



## ORIGINAL ARTICLE

# Isolation and Molecular Characterization of Marek's Disease Virus from Layer Chickens in Egypt

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### Abstract

Marek's disease virus is a lymphomatous neuropathic disease of domestic fowl. In Egypt, poultry flocks are suffering from recurrent uprising of MD outbreaks due to increased virulence of the virus. In this study, a total of 10 clinical samples were collected from 6-8 months old vaccinated layer chickens suffering from depression, ataxia and paralysis. These samples were examined for the presence of MDV by PCR using specific primers for ICP4 gene. MDV was detected in 5 out of 10 samples. One out of the 5 positive samples was isolated and partially sequenced for ICP4 gene (F1352-S4). The results proved that it was genetically characterized as very virulent strain of Marek's disease virus type 1 resembling Gallid herpesvirus 2 isolates from Europe, China, Africa and India. There was a characteristic serine residue at the coding region of ICP4 gene encoding the reverse complement protein found only in the Egyptian virus (F1352-S4) and EU-1 virus, IND-KA-02-06, JL/1404 and in Debre-zeit/01/2013 virus that is tracing for the original virus CV1988. The obtained results indicated the importance of continuous monitoring of MDV infection in chicken layer flocks.

**Keywords** Marek's disease virus, PCR, ICP4 gene, Egypt

## 1. Introduction

Marek's disease virus is a serious oncogenic herpesvirus of poultry. MDV causes considerable mortality rate in chickens, in addition of being a leading cause of immunosuppression in the infected chickens. MDV belongs to order *Herpesvirales*, family *Herpesviridae*, subfamily *Alphaherpesvirinae*, genus *Mardivirus*, which has three serotypes as MDV-1, MDV-2, and the serotype 3 herpes virus of turkeys (Dunn et al. 2013). The genome of MDV encodes more than 200 genes. Genome structure and gene content of each region are similar among all MDV serotypes, but there exist key differences; oncogenic

serotype 1 is defined by the presence of the Meq oncogene and other unique genes including pp38, vIL8, and vTR in repeat regions, particularly TRL. The viral genes that are unique to each particular avian herpesvirus are located in the repeat regions of the genome (Lee et al. 2000). The MDV ICP4 homolog gene maps to the BamHI-A fragment, present within the inverted repeat flanking the unique short region of the MDV genome (Cantello et al. 1994). ICP4 gene is located with a major category of genes in MDV, which grouped under genes homologous with alpha-herpes viruses. This is a broad category of genes which is divided into immediate early, early and late genes,

which are important in virus replication (**Handberg et al. 2001**). Imitation activity of MVD in tumor cells is due to the repeat regions (**Ross et al., 1997**). In these regions there are a limited number of genes, including viral telomerase RNA, viral IL-8, meq, pp38, and ICP4 (**Anderson et al. 1992**). Serotype 1 of MDV is the only serotype which can cause disease in chickens. The virus establishes an early cytolitic infection in both T and B lymphocytes, a latent infection and a transformation of T- lymphocytes. On the other hand serotype 2 (MDV-2) and serotype 3 herpes virus of turkeys (MDV-3) are avirulent (**Calnek 2001**).

Many disease syndromes in chickens are caused by MDV as lymphomatosis in skin, eyes, nerves, and visceral organs. In addition to lympho-degeneration in the immune system, transient paralysis and atherosclerosis in the blood vessels (**Witter 2003**). Although there is a strong vaccination strategy by CVI988 vaccine (**Rispens 1972**), the virulent MDV strains infection may occur due to many factors such as vaccination failure and increasing MDV strains virulence (**Witter 1997**).

Isolation of the virus and propagation in cell culture then identification or quantification by cytopathic changes is the standard approach adopted for the MDV diagnosis (**De Laney et al. 1998**). In the recent years, polymerase chain reaction is used as diagnostic tool, for serotype selectivity (**De Laney et al. 1998**) and can also be used for the differentiation between the vaccine and field strains of MDV serotype-1 (**Handberg et al. 2001**).

This study aimed to screen the presence of MDV in samples collected from layer flocks from different localities in Egypt. Characterization of such isolate by sequencing of ICP4 gene was done to differentiate between field and vaccine virus.

