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Isolation and Identification of Very Virulent Strains of Marek's Disease Virus from MDV-Vaccinated Flocks in Egypt

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Abstract
Marek's disease is still a major problem among Egyptian poultry flocks, despite the intensive vaccination programs against the disease. This study was conducted to investigate the prevalence of MDV infection among forty-four flocks of breeders and layers with ages ranged from 4-20 months. Feather follicle specimens were collected during 2012-2015 from 44 vaccinated chicken flocks showing emaciation and visceral tumors. The samples were tested by PCR using three pairs of primers. Nineteen flocks were positive for MDV using primers targeting the UL 19 gene with a percentage of 43.2%. Three flocks were shown to be positive by primers targeting the *meq* and 132 bp tandem repeat genes with a percentage of 6.8%. Inoculation of duck embryo fibroblast (DEF) and chick embryo fibroblast (CEF) showed CPE in the form of plaques formation within 5-14 days post inoculation. Sequencing of *meq* and 132 tandem repeat genes of the 3 samples revealed that the isolated strains exhibited 99% homology with the very virulent European, Chinese, American, Indian and Egyptian MDV isolates. In conclusion, although the availability of MDV vaccines especially HVT vaccine which is used in the examined flocks in the present study, the disease was recorded. Thus, indicating that HVT vaccines are unable to protect completely against more virulent strains. Therefore, there is a need to develop a new strategy and types of vaccination to be able to protect against new strains of virus.

Keywords: Marek's Disease, MDV, *meq* Gene, PCR

Introduction

Marek's disease (MD) is one of the most economically devastating infectious diseases of poultry caused by the highly infectious, cell associated MD virus (MDV) [1]. The MDV belongs to the genus *Mardivirus*, subfamily *alphaherpesvirinae* and there are three serotypes of MDV: MDV-1 (e.g., RB-1B, Md5, GA, and CVI988) able to induce disease in chickens, MDV-2 (e.g., SB-1 and HPRS24) that is considered non-pathogenic and used as vaccines, and MDV-3, which is also called herpesvirus of turkeys (HVT; e.g., FC126) that also considered as non-pathogenic and used as vaccines. Recently, serotype-1 is named as gallid herpesvirus II, serotype -2 as gallid herpesvirus III, and serotype -3 (HVT) as meleagrid herpes virus 1[1].

During early cytolytic infection, replication of the virus occurs in B and T lymphocytes then a latent infection is established in T lymphocytes, which may become transformed and form lymphomatous lesions in the visceral organs, peripheral nerves and skin [2]**.** The virus is capable of inducing many disease syndromes in chickens such as lymphomatosis in nerves, skin, eye, and visceral organs; lymph degeneration in the immune system; transient paralysis in the central nervous system; and atherosclerosis in the blood vessels [1]. Infection with the virulent MDV strains and subsequent vaccine breaks can still occur despite the intensive vaccination strategies with the widely used CVI 988 vaccine [3]. The vaccine breaks may be attributed to many factors such as increased virulence of MDV strains over the last four decades [4]. In addition, presence of immunosuppressive agents or the difficulties associated with the vaccine handling due to its cell-associated form may also cause vaccine breaks [5]. Polymerase Chain Reaction (PCR) is a suitable technique to determine serotype specificity and to differentiate between vaccinal and wild strains of MDV serotype-1 [6-9]. The aim of this study was the isolation and molecular identification of MDV strains from MDV-vaccinated layer and breeder chicken flocks, Egypt.

