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Highlight on Reticuloendotheliosis virus (REV) in commercial chicken flocks

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ABSTRACT

In this study, the reticuloendotheliosis (REV) infection status was monitored through antibody determination and molecular detection in chickens in different provinces of Egypt. We investigated the occurrence and genetic characteristics of REVs in chickens' flocks from January 2019 to March 2021 as a PCR test examined 39 commercial chicken farms (27 layers and 12 broiler breeders) from 11 provinces of Egypt. Results showed six farms (15.4%) were REV-positive in 3 provinces (AL Sharqia, Al Menya, and Al Behera). Four REV-positive farms were genetically sequenced.

24 of these 39 farms (18 layers and 6 broiler breeders) were examined by ELISA test for antibodies detection against REV and ALV (subtype A and B). 22 farms (91.7%) were REV antibodies positive in all 11 provinces tested (Al Sharqiyah, Al Monofiya, Al Daqahlia, Al Gharbia, Al Qualiobia, Al Beheira, Giza, Damietta, Kafr El Sheikh, Bani Suief and El Minya), 3 farms (12.5%) were ALV (subtype A and B) antibodies positive from Al Beheira governorates. Molecular detection of REV was differentiated from other neoplastic viruses MD, ALV (subtype A, B, C, D, and J), from 39 farms, seven farms were positive for MDV (5 of them were seropositive for REV), one farm positive for ALV.J (the farm was seropositive for REV), all farms were negative for ALV subtype (A, B, C and D0. Our results demonstrated that REV was extensively distributed in different regions of Egypt. Phylogenetic analysis of the partially sequenced envelope glycoprotein gene showed that REV was most closely related to strains from China, Taiwan, Thailand, and the USA. The REV strains were clustered into REV subtype III. This finding indicates that REV subtype III was predominantly circulated in Egyptian chicken flocks. Our findings raise awareness about REV-induced diseases as the causative agent of runting and oncogenic disease in chickens and highlight the incidence of REV infection among some commercial chicken flocks in Egypt.

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INTRODUCTION

Neoplastic diseases in chickens cause enormous financial losses to the poultry industry due to high mortality, sub-performance, and immunosuppression. Reticuloendotheliosis (REV) is a typical oncogenic, immunosuppressive virus. It belongs to the family Retroviridae, genus Gamma retrovirus **(Witter et al. 1979; Payne 1998; Barbosa et al. 2007; Xu et al. 2020).**

Chickens are exposed to REV both vertically and horizontally. Cloacal swabs, litter, and feces all contained the virus. Commercial vaccines infected with this virus have been demonstrated to transmit the disease into flocks of chickens (**Fadly et al. 1996; Wozniakowski et al. 2015; Abd El Hamid et al. 2008; Awad et al. 2010).** In domestic chickens and other avian species, REV can cause runting disease syndrome, immunosuppression, and neoplasia **(Witter et al. 2003).**

The group-specific antigen (gag), polymerase (pol), and envelope (env) genes make up the REV genome. According to Payne 1998, the REV-T, REV-A, chick syncytial virus (CSV), spleen necrosis virus (SNV), and duck infectious anemia virus are the representative REV strains. Even though there is only one serotype of REV known, it can be divided into three different subtypes, including subtypes I (170A), II (SNV), and III (CSV) (Chen et al. 1987). Chicken anemia virus (CAV), fowl poxvirus, Marek's disease virus (MDV), and ALV-J were frequently co-infected viruses in REV-infected birds **(Sun and Cui 2007; Li et al. 2021; Lu et al. 2022).**

REV can be diagnosed based on viral isolation, histological analysis of tumor tissues, serology, and molecular diagnosis by PCR test. Multiple virus infections might make a diagnosis based on virus isolation more difficult and time-consuming. However, it might be challenging to differentiate between distinct lymphoid tumor lesions caused by different viruses based on histological diagnosis. The use of an enzyme-linked immunosorbent assay (ELISA), which is more sensitive than indirect immunofluorescent-antibody testing and appropriate for veal flock screening, allowed for the successful detection of antibodies against REV **(Smith and Witter 1983; Hafez 2001**). The PCR test is a sensitive and precise approach to diagnosis that makes it possible to find numerous viral infections and REV **(Aly et al. 1993; Davidson 2009).**

Numerous investigations of REV diagnosis were published in Egypt **(Aly et al. 1998; Awad and Youssef 2008; Eid et al. 2019).** For the control of REV infections in chicken farms, there are no reliable medications or vaccinations available **(Yang et al. 2017).**

To update knowledge on tumor disease issues in chicken flocks that REV may cause, we have investigated REV occurrence in flocks of suspected tumor-bearing chickens collected between January 2019 and March 2021 from a variety of chicken flocks (broiler breeders and layers) in 11 Governorates of Egypt. We also characterize field isolates based on molecular diagnosis and serological diagnosis.

