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Trials for isolation and identification of Marek's disease virus (MDV) in layer chicken farms vaccinated with MDV vaccines exhibiting MDV-like symptoms in Egypt

Alaa H. Ibrahim[1*], Eman Mohamed Samy[2], Ayman S. El-Habbaa[1], Ehab M. El-Nahas[1]

[1]*Department of Virology, Faculty of Veterinary Medicine, Benha University, Po Box: 13736, Moshtohor, Toukh, Egypt*

[2]*Department of Pathology, Faculty of Veterinary Medicine, Benha University, Po Box: 13736, Moshtohor, Toukh, Egypt*

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ABSTRACT

Marek's disease virus (MDV) is a lymphoproliferative neuropathic disease affecting domestic fowl. Despite intensive vaccination, Egyptian poultry farms continue to experience recurrent infections of MDV. Our study aimed to investigate MDV in three Egyptian layer chicken farms that showed MDV-like symptoms, such as depression, ataxia, and emaciation. In this study, a total of nine field tissue samples (three from each farm) were collected from 7-10-month-old chickens vaccinated with CVI988 and HVT commercial MDV vaccines. The processed samples were inoculated on the chorioallantoic membrane (CAM) of SPF-ECEs for three serial passages, resulting in embryos with stunted growth, body curling, enlarged liver, and thickened CAM. Subsequently, the harvested isolates were tested for MDV using the agar gel precipitation test (AGPT), which confirmed that all these isolates tested negative. Furthermore, the isolates underwent conventional PCR for MDV using specific primers based on the *ICP4* gene, confirming that all of them were negative. Therefore, we used specific primers for other tumor viruses to determine which viruses caused tumors on these farms. Avian leukosis virus subgroup J (ALV-J) and Reticuloendotheliosis virus (REV) were tested based on the *gp85* and *LTR* genes, respectively. To our surprise, out of the three examined isolates, one tested positive for ALV-J, another was co-infected with ALV-J and REV, while the third was negative for both. Overall, this study underscored that the absence of MDV infection in vaccinated chickens demonstrates the efficacy of commercial MDV vaccines; however, tumors observed in the chicken farms studied were caused by ALV-J and REV infections.

1. INTRODUCTION

Marek's disease virus (MDV) poses a significant pathogenic threat to poultry industry, resulting in substantial and devastating economic impacts worldwide due to tumors and immunosuppression in affected birds (Witter and Schat, 2003). MDV is an avian neoplastic disease that causes significant economic losses in chickens and turkeys worldwide (Buckmaster et al., 1988). It is responsible for increased mortality in chicken flocks, as well as neuro-lymphoproliferative and immunosuppressive disorders (Calnek, 2001). It induces some clinical signs, including leg and wing paralysis, cloudy eyes, and solid visceral tumors in the liver, spleen, and ovaries (Emad et al., 2024; Meligy et al., 2023).

MDV belongs to the order *Herpesvirales*, family Herpesviridae, subfamily *Alphaherpesvirinae*, and is classified under the genus *Mardivirus* (Dunn et al., 2013). It is an enveloped virus with a linear, double-stranded DNA genome that is approximately 160–180 kb long. In addition to a characteristic tegument protein layer (Osterrieder et al., 2006). The MDV genome encodes more than 200 genes (Lupiani et al., 2004). Among them, Marek's EcoRI-Q *(Meq)* and the infectious cell protein-4 *(ICP4)* genes were identified as the most significant genes associated with the virus's virulence and oncogenicity. Mutations in the *Meq* gene play an essential role in determining the virulence, tumorigenic potential, and genetic diversity of MDV (Renz et al., 2012). Hence, MDV loses its oncogenicity when the *Meq* oncogene is deleted as described in MDV serotypes 2 and 3 (McPherson and Delany, 2008).

MDV is classified into three serotypes with different pathogenicity and virulence (OIE, 2010). MDV serotype 1 (CVI988) is defined to be the only oncogenic serotype that causes disease in chickens, whereas serotype 2 (SB-1) (Schat and Calnek, 1978). Serotype 3, known as turkey herpesvirus (HVT), is a naturally avirulent, non-oncogenic strain used for flock immunization (OIE, 2010). MDV serotype 1 was further subdivided into four pathotypes based on virulence: mild, virulent, very virulent, and very virulent plus (Witter et al., 2005).