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Table 1: Isolates of MDV published on GenBank used in comparison and phylogenetic analysis of *meq* gene

No	Isolate	0rigin	Accession No
1	Gallid herpesvirus 2 isolate LDH-2483 Meq oncoprotein (Meq) gene	India	KF895031.1
2	Gallid herpesvirus 2 isolate ATE, Meq gene, complete cds	USA	AY571784.1
3	Gallid herpesvirus 2 isolate MDJ4/1303, MEQ, complete cds	China	KP888850.1
4	Gallid herpesvirus 2 isolate HL/1111, complete cds	China	KP888821.1
5	Gallid herpesvirus 2 isolate 04 meq protein,Meq gene, partial cds	India	KT246103.1
6	Gallid herpesvirus 2 isolate LDH-2614, Meq gene	India	KF895032.1
7	Gallid herpesvirus 2 isolate Egypt 2 Meq gene, partial cds	Egypt	JX467679.1
8	Gallid herpesvirus 2 isolate Egypt_4 Meq gene, partial cds	Egypt	KC161220.1
9	Gallid herpesvirus 2 isolate DL/1311 MEQ gene	China	KP888845.1
10	Gallid herpesvirus 2 isolate DL/1112 MEQ gene	China	KP888818.1
11	Gallid herpesvirus 2 isolate LDH(JL/07/I), Meq gene	China	HQ658614.1
12	Gallid herpesvirus 2 strain 617 A meq oncoprotein	USA	AY362712.1
13	Gallid herpesvirus 2 isolate YC/1210 MEQ gene	China	KP888840.1
14	Gallid herpesvirus 2 strain N MEQ (meq) mRNA	China	AF493557.1
15	Gallid herpesvirus 2 isolate CF/1312 MEQ gen	China	KP888844.1
16	Gallid herpesvirus 2 isolate HT/1207 MEQ gene	China	KP888837.1
17	Gallid herpesvirus 2 isolate LLY MEQ gene	China	KP888822.1
18	Gallid herpesvirus 2 strain YL 040920 Meq gene	China	DQ174459.1
19	Gallid herpesvirus 2 isolate SJZ/1208 meq gene	China	KP888828.1
20	Gallid herpesvirus 2 isolate LNCY/1205 Meq gene	China	KP888835.1
21	Gallid herpesvirus 2 isolate FY/1303 Meq gene	China	KP888847.1
22	Gallid herpesvirus 2 isolate NT/1211 MEQ gene	China	KP888836.1
23	Gallid herpesvirus 2 isolate QD/1311 meq gene	China	KP888856.1
24	Gallid herpesvirus 2 isolate SD2012-1 MEQ gene	China	KC511815.1

Material and Methods

Sample collection

The examined samples were collected during 2012-2015 from 44 vaccinated chicken flocks located in four different Governorates in the Egyptian Delta: Sharkia (5 flocks), Dakahlia (25 flocks), Damietta (10 flocks) and Ismalia (4 flocks). Two to three birds per flock were sampled according to availability. The samples collected from the wing of each bird, consisted of three or four feather tips. For preparation of feather tips, feathers were pulled from all major feather tracts, and 3-5 mm parts of the tips were cut with scissor, diluted 1:5 (W/V) with sucrose-phosphate-glutamine-albumin buffer containing sodium ethylene diaminetetraacetic acid (SPGA-EDTA) buffer, and sonicated for 2-3 min. From each flock, pooled feathers from the examined birds were used for PCR.

Molecular identification

Total DNA was extracted using QIAamp DNA Mini Kit (QIAGEN). The procedures were carried out according to the manufacturer's instructions. Three pairs of primers specific for UL19, meq gene and 132 bp tandem repeat genes were used. The sequences of primers are: UL19: F (5´CCC GAT ATT ATC ATT TCA CC-3´) R (5´CTC GCA TTA TTA TCT GAA GT-3´) producing 521 bp [10], meq: F (5´- GCA CTC TAG AGT GTA AAG AGA TGT CTC AG-3´) R (5´- TAA CTC GAG GAG AAG AAA CAT GGG GCA TAG-3´) producing 1060 bp and 132bp tandem repeat: F (5´- TAC TTC CTA TAT ATA GAT TGA GAC GT-3´) R (5´- GAG ATC CTC GTA AGG TGT AAT ATA-3´) producing 434 bp [11].