## 2. Materials and methods

### 2.1. Specimen collection

Ten specimens (ten chickens for each sample) were collected from 6 to 8 months-old, HVT vaccinated Isabrown layer chicken flocks from 6 governorates: 2 samples from Gharbia, 2 samples from Sharkia, 2 samples from Dakalia, 1 sample from Domyatt, 1 sample from Kaliobeya and 2 samples from Giza during the period from Jan. 2019 to Nov. 2020. Liver, spleen and ovary showing diffuse swelling were

collected from affected birds suffering from depression, ruffled feathers, and paralysis in legs and in some cases deaths due to inability to feed. These samples were processed as the following: 0.5g of each collected tissue sample (proventriculus, liver, spleen) was placed separately into a sterile mortar, minced into small pieces by using sterile scissors and forceps, then a small quantity of a sterile sand was added followed by addition of 4.5 ml Damsco then centrifuged at 2000 rpm for 10 min at 20 °C and stored at -20 °C till being examined.

### 2.2. Isolation and propagation of MDV on primary duck embryo fibroblasts

Duck embryo fibroblasts were prepared from 10-11 day-old duck embryos as previously described (**Freshney 2016**). Briefly, embryos were removed from the eggs and washed three times in Phosphate buffered saline (PBS) after removal of the head, wings, legs and visceral organs. Embryonic tissues were cut into smaller fragments using scissors. Embryonic fragments were washed three times in PBS solution and the contents were transferred into flask then adding 5 ml of 0.25% trypsin per embryo and stirred on a magnetic stirrer at 37 °C for 5 min. Three further cycles of trypsinization for harvesting of cells, after which the supernatant fluid was collected and held in an ice bath or few drops of fetal bovine serum were added to stop the action of trypsin. The pooled harvest of cells was then filtered through a beaker with muslin cloth and then centrifuged at 1500 rpm for 5 min. The resulting packed cells were re-suspended with complete growth medium containing 10% fetal calf serum, then cells were diluted to a final concentration of 3-4.5 X 10<sup>5</sup> viable cells/ml in growth medium. The cell suspension was then transferred to culture flasks of 25 cm<sup>2</sup> after that incubated at 37°C for 2-3 days at 5% CO<sub>2</sub> in a humidified atmosphere.

The standard method of isolation of the MDV (**OIE 2010**) was applied in this study. Briefly, the supernatant of the sample suspension was initially filtered through 0.45 µm syringe filter. 0.5 ml of the inoculum was inoculated on to DEFs grown in 25cm<sup>2</sup> tissue culture flask with more than 70% confluency and incubated at 37°C for one hour. The cells were then rinsed, and 8 ml of maintenance medium were added. Un-inoculated monolayer was maintained as a control negative. Both the inoculated and un-inoculated cultures were subsequently incubated at

37°C in an incubator with 5% CO<sub>2</sub> atmosphere for 3-5 days. Three successive serial passages were made on DEF monolayers and at every passage the infected DEFs were checked for cytopathic effects and preserved at -20°C for further passages.

### 2.3. Genomic DNA extraction

The collected samples were homogenized, and the nucleic acid was extracted using QIAamp<sup>®</sup> DNA Mini kit according to the manufacturer's instructions. In brief, visceral tumor samples were minced then, digested for 2 hrs using digestion buffer solution which contain 20µl proteinase K. After 2hrs, the digestion mixture was mixed with 200µl of lysis solution to obtain homogeneous mixture, and then the mixture was transferred into a spin column and centrifuged at 6000g for 1min. The column was washed twice, after that DNA was eluted by addition of 200µl elution buffer then centrifugation at 8000g for 1min.

### 2.4. Detection of MDV by polymerase chain reaction (PCR)

Amplification of ICP4 partial gene was performed using F-3' GGATCGCCCACCACGATTACTACC 5' and R- 3'ACTGCCTCACACAACCTCATCTCC 5' as previously described RT-PCR Primers set used followed (**Handberg et al. 2001**). The PCR was performed using Transcript PCR SuperMix (**Thermo UK**) in a total of 50µl using the following thermal cycler profile: 94 °C for 5 min followed by 40 cycles at 94 °C for 30 sec, 52 °C for 30 sec, and 72 °C for 1.5 min, with a final extension at 72 °C for 10 min. The amplified fragments were separated by gel electrophoresis using 1.5% agarose gel stained with Ethidium at the rate of 10ul/100 ml for visualization of the PCR products under ultraviolet light. The obtained fragments were purified using QIA quick gel extraction kit, according to the manufacturer's instructions.