The classical form of MDV was first reported in Egypt in 1954 (Soliman et al., 1954). Moreover, MDV was first isolated in Egypt at the beginning of this century (Awad, 2002). MDV-3 (HVT) vaccines cannot provide comprehensive protection against virulent MDV strains (Lebdah et al., 2017). Thus, it was recommended to apply the bivalent vaccines to Egyptian breeder flocks to enhance protection against infection (Hussein et al., 2004). Although intensive vaccination against MDV is used, recurrent outbreaks are still documented in both vaccinated and non-vaccinated chicken flocks worldwide (OIE, 2010). This study aims to conduct MDV isolation trials from three Egyptian layer chicken farms in Qalyubia, Menoufia, and Ismailia governorates between August 2024 and February 2025. However, these farms were vaccinated with CVI988 and HVT commercial MDV vaccines but also exhibited MDV-like symptoms. Histopathological investigation of the original sampled tissues was also performed. We also

* Correspondence to: alaa.hussein@fvtm.bu.edu.eg

conducted serological identification of MDV using AGPT, followed by molecular identification by conventional polymerase chain reaction (PCR) targeting the *ICP4* gene. This investigation will evaluate and update insights into the field efficacy of MDV vaccination programs.

2. MATERIAL AND METHODS

Ethical approval

This study was conducted in accordance with the guidelines of our institution's Animal Welfare Committee. The protocols were approved by the Research Ethics Committee of the Faculty of Veterinary Medicine at Benha University, Egypt, with the approval number BUFVTM09-08-24.

Sample collection and preparation

From August 2024 to February 2025, nine field tissue samples were collected from three-layer chicken farms (three samples from each farm) aged 7–10 months. Although the studied farms were vaccinated with both CVI988 and HVT commercial MDV vaccines, the chickens exhibited MDV-like signs, including lethargy, ataxia, and emaciation, as well as 5–10% decline in egg production. The collected samples included the liver and ovaries. Liver samples were collected for analysis from the first farm, which was 232 days old and located in Qalyubia Governorate in January 2025, and the second farm, which was 230 days old and located in Menoufia Governorate in February 2025. Ovary samples were collected from the third farm, which was 300 days old and located in Ismailia Governorate in December 2024.

Necropsy of the examined chickens revealed an enlarged, friable liver with diffuse, raised, multifocal whitish nodules (4–5 mm in diameter). In addition, the ovary had a cauliflower-like appearance (Figure 1). Pooled liver and ovarian tumor samples were collected from the freshly dead chickens under hygienic conditions. Each pooled sample was composed of tissues from three birds belonging to the same farm (one pooled sample per farm). Each sample was divided into two portions; the first was fixed in 10% neutral-buffered formalin for later histopathological examination according to routine techniques (Suvarna et al., 2018), and the other was kept at −20 °C until used for virus isolation and identification.

Sterile phosphate-buffered saline (PBS) supplemented with antibiotics was prepared at 100 IU/ml for penicillin and 1,000 µg/ml for streptomycin. 20% (w/v) suspension of each pooled sample was prepared (WOAH, 2023). Each processed tissue suspension was centrifuged at 3,000 rpm for 15 min at 4 °C to obtain the supernatants. Supernatants from each pool were filtered through a 45 µm pore-size Millipore filter. The obtained filtrate was then transferred into clean, sterile 2 mL cryotubes and stored at −20 °C for subsequent testing (Demeke et al., 2017; WOAH, 2023).