Table 2: Isolates of MDV published on GeneBank used in comparison and phylogenetic analysis of 132 bp tandem repeat gene

No	Isolate	0rigin	Accession No
1	Gallid herpesvirus 2 isolate CVI988, internal repeat long region, partial cds	USA	DQ534538.1
2	Gallid herpesvirus 2 isolate CU-2, internal repeat long region, partial cds	USA	DQ534537.1
3	Gallid herpesvirus 2 isolate R2/23 internal repeat long region, partial cds	USA	DQ534540.1
4	Gallid herpesvirus 2 isolate 571 internal repeat long region, partial cds	USA	DQ534531.1
5	Marek's Disease Virus (Strain BC-1), tandem direct repeat, from IR-L region	USA	M12619.1
6	Gallid herpesvirus 2 isolate RB1B, internal repeat long region, partial cds	USA	DQ534541.1
7	Marek's Disease Virus BamI-H fragment DNA from MSB-1	USA	M26392.1
8	Gallid herpesvirus 2 isolate CVI988-BP5 internal repeat long region, partial cds	USA	DQ534536.1
9	Gallid herpesvirus 1 DNA, Tumor associated region	USA	D10488.1
10	Gallid herpesvirus 2 isolate RM.1 internal repeat long region, partial cds	USA	DQ534542.1
11	Gallid herpesvirus 2 isolate JM\102 W internal repeat long region, partial cds	USA	DQ534539.1
12	Gallid herpesvirus 2 isolate 549 a internal repeat long region, partial cds	USA	DQ534530.1
13	Gallid herpesvirus 2 isolate 686 internal repeat long region, partial cds	USA	DQ534535.1
14	Gallid herpesvirus 2 isolate 595 internal repeat long region, partial cds	USA	DQ534533.1
15	Gallid herpesvirus 2 strain 648 a isolate p61	USA	JQ809692.1
16	Gallid herpesvirus 2 strain CVI988, complete cds	USA	DQ530348.1
17	Gallid herpesvirus 2 strain RB-1B, complete cds	USA	EF523390.1
18	Gallid herpesvirus 2 strain WK-2014 non-coding repeated region, complete cds	Poland	KP840550.1
19	Gallid herpesvirus 2 isolate LMS, complete cds	China	JQ314003.1
20	Gallid herpesvirus 2 virus, complte cds	USA	AF147806.2
21	Gallid herpesvirus 2 isolate GX0101, complete cds	China	JX488666.1
22	Gallid herpesvirus 2 isolate Md5, complete cds	USA	AF243438.1
23	MDV-Anand3 BamI-H fragment repeat region, partial cds	India	DQ296003.1

PCR amplification of MDV genes

The amplification was carried out in 50 µL reaction volume containing 5 µL of PCR buffer (500 mM KCL, 100 µM Tris HCL, 15 µM Triton X-100), 2 µL of total DNA (1 µg/mL), 2 µL of each primer, 2 µL of dNTP (0.2 Mm), 2 µL of MgCL2 (2mM) , 1 µL of thermostable polymerase DNA and 34 uL of sterile water.

The following conditions were applied for UL19 PCR: 35 cycles with initial denaturation at 94°C for 5 min, denaturation at 94°C for 40 sec, annealing at 52°C for 1 min, elongation at 72°C for 65 sec and final elongation at 72°C for 7 min [10]. While, the conditions applied for meq PCR were 35 cycles with initial denaturation at 94°C for 5 min, denaturation at 94°C for 40 sec, annealing at 60.2°C for 1 min, elongation at 72°C for 65 sec and final elongation at 72°C for 7 min. Finally, the conditions for 132 bp PCR were 35 cycles with initial denaturation at 94°C for 5 min, denaturation at 94°C for 40 sec, annealing at 47°C for 1 min, elongation at 72° C for 65 sec and final elongation at 72°C for 7 min [11]. PCR products were visualized by agarose gel electrophoresis in 1.5% agarose gel, stained with Ethidium bromide and photographed under ultraviolet light.