### 2.5. Sequencing and phylogenetic analysis

The purification of PCR product was carried out using QIA quick Gel Extraction Kit (Qiagen, Hilden, Germany) using the DNA fragment excised from the agarose gel with 3 volumes of Buffer QG to 1 volume of gel (100mg/100µl). Then it was incubated at 50 °C for 10 min (or until the gel slice has completely

dissolved). Then it was placed in a QIA quick spin column and centrifuged for 1 min at 8000 rpm. Then 500ul of Buffer QG were added to QIA quick column and centrifuge for 1 min. Then 750 ul of PE buffer was added to QIA quick column and centrifuged for 1 min at 8000 rpm. Finally, the QIA quick column was placed into clean 1.5 ml micro centrifuge tube to elute DNA using 30µl of Buffer EB by centrifugation for 1 min at 12000 rpm.

Sequencing of the purified PCR product was done for the forward and reverse directions using a ready reaction Bigdye Terminator V3.1 cycle sequencing kit (Cat. No. 4336817, Applied Biosystems, Foster City, CA). Briefly 2µl of Bigdye terminator v.3.1 kit was used with 1µl Primer and 6 µl of DNA template according to concentration of the DNA then completed to total volume of 20µl with deionized water (PCR grade). The sequence reaction was done using ABI-2700 Thermal cycler with Thermal profile as follows: 96 °C/1 min then 25 Cycles of 96 °C/10 sec, 50 °C/5 sec and 60 °C/2 min.

The purification of the sequence reaction was done based on the manufacture instructions using Centrise spin column (Cat. No. CS-901). BLAST analysis (Basic Local Alignment Search Tool) was initially performed for establishing sequence identity to GenBank accessions (**Altschul et al. 1990**). Sequence alignments of nucleotide and amino acid sequence were performed in comparison with other related strains obtained from Gene Bank database using BioEdit software version 7.1.7 using the CLUSTAL-W program. Phylogenetic tree was created using the (N-J) tree of MEGA version 6 (**Tamura et al. 2013**) assessed with 1000 boot-strap replicates and the pairwise nucleotide percent identity was calculated by the CLUSTAL-W algorithm in the MegAlign program of Lasergene software suite (DNASTAR, Madison, Wisconsin).

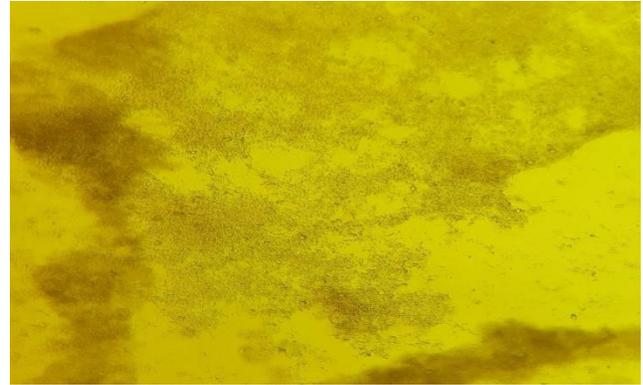
## 3. Results

### 3.1. MDV isolation and identification

The CPEs were observed in after 3 successive passages that appeared in the form of clumping of the cells (**Fig. 1**).

### 3.2. PCR for MDV detection

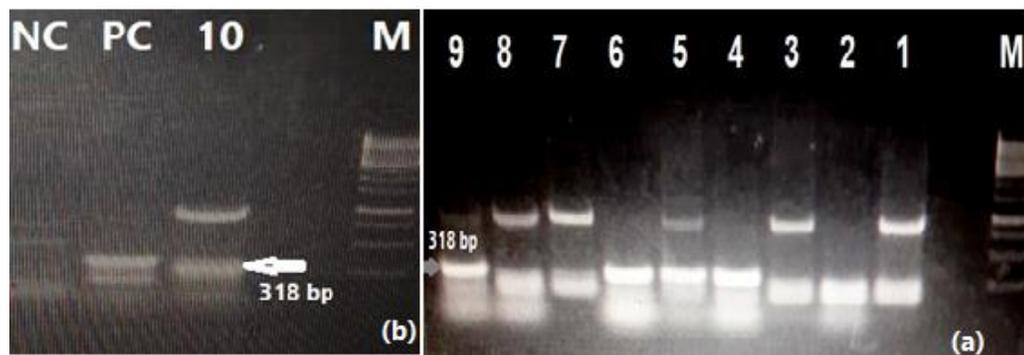
In this study, five out of 10 samples showed 318 bp amplicons. The collected samples included Eg- F1352-S1-2020, Eg- F1352-S2-2020, Eg- F1352-S3-2020, Eg- F1352-S4-2020, Eg- F1352-S5-2020, Eg- F1352-S6-2020, Eg- F1352-S7-2020, Eg- F1352-S8-2020, Eg- F1352-S9-2020, Eg- F1352-S10-2020 (Table 1, Fig. 2).