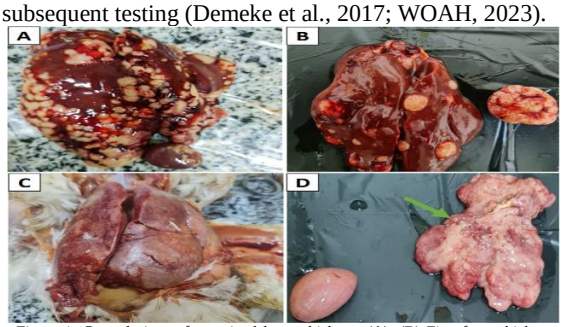

Figure 1: Gross lesions of examined layer chickens. (A), (B) First farm chickens showed enlarged liver with diffuse, raised, and multifocal whitish nodules. (C) Severe enlargement of the liver, friable in consistency, in chickens of the second farm. (D) Third farm chickens showed ovaries with a cauliflower-like appearance (green arrow).

Histopathological study

A portion of the liver and ovary tissue samples were dissected carefully and immersed immediately in 10% neutral-buffered formalin and kept for 72 h for proper fixation. The tissues were then washed, dehydrated in a series of ascending alcohols, cleared in xylene, and finally embedded in paraffin wax. These tissues were then sectioned into 5µm-thick paraffin sections using a rotary microtome. For routine histopathological examination, the slides were stained with hematoxylin and eosin (H&E) stain and examined under a light microscope (Bancroft and Layton, 2019).

Inoculation of the processed samples on specific pathogen-free embryonated chicken eggs (SPF-ECEs)

Three serial passages of pooled samples were conducted on SPF-ECEs sourced from the Koum Oshiem SPF chicken farm in Fayoum, Egypt. A total volume of 0.2 mL of pooled sample supernatant was inoculated on the chorioallantoic membrane (CAM) route in 11-day-old SPF-ECEs. In contrast, the mock control group received 0.2 mL of PBS. The inoculated eggs were incubated at 37 °C, with daily candling. Subsequently, dead embryos 3-5 days post-inoculation (p. i.) and all surviving embryos after seven days were chilled at 4 °C for 6 h, then harvested aseptically and stored at −20 °C for further identification, with examination of the embryos and CAMs for viral characteristic signs (Begum et al., 2016; Biggs and Milne, 1971; Burleson et al., 2014).

Reference MDV

FC-126 strain of the Herpesvirus of Turkey (HVT) tissue culture-adapted vaccine was purchased from FaTRo Veterinary Pharmaceutical Industry (Bio Marek HVT®). It was delivered in lyophilized vials, each containing 1,000 doses and a virus titer of 1,500 PFU/mL. It was used for the preparation of hyperimmune serum against MDV and set as a positive control during virus identification by AGPT and PCR.

Preparation of hyperimmune serum against the reference MDV

This was conducted according to the procedures of Mikami and Bankowski (1971). Four weaned, healthy, and young New Zealand white rabbits were used to prepare a hyperimmune serum against the reference MDV (Bio Marek HVT®). They received four subcutaneous (S/C) injections of the reconstituted HVT vaccine with Freund's adjuvant at two-week intervals. This detailed protocol is presented in Table 1. The prepared hyperimmune serum was centrifuged at 3000 rpm for 10 min at 4 °C, then stored at -20 °C until use. The reactivity of the prepared serum was tested against the reference MDV using the agar gel precipitation test (AGPT), which revealed antibody presence. This serum was later used to identify MDV in egg-passaged isolates, with the formation of white precipitin bands.

Table 1: A schedule illustrates the protocol followed for the preparation of hyperimmune serum against the reference MDV

Day of inoculation	Inoculum	Route	Total inoculum
0	1ml of reconstituted HVT vaccine +1ml Complete Freund's adjuvant	S/C	2ml
14	1ml Reconstituted HVT vaccine + 1ml Incomplete Freund's adjuvant	S/C	2ml
28	1ml Reconstituted HVT vaccine + 1ml Complete Freund's adjuvant	S/C	2ml
42	1ml Reconstituted HVT vaccine + 1ml Incomplete Freund's adjuvant	S/C	2ml
56	Slaughtering of rabbits and blood collection		

Agar gel precipitation test (AGPT)

Identification of suspected isolates of MDV in inoculated eggs was performed using AGPT as described by Mikami and Bankowski (1971) and OIE (2010). The prepared hyperimmune serum was loaded into the central well. In contrast, the peripheral wells were loaded with egg-passaged isolates (embryos ground together with CAMs), and the standard MDV vaccine was used as the positive control. The dishes were incubated in a humidified incubator at 37°C for 5 days and subsequently examined for the presence of white precipitating bands, indicating a positive result.