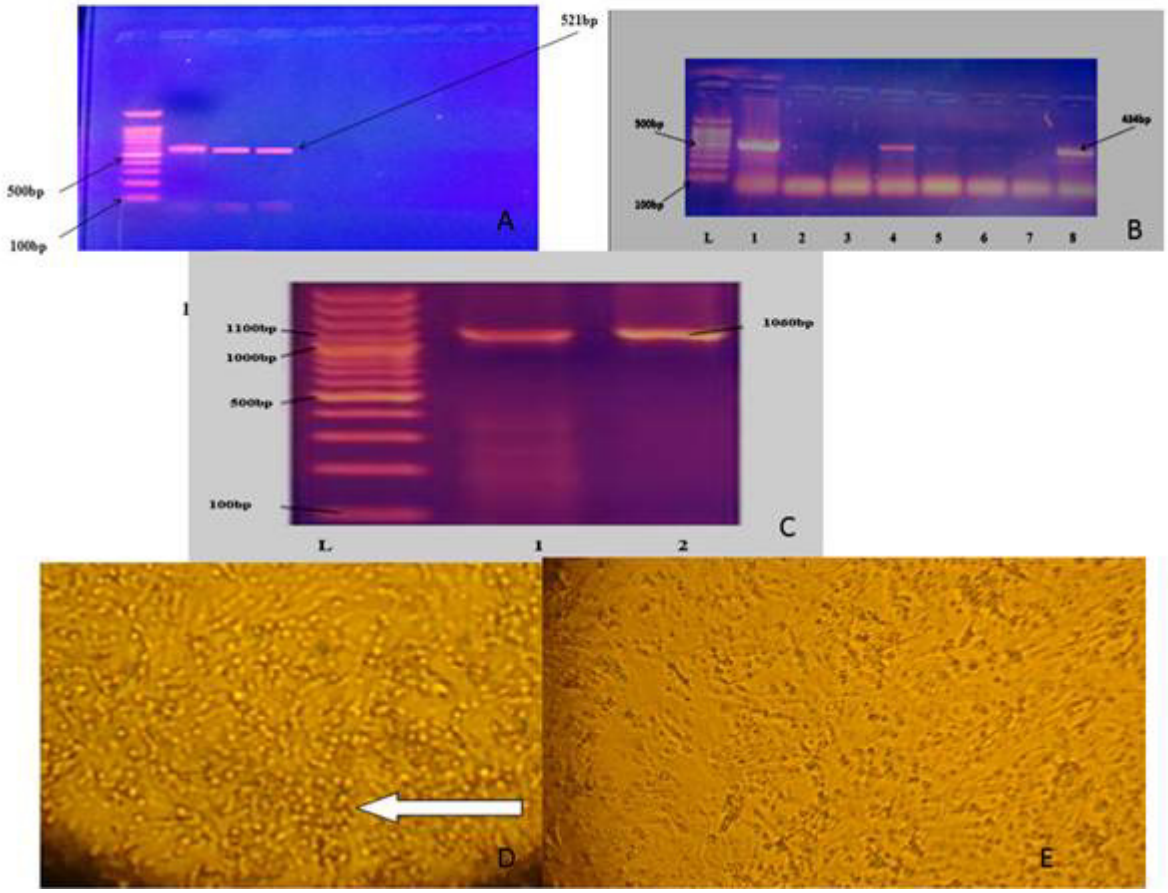

Figure 1: (A): Showing gel electrophoresis of 521bp products amplified by PCR using UL19 primer. Lane M= 100bp ladder, Lane 1 – 3 represent samples, samples 1,2,3 are positive samples which represent flock No. 9, 10 and 11. (B): Showing gel electrophoresis of 434 bp products amplified by PCR using 132 bp tandem repeat primer. Lane M= 100bp ladder, Lane 1 – 8 represent samples, samples 1, 4 and 8 are positive samples represent flock No. 24, 25 and 42. (C): Showing gel electrophoresis of 1.06 bp products amplified by PCR using meq primer. Lane M= 100bp ladder, Lane 1-2 represent samples. Samples 1,2 are positive. (D): CPE of MDV on CEF (5-8dpi). (E): CPE of MDV on DEF (13 dpi).