**Fig. 1.** The cytopathic effects (CPE) of MDV in duck embryo fibroblast (DEF) cells that appeared in the form of cell aggregation and clumping of the cells (magnification power 40x).

**Table 1.** Result of PCR from layer flocks.

Sample code	Age	Governorate	Collection date	Result of PCR
Eg- F1352-S1-2020	6 months	Gharbia	10-2019	-
Eg- F1352-S2-2020	6 months	Gharbia	10-2019	-
Eg- F1352-S3-2020	6 months	Dakalia	10-2019	-
Eg- F1352-S4-2020	8 months	Dakalia	12-2019	+
Eg- F1352-S5-2020	8 months	Domyatt	12-2019	+
Eg- F1352-S6-2020	8 months	Sharkia	12-2019	+
Eg- F1352-S7-2020	6 months	Gharbia	8-2019	-
Eg- F1352-S8-2020	6 months	Gharbia	8-2019	-
Eg- F1352-S9-2020	7 months	Giza	2-2020	+
Eg- F1352-S10-2020	7 months	Giza	2-2020	+

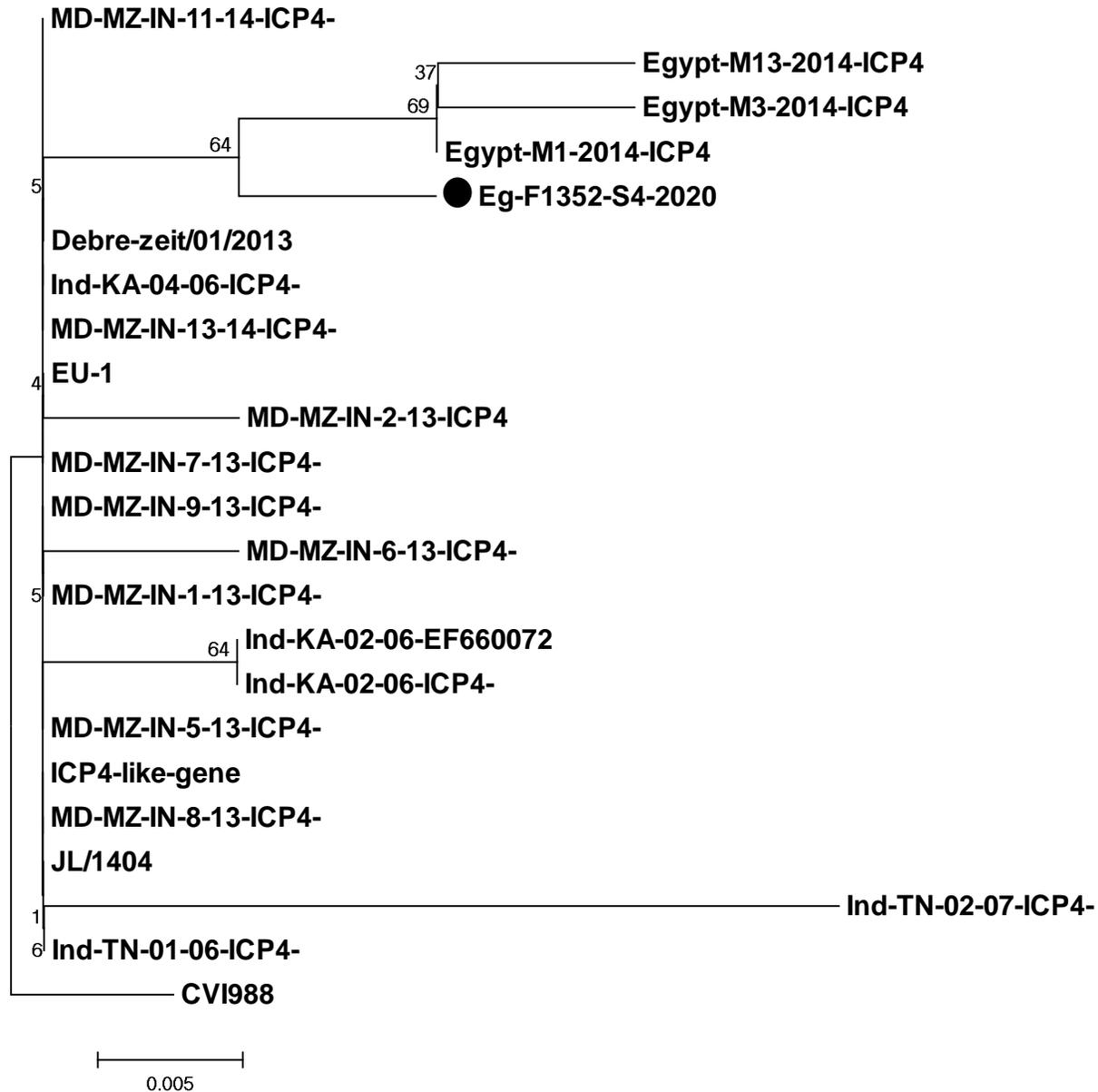


**Fig. 2.** PCR of MDV showing positive samples (318 bp.) (a): samples from 1-9 with positive band at 318 bp in samples 4, 5, 6, and 9 against DNA ladder (M). (b): sample 10 with positive band at 318 bp, positive control sample (PC) with positive band at 318 bp and negative control (NC) with no bands against DNA ladder (M).

### 3.3. Genetic characterization and phylogenetic analysis

The phylogenetic analysis of ICP4 of the MDV strain revealed that it is closely related to very virulent strain of Marek's disease virus type 1 resembling Gallid herpesvirus 2 isolates from Europe, China, Africa and India. The sequence of the MDV isolate was deposited in the GenBank under accession number MW194840.

The partial ICP4 gene sequencing of the Egyptian virus indicates its similarity with other related viruses from Egypt, Ethiopia, India and Europe (**Fig. 3**). There was a characteristic serine residue at the coding region of ICP4 gene encoding the reverse complement protein found only in the Egyptian virus (F1352-S4), EU-1 virus, JL/1404, Debre-zeit/01/2013 virus that is tracing for the original virus CV1988 (**Fig. 5**).



**Fig. 3.** Phylogenetic analysis of partial ICP4 gene of MDV of the positive isolate from Egypt (Eg-F1352-S4/2020) in comparison to other field and vaccine strains: the virus (Eg-F1352-S4/2020) was placed with recent viruses from Egypt 2014 and grouped with vvMDV from Europe, China, Africa and India.