MATERIALS AND METHODS

Flocks' history:

39 flocks of chickens (27 layers and 12 breeding flocks) were tested for REV by PCR, and 24 flocks from these farms (18 layers and 6 breeder flocks) were tested by ELISA for the presence of REV antibodies.

The samples were collected between January 2019 and March2021, and their ages varied from 7 to 64 weeks for layer breeder flocks and 29 to 61 weeks for broiler breeder flocks. According to Table 1, the farms are spread across 11 Egyptian provinces: Al Sharqia, Al Monofiya, AL Daqahylia, AL Gharbia, AL Qalyoubia, Al Beheira, Giza, Damietta, Kafr El Sheikh, Bani Suief, and El Menya. The Reference Laboratory for Veterinary Control on Poultry Production (RLQP, Giza, Egypt) received apparent healthy and sick birds for disease diagnosis. Runting, pallor of the face, swelling around the head, lameness, aberrant feathering, and lesions that seemed to be tumors in the skin, liver, and spleen were all collected in the clinical diseased chickens.

Table 1. Numbers of farms examined for detection of REV by PCR and ELISA test.

Governorates	Total No. of farms	No # of examined flocks (Positive/Total)			
		PCR test		ELISA test	
		Farm No#	No. of Positive	Farm No#	No. of Positive
Al Sharqia	10	10	4/10	5	5/5
Al Monofia	5	5	0/5	2	2/2
Al Daqahylia	2	2	0/2	1	1/1
Al Gharbia	4	4	0/4	3	3/3
Al Qalyoubia	3	3	0/3	2	2/2
Al Beheira	7	7	1/7	5	3/5
Giza	2	2	0/2	2	2/2
Demietta	1	1	0/1	1	1/1
Kafr El-Sheikh	3	3	0/3	2	2/2
Bani Suief	1	1	0/1	0	0
Al Menya	1	1	1/1	1	1/1
Total	39	39	6/39 (15.4%)	24	22/24 (91.7%)

Samples for laboratory investigation

For molecular detection by PCR test, various organs, including the thymus, bursa of Fabricius, liver, intestine, and spleen, were sampled. Additionally, 236 sera samples (about 8–10 blood samples per flock) from 24 chicken farms were obtained for serological testing. The sera samples were stored at -20 ºC until testing.

Serological detection using the ELISA test

Using a commercial ELISA test, 236 serum samples were collected and tested for antibodies against REV and avian leukosis virus subgroups A and B (ALV). The ELISA test was performed according to the manufacturer's instructions (IDEXX Laboratories, Inc).

Molecular detection

The tissue samples from 39 chicken flocks were collected, prepared, and tested by PCR test for different tumor viruses (REV, MDV, and ALV subtypes A, B, C, D, and J).

Extraction of viral Nucleic acid

39 samples were subjected to whole nucleic acid extraction using the QIA amp Mini Elute virus spin kit (Qiagen, Germany, GmbH). In a nutshell, for 15 minutes at 56°C, 200 µl of the sample suspension was treated with 25 µl of Qiagen protease and 200 µl of AL lysis solution. 250 µl of 100% ethanol was added to the lysate after incubation. After that, the sample was cleaned and centrifuged following the manufacturer's instructions. With the help of 100 µl of elution buffer, DNA was extracted. DNA extracts were stored at -20°C for additional investigation.