Gene Sequencing of meq and 132 bp tandem repeat genes

The PCR products of *meq* and 132 bp tandem repeat genes (two, each) were purified from agarose gel using QIAquick® PCR Purification kit (Qiagen), and then sequenced by Sanger dideoxy sequencing method (Sigma Company). The sequences were submitted to the GenBank and the accession numbers KU229905 -KU229906 -KU229907 - KU229908 were provided.

Sequence and phylogenetic analysis

Alignment of the sequences was carried out by the Clustal W method using MegAlign module of DNA star software (Lasergene, version 7.2, DNA STAR, WI, USA). The nucleotide sequences were compared with vvMDV sequences available on the GenBank (Tables 1,2). Construction of the phylogenetic tree was performed using neighbor-joining method in MEGA 5 (www.megasoftware.net) and the tree topography was evaluated by 1000 bootstrap analysis.

Isolation of MDV on cell culture

Duck embryo and Chick embryo fibroblasts were prepared according to Schat and Purchase [12]. Three positive samples by *meq* and 132 bp tandem repeat were inoculated into primary duck embryo fibroblast (DEF) prepared from 11-14 days old embryos and then they were incubated at 37°C with 5% CO_2 for 7-14 days. The supernatant of DEF was then used to inoculate chick embryo fibroblast (CEF) prepared from 9-11 days old embryos, and incubated at 37°C with 5% CO_2 for 7-14

days. The inoculated CEF was observed daily for cytopathic effect (CPE).

Results

Clinical examination and necropsy

The samples were obtained from chickens between 120 and 450 days of age. In the 44 examined flocks, the chicken necropsy revealed visceral tumors that were mainly in liver, spleen and gonads in the form of diffuse or localized lymphomas.

Detection of MDV genome by PCR

Out of 44 pooled samples, only 19 (43.2 %) were positive by primer sets targeting the UL19 gene (Figure 1A). Only 3 out of the positive samples by UL19 PCR were positive for 132 bp tandem repeats and *meq* PCR (Figures 1B and 1C).

Sequence and phylogenetic analysis

Comparison of the nucleic acid sequences of *meq* and tandem repeat genes of the isolates were aligned together with other MDV strains on Gene Bank. The results presented that the isolates obtained in the current study are very virulent MDV strains and with high similarity (99%) with very virulent Chinese, Indian, European ATE and Egyptian strains (Figures 2 ,3).