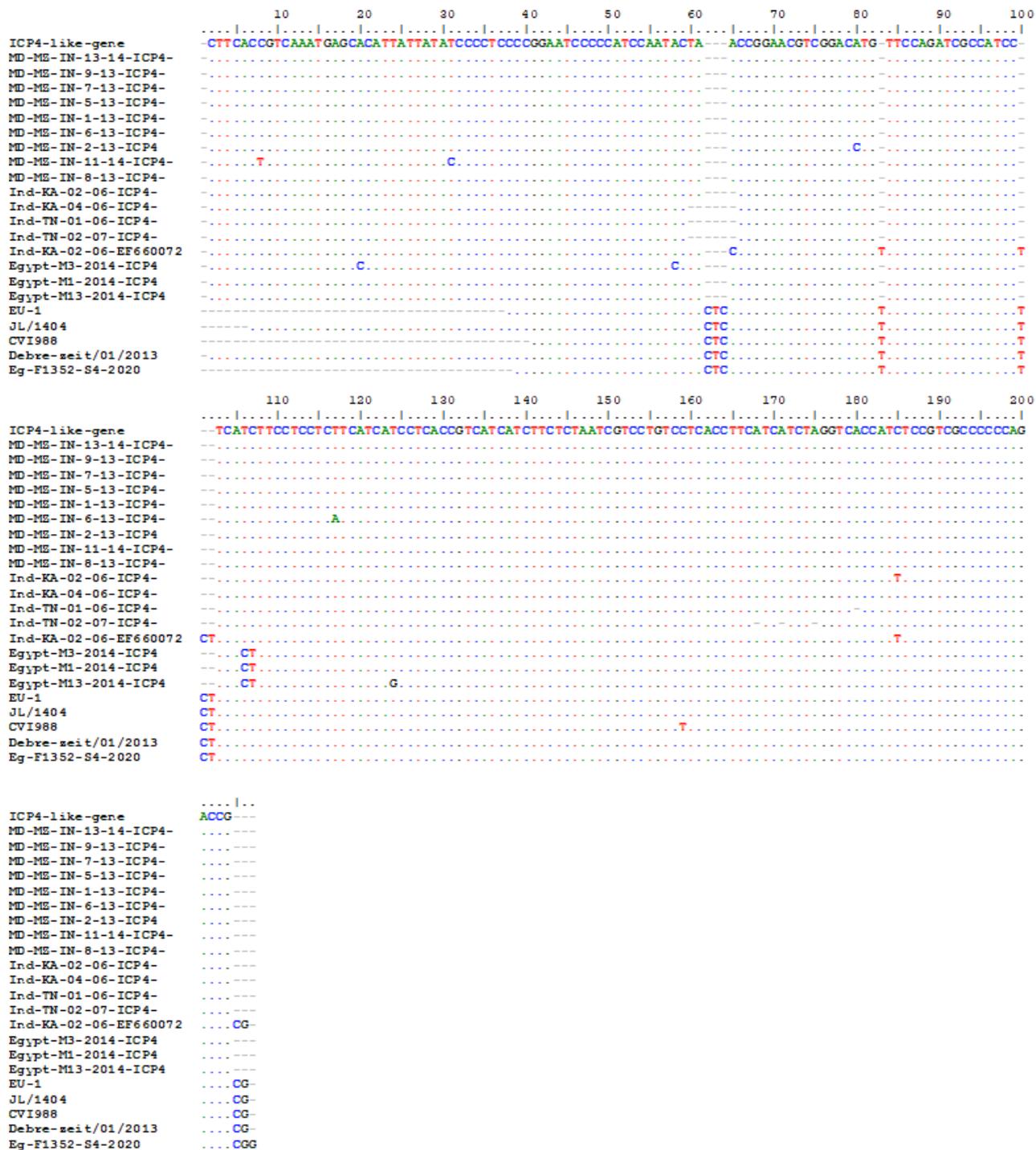