Genomic DNA extraction and Conventional Polymerase Chain Reaction (PCR) analysis

Genomic DNA was extracted from each isolate using the QIAamp MinElute Virus Spin Extraction Kit (Qiagen, GmbH, Hilden, Germany) according to the manufacturer's protocol. The PCR was performed using Transcript PCR SuperMix® (Thermo Fisher Scientific, UK). The PCR reaction mixture was adjusted to 25 µl, as recommended by the kit manual. Importantly, we conducted conventional PCR analysis for MDV in the obtained isolates, and subsequently, PCR was also conducted for ALV-J and REV.

The amplification employed for MDV used the forward primer MDV-F: 5-GGATCGCCCACCACGATTACTACC-3 and the reverse primer MDV-R: 5-ACTGCCTCACACAACCTCATCTCC-3, which specifically targets the *ICP4* gene, yielding an expected amplicon size of 318 bp. The optimum thermal profile conditions for this reaction included primary denaturation at 95 °C for 10 min. This was followed by 35 cycles that included secondary denaturation at 94°C for 30 sec, annealing at 58°C for 45 sec, extension at 72°C for 45 sec, and a final extension at 72°C for 10 min (Handberg et al., 2001).

Furthermore, the amplification employed for ALV-J used the forward primer ALV-J-H5-F: 5-GGATGAGGTGACTAAGAAAG-3 and the reverse primer ALV-J-H7-R: 5-CGAACCAAAGGTAACACACG-3, which specifically targets the *gp85* gene, yielding an expected amplicon size of 545 bp. The optimum thermal profile conditions for this reaction included primary denaturation at 95°C for 5 min. This was followed by 30 cycles that included secondary denaturation at 93°C for 1 min, annealing at 48°C for 1 min, extension at 72°C for 1 min, and a final extension at 72°C for 10 min (Smith et al., 1998).

Finally, the amplification employed for REV used the forward primer REV-P5-LTR-F: 5-ACCTATGCCTCTTATTCCAC-3 and the reverse primer REV-P6-LTR-R: 5-CTGATGCTTGCCTTCAAC-3, which specifically targets the *LTR* gene, yielding an expected amplicon size of 370 bp. The optimum thermal profile conditions for this reaction include primary denaturation at 94°C for 2 min. This was followed by 35 cycles that included secondary denaturation at 94°C for 1 min, annealing at 55°C for 2 min, extension at 72°C for 1 min, and a final extension at 72°C for 6 min (Biswas et al., 2011).

The amplified fragments were analyzed by gel electrophoresis using a 1.5% agarose gel stained with ethidium bromide at a rate of 10 µL/100 mL to detect the PCR products, compared with a DNA ladder, under an ultraviolet (UV) transilluminator. The gel was then photographed.

3. RESULTS

Histopathological findings

Examination of liver tissue under a microscope revealed significant congestion in the portal blood vessels, central veins, and hepatic sinusoids. The hepatic parenchyma appeared disorganized, with hepatocytes forming isolated islets among the lymphoblastic cells. Vesicular nuclei with clumped chromatin and numerous mitotic patterns were observed in the neoplastic cells. Microscopic examination of the ovarian tissue revealed the presence of pleomorphic neoplastic cells that replaced the ovarian stroma, either diffusely or focally. These cells exhibited numerous mitotic figures and large, eccentric, hyperchromatic oval nuclei. The granulosa cells, the interfollicular spaces, and the area around the ovarian blood vessels were the main sites of infiltration. Follicles became atrophied or malformed as a result of the disruption of the ovarian tissue architecture (Figure 2).