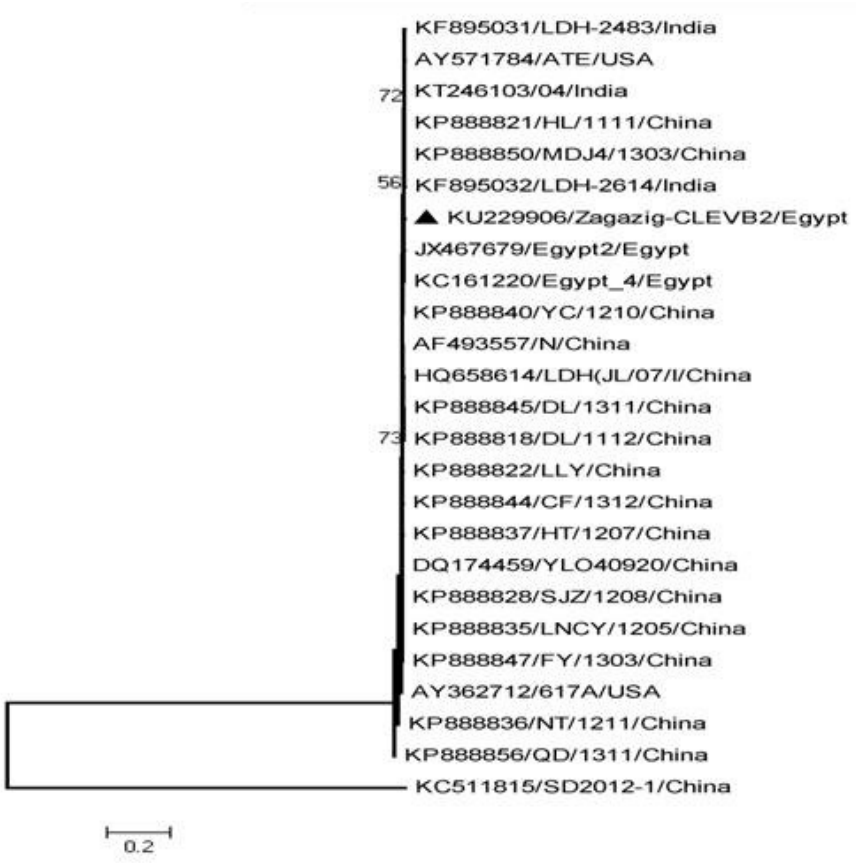

Figure 2: Phylogenetic analysis of *meq* gene nucleotide sequences of vvMDV isolated from Egyptian poultry flocks and other sequences available on GeneBank.

Isolation of MDV on cell culture

The MDV isolation trials revealed variable degrees of CPE in form of plaques formation in the three positive samples. Infected cell cultures developed discrete focal lesions, which consisted of clusters of rounded, refractile cells (foci or plaques) (5- 14 days post infection). The CPE on DEF appeared 13

dpi (Figure 1D), while on CEF appeared within 5-8 dpi (Figure 1E).

Discussion

In spite of intensive vaccination strategy against MDV using HVT or HVT+CVI988 in chicks at one day of age, the flocks are still having cases of neural and or visceral tumors [13,14]. This may be due to vaccination failure which is caused by several reasons mainly the emergence of new more virulent strains of the virus.

Out of 44 specimens, 19 (43%) were positive for MDV serotypes (1,2 and 3) using UL19 primers that gave 521 bp amplicon. Similarly, Ottiger [10] stated that UL19 primer is helpful in detecting any of the three MDV serotypes [10]. There is a close correlation between the number of 132 bp sequence repeats and pathogenicity and in turn oncogenicity of the strains, therefore, this sequence can be used for differentiation between vaccinal and field strains [7,15]. The 19 MDV 19 isolates were tested using 132 bp tandem repeats primer, 3 positive oncogenic viruses of serotype 1 were confirmed by production of 434 bp amplicon. These results were supported by previous studies [7,15] reported that only one product is observed in the case of oncogenic (serotype 1) field strains, while, multiple copies of 132 bp repeats were identified in vaccine CVI 988 Rispens (serotype 1). In addition, in the serotype 3 vaccinal strain (HVT) virus, no bands were recorded. These findings could be attributed to an increase in the number of copies of 132 bp repeats within the BamHI-H region of IR (inverted repeats) as a result of attenuation by cell culture passage for vaccine preparation [16].