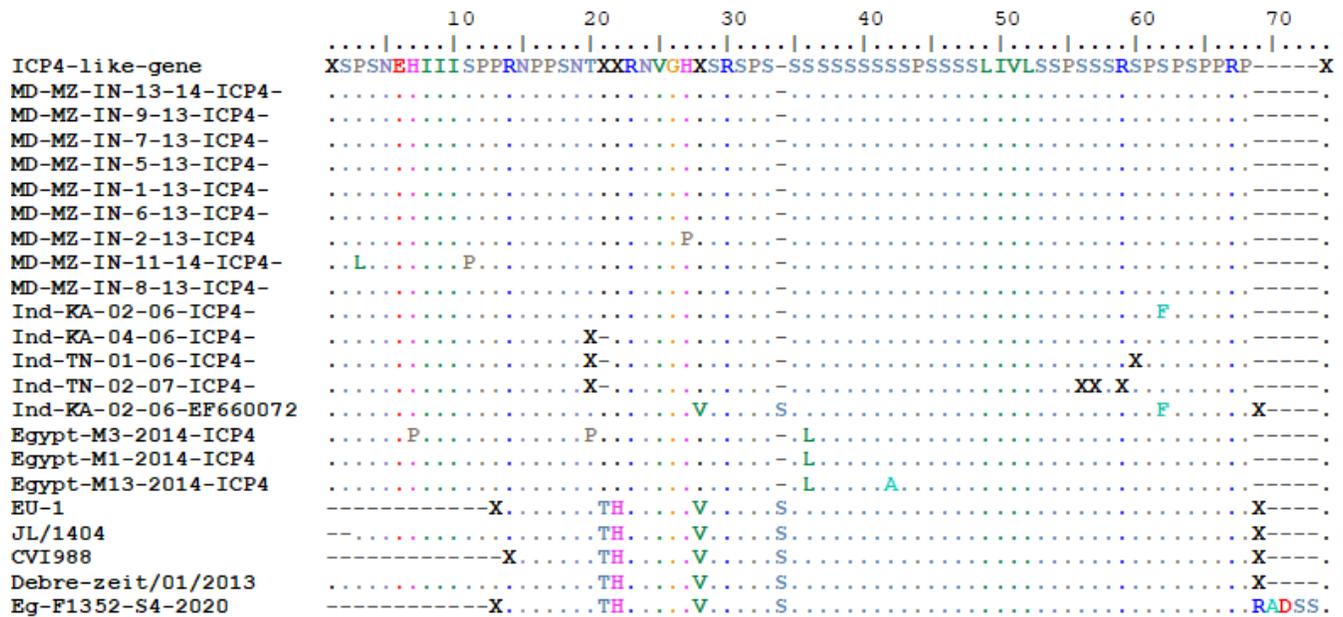


