, Abdallah F, El-Araby IE. Molecular characterization and phylogenetic analysis of Marek's disease virus from clinical cases of Marek's disease in Egypt. Avian diseases. 2013. 57 (2s1). 555-61.
18. Murata S, Hashiguchi T, Hayashi Y, Yamamoto Y, Matsuyama-Kato A, Takasaki S, Isezaki M, Onuma M, Konnai S, Ohashi K. Characterization of Meq proteins from field isolates of Marek's disease virus in Japan. Infection, Genetics and Evolution. 2013. 16. 137-43.
19. Shamblin CE, Greene N, Arumugaswami V, Dienglewicz RL, Parcells MS. Comparative analysis of Marek's disease virus (MDV) glycoprotein-, lytic antigen pp38-and transformation antigen Meq-encoding genes: association of meq mutations with MDVs of high virulence. Veterinary microbiology. 2004. 102 (3-4). 147-67.
20. Gimeno, I.M. and Wakenell, P.S. Marek's disease. In S.M. Williams, L. Dufour-Zavala, M.W. Jackwood, M.D. Lee, B. Lupiani, & W.M. Reed (Eds.), A Laboratory Manual for the Isolation, Identification and Characterization of Avian Pathogens. Jacksonville, FL: American Association of Avian Pathologists. 2016 (pp. 249–258).
21. Fabricant, J., Calnek, B. W., Schat, K. A. and Murthy, K. K. Avian Diseases. 1978. 22 (4). 646-658
22. Gong, Z., L. Zhang, J. Wang, L. Chen, H. Shan, Z. Wang, and H. Ma. Isolation and analysis of a very virulent Marek's disease virus strain in China. Virol. J. 2013. 155. 1 – 8.
23. Lee LF, Zhang H, Heidari M, Lupiani B, Reddy SM. Evaluation of factors affecting vaccine efficacy of recombinant Marek's disease virus lacking the Meq oncogene in chickens. Avian Dis. 2011. 55 (2). 172-9.

Online ISSN: 2537-1045
Print ISSN: 1110-1423

Table 1: Detailed history of thirty one affected layer flocks under study between November 2015 to October 2019 as follows:

Government	Number of flocks	Type of collected samples
Banha (Toukh)	8 flocks	Spleen, liver, proventriculus, kidney, heart and nerves
Sharkia	3 flocks	Spleen, liver, proventriculus, kidney, heart and nerves
Gharbia	2 flocks	Spleen,liver, proventriculus,kidney,heart and nerves
Giza	3 flocks	Spleen,liver, proventriculus,kidney,heart and nerves
Dakahlia	3 flocks	Spleen,liver, proventriculus,kidney,heart and nerves
Alexandria	4 flocks	Spleen, liver, proventriculus, kidney, heart and nerves
Behira	4 flocks	Spleen, liver, proventriculus, kidney, heart and nerves
Fayoum	4 flocks	Spleen,liver, proventriculus,kidney,heart and nerves
Total number of flocks	31 flocks	