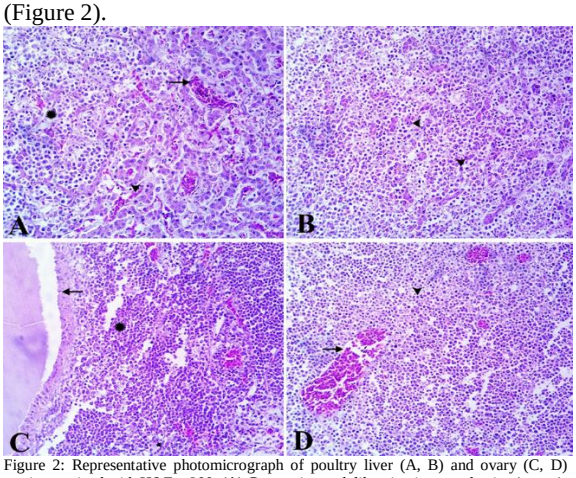

Figure 2: Representative photomicrograph of poultry liver (A, B) and ovary (C, D) sections stained with H&E, x200. (A) Congestion and dilatation in central veins (arrow), and hepatic sinusoids (arrowhead) with lymphoblastic cell infiltration (asterisk). (B) Neoplastic cells showing vesicular nuclei with clumped chromatin and numerous mitotic patterns (arrowhead). (C) Pleomorphic neoplastic cells (asterisk) surrounding the ovarian follicle (arrow). (D) Congested blood vessels (arrow) with massive neoplastic lymphoblast cells infiltration (arrowhead).

Virus isolation on CAMs of SPF-ECEs

Embryos harvested from the inoculated SPF-ECEs manifested pathognomonic signs following three serial passages, as shown in Figure 3. These signs were observed as embryonic death after 48 h p. i. In addition, the surviving embryos until the seventh d. p. i showed notable dwarfism, pronounced stunting, and body curling in comparison to the mock control embryos. Additionally, anomalies, widespread congestion with a hemorrhagic body, and an enlarged, congested liver were observed. In addition, the CAM exhibited marked thickening. Notably, the signs and embryo mortality increased with serial passaging.

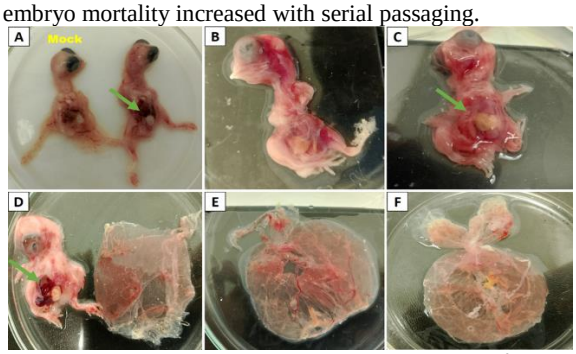

Figure 3: Macroscopic lesions exhibited on the harvested embryos and CAM of inoculate SPF-ECEs. (A) Chick embryo showing widespread hemorrhage with enlarged an congested liver compared to the mock control embryo (green arrow). (B), (C) Chic embryo with stunting, body curling, widespread hemorrhage, and congestion. (D) Chic embryo exhibiting an enlarged liver with notable congestion (green arrow). (E), (F) CAM showing marked thickening.

Agar gel precipitation test (AGPT)

All peripheral wells containing the harvested isolates from SPF-ECEs showed negative results against the prepared hyperimmune serum of HVT, which was in the central well. A white precipitin band was formed between the wells containing the reference virus and the well containing the prepared hyperimmune serum (Figure 4).

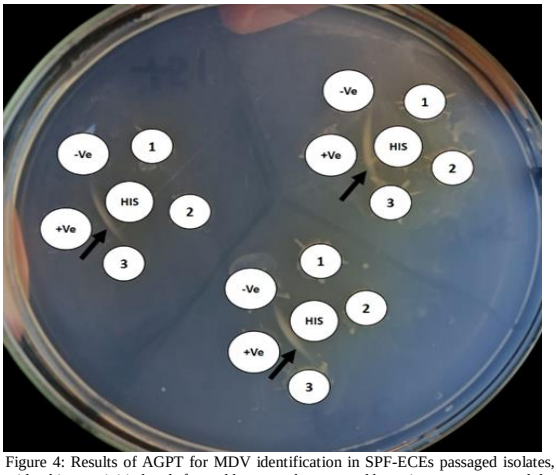

Figure 4: Results of AGPT for MDV identification in SPF-ECEs passaged isolates, with white precipitin bands formed between the prepared hyperimmune serum and the reference virus (black arrow). HIS well: Hyperimmune serum. +Ve well: control positive (reference virus). -Ve well: control negative (PBS). Both positive and negative controls were involved in the reaction to ensure the reliability of the result.