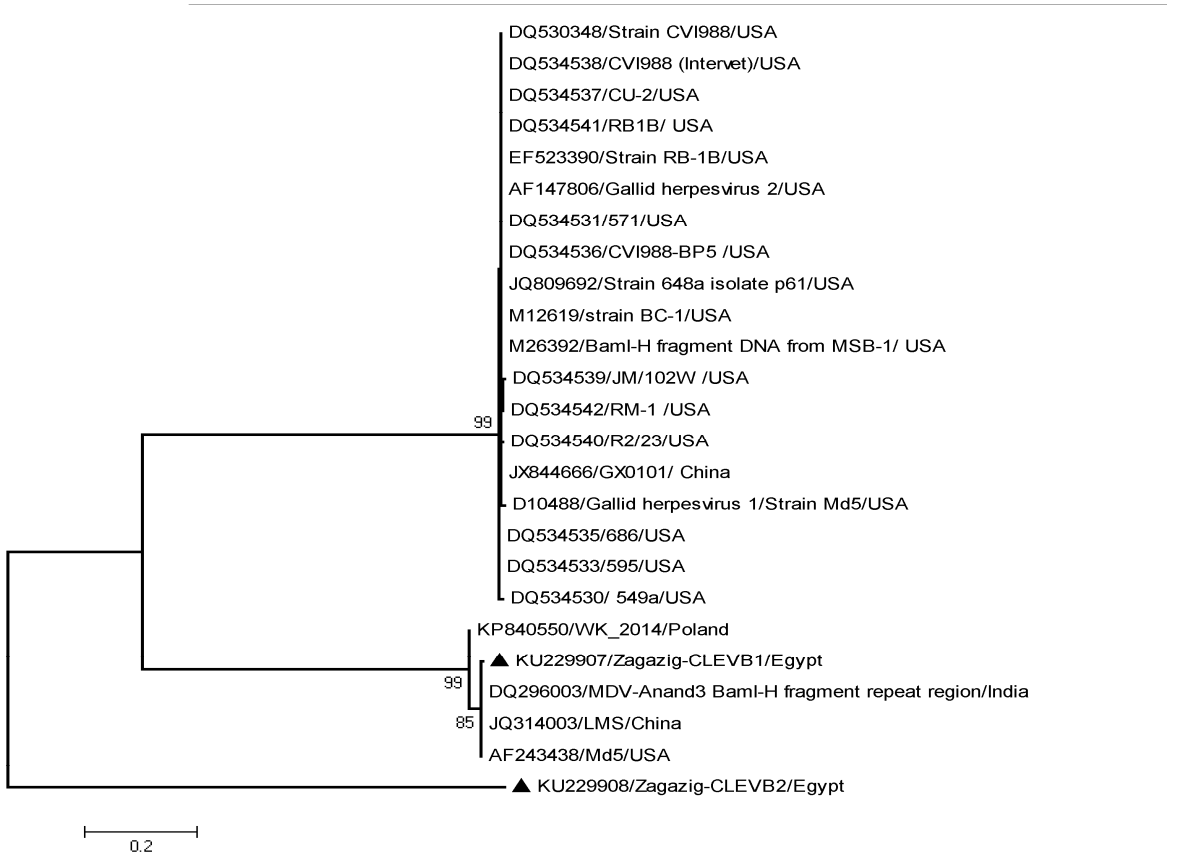

Figure 3: Phylogenetic analysis of 132bp tandem repeat gene nucleotide sequences of vvMDV isolated from Egyptian poultry flocks and other sequences available on GenBank.

Further confirmation of the oncogenicity of the three MDV-1 isolates was conducted by the amplification of the *meq* gene. The three isolates produced 1060 bp amplicon. Chang *et al*. [17] explained that during the attenuation, the 1.06 kb sequence is inserted into the gene, resulting in an additional PCR product (L-meq) in the case of vaccine strains [17]. Therefore, these primers were used in the present study to differentiate vaccinal and field strains of MDV serotype 1. However, the serotype 3 vaccinal strain (HVT) produced no bands by these primers. Krol *et al.* [11] reported similar findings.

In the current study, sequence analysis of the PCR products of the three oncogenic field viruses and alignment of the obtained sequences of 132 bp tandem repeats and *meq* gene of several isolates showed 99% similarity to very virulent MDV. According to phylogenetic tree and identity, our isolates were more similar to very virulent strains of China, Egypt, USA, India and Poland.