Online ISSN: 2537-1045
Print ISSN: 1110-1423

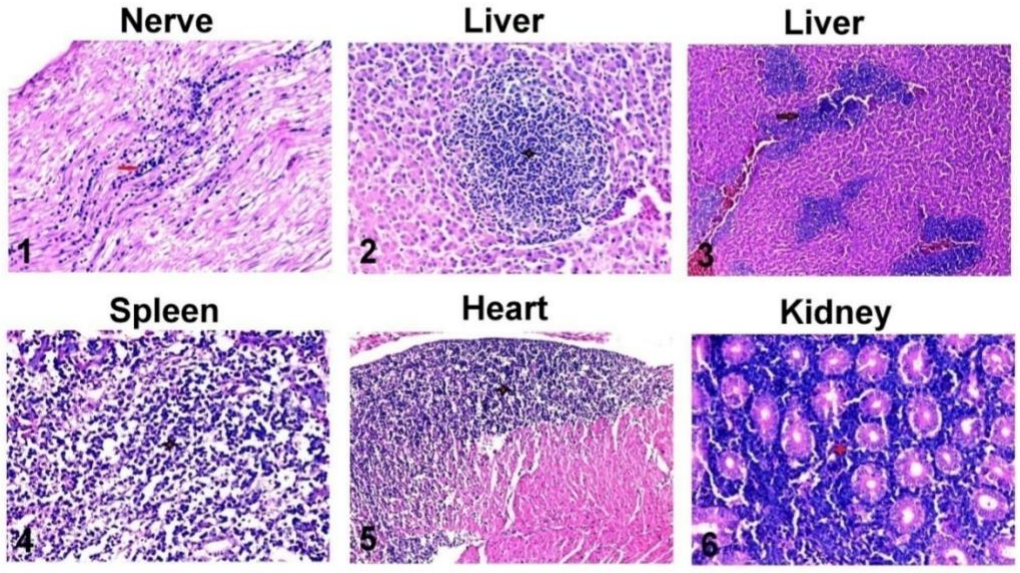

Fig. 1. Histopathological examination of chicken organs affected with typical lesion of MD. (1) Sciatic Nerve showing demyelination and edema of very few small lymphocytes infiltration (arrow) (H&E X 400). (2) Liver showing focal nodule of pleomorphic lymphocytes infiltration and lymphoblasts (star) (H&E x400). (3) Liver showing perivascular multifocal pleomorphic lymphocytes and infiltration (arrow) (H&E x100). (4) Spleen showing diffuse infiltration of different size lymphocytes (H&E X 400). (5) Heart showing massive diffuse infiltration of pleomorphic lymphocytes in myocardium (star) (H&E x200). (6) Kidney showing inter tubular pleomorphic lymphocytes aggregation (star) (H&E x400).

Online ISSN: 2537-1045
Print ISSN: 1110-1423

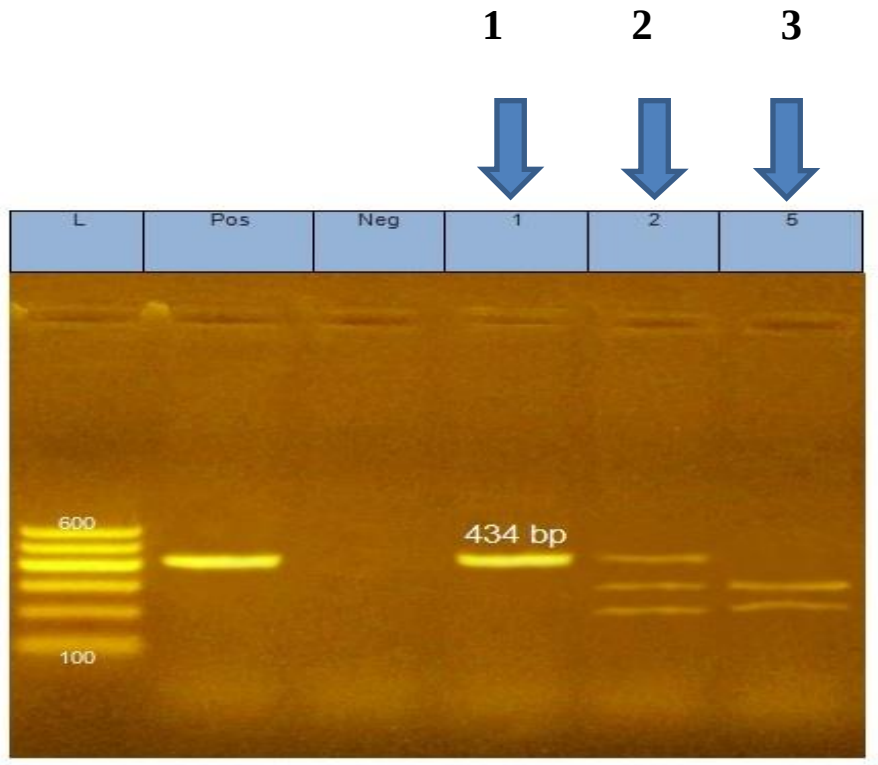

Fig. 2. Showed positive field isolate of MDV with specific DNA product (434 bp) with using of oligo nucleotide primers in sample Lane1, whereas lanes 2 and 3 were of the vaccine strains detected in the study (132 tandem repeats) [13] along with specific ladder.