Conventional PCR analysis

The isolates obtained from the three farms in Qalyubia, Menoufia, and Ismailia governorates were tested for MDV using conventional PCR targeting the *ICP4* gene, and all three isolates were negative. Surprisingly, the PCR testing for other common avian tumor viruses, such as Avian leukosis virus subgroup J (ALV-J) and Reticuloendotheliosis virus (REV), revealed that two isolates from the first and third farms were positive for ALV-J, yielding 545 bp amplicons. In addition, the isolate from the third farm tested positive for REV, yielding a 370 bp amplicon. Notably, the harvested isolate, which was originally sampled from the second farm, tested negative for both ALV-J and REV. The detailed electrophoretic analysis of PCR products is shown in Figure 5.

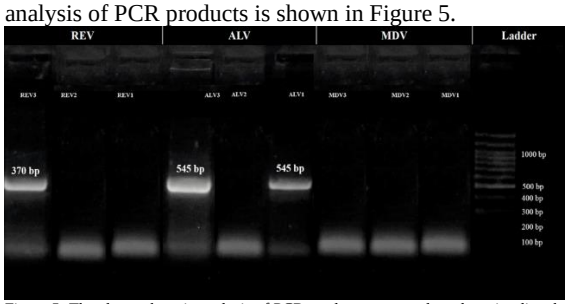

Figure 5: The electrophoretic analysis of PCR products was conducted to visualize the size of amplicons. In this assessment, all three isolates showed a negative result for MDV based on the *ICP4* gene. The amplicon corresponding to the *gp85* gene in ALV-J was identified at 545 bp in both isolates from the first and third farms. Additionally, the amplicon for the LTR region in REV was found to measure 370 bp in the isolate from the third farm, which was sampled from the ovaries. Also, the isolate of the second farm tested negative for both ALV-J and REV. The first lane represents a 100 bp ladder as a size standard. The results were visualized on a 1.5% agarose gel.

4. DISCUSSION

Marek's disease virus is a major threat to the poultry industry worldwide, causing significant economic losses (Ewies et al., 2020). However, the hatcheries in Egypt follow an intensive vaccination strategy against Marek's disease virus (MDV) using CVI988 and Herpesvirus of Turkey (HVT), or CVI988 in one-day-old chicks; the chicken farms still suffer from recurrent outbreaks of tumors. Interestingly, a highly virulent MDV strain was previously isolated from various MDV-vaccinated Egyptian chicken flocks (Hussein et al., 2004).

In the present study, trials for the isolation, serological, and molecular identification of MDV were performed on pooled samples collected from three Egyptian chicken layer farms in Qalyubia, Menoufia, and Ismailia governorates. These farms were vaccinated with CVI988 and HVT MDV vaccines; otherwise, they exhibited MDV-like signs, including emaciation, depression, ataxia, and reduced egg production. A recorded morbidity rate of approximately 5–20% and a mortality rate of approximately 2–10% were observed. Necropsy revealed an enlarged liver with raised multifocal whitish nodules. In addition, the ovaries were markedly enlarged and had a cauliflower-like appearance. These findings matched the common symptoms and lesions observed in flocks infected with avian tumor viruses. These findings are consistent with those of Emad et al. (2024) and Ewies et al. (2020).

This study focused on trials for isolating MDV from the liver and ovaries on CAMs of SPF-ECEs, followed by serological identification using AGPT and molecular identification by conventional PCR with specific primer sets. This is consistent with El-Kenawy et al. (2019). In addition, a histopathological study was carried out.