The three oncogenic strains confirmed by PCR were propagated on cell culture using DEF and CEF and were observed daily for appearance of CPE within 5-14 dpi. Primary propagation on DEF then propagation on CEF, CPE appeared within a week dpi. Chick embryo fibroblast (CEF) are common cells for the production of MD vaccines, but not suitable for primary isolation of MDV. The CKC or DEF is more suitable cells for primary propagation of MDV isolates [18-20]. While, CEF can provide an alternative substrate for isolation of attenuated strains of MDV-1, also, there have been reports of successful propagation of MDV in some continuous cell lines, such as OU2.2, OU2.1, QM7 and SOgE, their use for primary isolation was not described [21- 23]. The obtained results of MDV isolation on CEF and DEF were strengthened by De Laney *et al.* [24] who stated that cell culture isolation could be a helpful tool in identification of MDV virulence.

Our virulent MDV field isolates showed propagation in cell culture, with the CPE in form of small to medium-sized plaques of rounded refractile cells or syncytia appearing 5-8 days post-inoculation. Continuous passage of the virus in cell culture leads to its adaptation and faster replication *in vitro*, attenuation of the oncogenic potential of the virus and structural and gene expression changes [25-30]. Attenuated strains produce larger plaques and CPE usually occurs 2-4 dpi.

Conclusion

In conclusion, although the availability of MDV vaccines especially HVT vaccine which is used in the examined flocks, the disease was recorded. Thus, indicating that HVT vaccines are unable to protect completely against more virulent strains. Therefore, there is a need to develop a new strategy and types of vaccination to be able to protect against new strains of virus.

Conflict of interest

All the authors have no conflict of interest to declare.

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الملخص العربي

عزل وتصنيف عترات شديدة الضراوة من فيروس الماريك من القطعان المحصنة ضد مرض الماريك فى مصر

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[٢]المعمل المركزى للرقابة على المستحضرات البيولوجية البيطرية – العباسية[٢]

مازال مرض الماريك يمثل مشكلة رئيسية بين قطعان الدجاج المصرية على الرغم من برامج التحصين المكثفة ضد المرض . اجريت هذه الدراسة للتحقق فى انتشار عدوى فيروس الماريك فى اربع واربعين قطيع بياض وامهات ذات اعمار تتراوح بين ٤-٢٠ شهرا . تم جمع عينات من بصيلات الريش خلال الفترة من ٢٠١٢-٢٠١٥ من ٤٤ قطيع من قطعان الدجاج المحصنة ضد المرض والتى ظهر عليها هزال واورام حشوية وتم اختبار العينات بواسطة اختبار البلمرة المتسلسل باستخدام ثلاث ازواج من البريمرات . وكان ١٩ قطيع ايجابيا ل الماريك باستخدام بريمر UL 19 بنسبة ٤٣.٢%. واظهرت ثلاث قطعان ايجابية باستخدام بريمرات meq و132bp tandem repeat بنسبة ٦.٨%. واظهرت نتائج حقن خلايا الفيبروبلاست من اجنة البط واجنة الدجاج تجمعات للخلايا خلال ٥- ١٤ يوم بعد الحقن. وكشف التسلسل الجينى ل meq و132 bp tandem repeat للعينات الثلاثة ان السلالات المعزولة اظهرت ٩٩% تطابق مع المعزولات الاوروبية والأمريكية والصينية والهندية والمصرية الشديدة الضراوة . فى الختام على الرغم من توافر لقاحات ضد المرض خصوصا لقاح HVT التى تم استخدامه فى القطعان التى تم فحصها فى هذه الدراسة الا انه تم تسجيل المرض فى هذه الحالات . وبالتالى هذا يشير الى ان هذه اللقاحات غير قادرة على الحماية التامة ضد السلالات الاكثر ضراوة لذلك هناك حاجة لوضع استراتيجية جديدة وانواع من التحصينات لتكون قادرة على الحماية ضد العترات الجديدة من الفيروس .