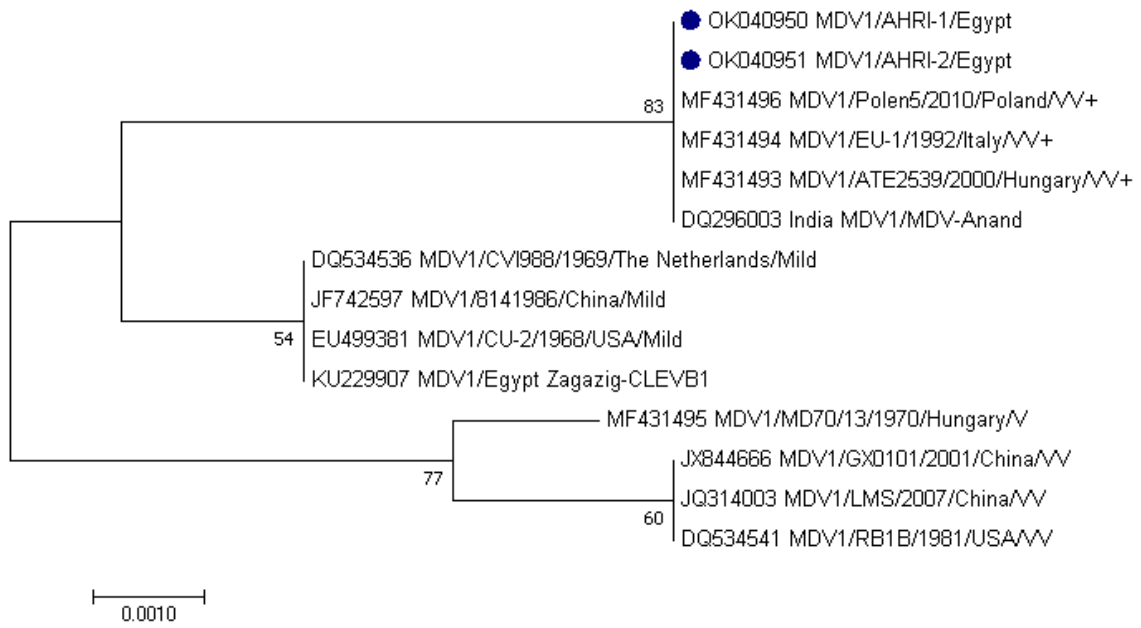

Fig. 3. Phylogenetic analysis of nucleotide sequences of positive field MDV isolates from Egyptian poultry flocks in this study with accession numbers (OK040950 MDV1/AHRI-1/Egypt and OK040951 MDV1/AHRI-2/Egypt)in comparison with other sequences on gene bank (MF431496 MDV1/Polen5/2010/Poland/VV+ , MF431494 MDV1/EU-1/1992/Italy/VV+, MF431493 MDV1/ATE2539/2000/Hungary/VV+, DQ296003 India MDV1/MDV-Anand, JX844666 MDV1/GX0101/2001/China/VV, JQ314003 MDV1/LMS/2007/China/VV, DQ534541 MDV1/RB1B/1981/USA/VV, MF431495 MDV1/MD70/13/1970/Hungary/V, DQ534536 MDV1/CVI988/1969/Netherlands/Mild, JF742597 MDV1/8141986/China/Mild , EU499381 MDV1/CU-2/1968/USA/Mild and KU229907 MDV1/Egypt Zagazig-CLEVB1).

Online ISSN: 2537-1045
Print ISSN: 1110-1423