Histopathological findings showed neoplastic proliferation and tissue disorganization in the liver and ovaries, consistent with lesions induced by ALV and REV. Hepatic congestion, lymphoblastic infiltration, and numerous mitotic figures indicate hepatic lymphoid leukosis, while pleomorphic neoplastic cells replacing the ovarian stroma and follicular atrophy suggest ovarian lymphoid tumors. Similar findings were reported by Ramoutar et al. (2022). Hepatic and ovarian tissue involvement may suggest viral coinfection, which enhances the neoplastic transformation process (Wen et al., 2018).

Concerning the inoculation of processed pooled samples on CAMS of SPF-ECEs for three serial passages, the exhibited pathognomonic signs were mainly embryo dwarfism, stunted growth, body curling, enlarged liver, widespread congestion, and clear thickening of CAMs. Embryo mortality increased with passage, reaching its highest level in the third passage. These observed lesions may also be due to the direct effect of ALV-J, as it has been previously recorded to cause severe hemorrhage in inoculated embryos, followed by death after 48 h and sometimes within 4–5 days. Our results are consistent with those of Fotouh et al. (2024).

Subsequently, AGPT was performed on the isolates harvested from SPF-ECEs for all three passages against the prepared MDV hyperimmune serum. Our findings proved that all samples were MDV-negative. Lesions caused by avian oncoviruses often appear similar, making it challenging to distinguish between them, especially with non-distinct clinical symptoms. Thus, the differential diagnosis between these viruses is achieved through serological and molecular identification (Gimeno et al., 2005; Wang et al., 2014). PCR has emerged as the diagnostic technique of choice for these viruses because of its ability to overcome many challenges associated with classical differential diagnostic methods and detect multiple viral infections (Davidson, 2009).

Despite evidence of virus growth on CAMs and SPF-ECEs embryos, PCR is still regarded as the gold standard method used for investigating MDV in chicken farms, as previously revealed by Raja et al. (2009). The isolates underwent conventional PCR analysis for MDV using primers

targeting the *ICP4* gene, with an expected amplicon size of 318 bp. To our surprise, all the tested isolates were confirmed to be negative. Therefore, specific primers for ALV-J and REV were used to identify the viruses causing tumors on these farms. PCR in this study showed high specificity and sensitivity, with a unique band of the expected amplicon size of 545 bp corresponding to the *gp85* gene in ALV-J. The amplicon for the *LTR* gene in REV was found to measure 370 bp. Our molecular identification was consistent with that of Fotouh et al. (2024).

Surprisingly, PCR results revealed that of the three examined isolates, one tested positive for ALV-J, another was co-infected with ALV-J and REV, and the third was negative for both ALV-J and REV. To the best of our knowledge, this is the first statement describing the co-detection of ALV-J and REV in the ovaries of Egyptian layer chickens. This finding indicates that there should be further investigation into the potential effects of such co-infections on reproductive organs and virus transmission strategies among layer chickens.

Our results revealed that ALV-J and REV were the main causes of visceral tumors in layer chickens in the present study. Importantly, our results suggest that the ALV-J and REV isolates may have a common ancestor. This investigation resulted in a prominent detection rate attributed to the vertical transmission of these viruses. Fotouh et al. (2024) and Shosha et al. (2024) support these findings. Our study highlighted that the absence of MDV infection in vaccinated chicken farms demonstrates the efficacy of commercial MDV vaccines used in these farms. Further research is recommended to understand the effects of avian oncogenic viruses on pathogenesis and pathogenicity, as well as the implications of inadequate vaccination protection against MDV.

5. CONCLUSIONS

In conclusion, our study highlighted that MDV infection was not recorded in chicken layer flocks with MDV-like symptoms vaccinated with CVI988 and HVT commercial vaccines. This indicates the efficacy of the aforementioned vaccines in providing complete protection against more virulent MDV strains. The authors also highlighted the potential coexistence of ALV-J and REV in our isolates, drawing attention to their presence in Egyptian poultry farms. Moreover, PCR is the technique of choice for rapid differential diagnosis of avian oncogenic viruses and for detecting coinfections under field conditions. Ultimately, further research is required to evaluate the protection rates provided by the current MDV vaccines used in Egypt and to determine the evolutionary traits of both ALV-J and REV in the Egyptian poultry sector.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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