Fig. 4. Alignment of partial ICP4 gene nucleotide sequence from field virus (Eg-F1352 S4/2020) in comparison to other field and vaccine strains.



**Fig. 5.** Alignment of partial ICP4 amino acids sequence from field virus (Eg-F1352-S4/2020) in comparison to other field and vaccine strains.

#### 4. Discussion

Marek's disease causes severe economic losses in poultry commerce worldwide. The classical form of MD usually affects 12 to 30 weeks age group of layer flocks with manifestation of paralysis of wings and legs. The mortality rates ranged between 10 to 15% in the affected flocks. Subsequently there was incidence of more virulent strains of MDV which could not be controlled by using HVT vaccination. The gross lesion was diffuse or focal tumors in visceral organs. Imitation activity of MVD in tumor cells is due to the repeat regions (Ross et al. 1997). In these regions there are a limited number of genes, including viral telomerase RNA, viral IL-8, meq, pp38, and ICP4 (Anderson et al. 1992). ICP4 gene located with a major category of genes in MDV, which grouped under genes homologous with alpha-herpes viruses. This is a broad category of genes which is divided into immediate early, early and late genes, which are important in virus replication (Handberg et al. 2001). In Egypt, in spite of the hatcheries follow an intensive MDV vaccination strategy using CVI988 + HVT or CVI988 at one day old chick, the flocks are suffering from either outbreaks or sporadic cases of neural and/or visceral tumors. Previously, a very virulent strain of MDV was isolated from different MDV vaccinated Egyptian poultry flocks (Hussein et al. 2004). The failure of MDV vaccination is due to either, presence of immunosuppressive pathogen as

chicken anemia virus (Otaaki et al. 1988), or the emergence of new virulent MDV strains (Witter 1997).

In the present study there was an involvement of liver, spleen, proventriculus and kidneys. The affected organs showed enlargement with discrete grayish white nodules. Presence of lymphomas in different visceral organs of MDV affected birds. Furthermore, the presence of gross lesions only is not sufficient to confirm the positive result so that, using of PCR targeting ICP4 gene to measure viral genome titers in lymphocytes isolated from the blood was initially started (Bumstead et al. 1997).

In the present study, 5 out of 10 samples collected from suspected MDV outbreaks were found to be positive in PCR. All the samples yielded a 318 bp product in PCR. The PCR product targeting 318 bp tandem repeats of one positive field sample was extracted from agarose gel, then the purified DNA was sequenced for the confirmation of MDV serotype. The nucleotide sequence was verified by NCBI Blast for analysis of nucleotide homology and it was found that the sequence alignment of 318 bp sequences showed that the representative field isolate of this study has maximum homology as predicted due to the highly conserved region.

The alignment of the 318 bp sequence of MDVICP4 with sequences which were published of MDV showed that the representative isolate contains a genome of Marek's disease virus serotype 1 only.

## 5. Conclusion

The partial ICP4 nucleotide sequence alignment of the Egyptian virus (F1352-S4) of MDV ICP4 gene with the sequences which were previously published proved that the sample has a genome of MDV-1 resembling Gallid herpesvirus 2 isolates from Europe, China, Africa, and India. There was a characteristic serine residue at the coding region of ICP4 gene encoding the reverse complement protein that found only in the Egyptian virus (F1352-S4) and EU-1 virus, INd-KA-02-06, JL/1404 and in Debre-zeit/01/2013 virus that is tracing for the original virus CV1988.

## 6. Acknowledgement

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## 7. Conflict of interest

Authors declared no conflict of interests exist.

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