Amplification of viral nucleic acid using conventional PCR

PCR was performed using specific Primers supplied by Metabion (Germany). The nucleotide sequences are listed in **Table 2** as follows

Table 2. The nucleotide sequences

Agent	Primer sequence (5'-3')	Amplified product (bp)	Reference
ALV- A	H5-F GGATGAGGTGACTAAGAAAG EnvA-RAGAGAAAGAGGGGYGTCTAAGGAGA	694	Fenton et al. 2005
ALV-B and D	BD-F CGAGAGTGGCTCGCGAGATGG BD-R AGCCGGACTATCGTATGGGGTAA	1100	Silva et al. 2007
ALV-C	C-F CGAGAGTGGCTCGCGAGATGG C-R CCCATATACCTCCTTTTCCTCTG	1400	Silva et al. 2007
ALV-J	H5-F GGATGAGGTGACTAAGAAAG H7-R CGAACCAAAGGTAACACACG	545	Smith et al. 1998
MDV	ICP4 F GGATCGCCCACCACGATTACTACC ICP4 RACTGCC TCACACAACCTCATC TCC	434	Handberget al.2001
REV	env-F AGCTAGGCTCGTATGAA env-R TATTGACCAGGTGGGTTG	438	Wei et al. 2012

PCR amplification.

A 25µl reaction containing 12.5 µl of Emerald Amp Max PCR Master Mix (Takara, Japan), 1 µl of each primer at a concentration of 20 pmol, 5.5 µl of water, and 5 µl of DNA template was used to test the primers. Thermal cycler 2720 from Applied Biosystems was used to carry out the process. **ALV A, B, C, and D:** Initial denaturation was performed at 94°C for 4 min. After that, 35 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 30 s each were carried out, followed by a final elongation at 72°C for 10 min. **ALV J:** The initial denaturation was conducted for 5 min at 95°C, followed by 35 cycles of 95°C for 30 s, 60°C for 30 s, and 72°C for 30 s, followed by a final extension for 10 min at 72°C. **MDV:** The first denaturation step was place at The PCR conditions include one cycle of initial denaturation at 95°C for 15 minutes, 40 cycles of denaturation at 95 °C for 45 seconds, annealing at 50°C for 45 seconds, extension at 72°C for 1 minute, and final extension at 72°C for 10 minutes. **REV:** The PCR conditions are 95°C for 15 minutes of initial denaturation, 1 cycle of 95°C for 30 seconds of denaturation, 1 cycle of 54°C for 30 seconds of annealing, 1 cycle of 72°C for 30 seconds of extension, and 1 cycle of 72°C for 10 minutes of final extension. The PCR products were separated using 5V/cm gradient electrophoresis on 1.5 percent agarose gel (Applichem, Germany, GmbH) at room temperature. Each slot received 15µl of the goods for the gel analysis. The sizes of the fragments were determined using a gene ruler 100 bp ladder (Fermentas, Germany). A gel documentation system (Alpha Innotech, Biometra) took pictures of the gel, and computer software was used to analyze the information.

Sequence and phylogenetic characterization:

DNA bands of the anticipated size were removed from the gel per the manufacturer's instructions and purified using the QIA quick Gel Extraction Kit (Qiagen, Hilden, Germany). PCR products that had been purified were directly sequenced using ABI PRISM Big Dye Terminators v3.1 Cycle Sequencing Kits (Applied Biosystems, Foster City, CA, USA). ABI PRISM3500 xl genetic analyzer (Applied Biosystems, Foster City, CA) Centrisep purification kit was used to clean up the sequencing reaction products before sequencing them (Applied Biosystems). The original purpose of BLAST® analysis (Basic Local Alignment Search Tool) was to verify sequence identities (Badial et al. 2018). The MegAlign module of the Laser gene DNA Star version 12.1 program created a phylogenetic tree using the greatest likelihood approach (**Kumar et al. 2018).**

RESULTS

Clinical and necropsy findings:

Chickens with the condition ranted, had pale faces, had swelling around their heads, and had unusual feathering were investigated. The postmortem examination of dead and sac-

rificed birds showed moderately to significantly underweight carcasses, tumor development in the head area, engorged and enlarged liver, spleen, proventriculus, and gizzard, as well as whitish nodular infiltrations in the liver and spleen that were frequently seen. Neoplastic nodules were also found in the mesentery, pancreas, and intestine.

Serological investigation:

Using a commercial ELISA test, 236 sera samples from 24 farms' of commercial chicken flocks, (18 layers, and 6 broiler breeders) were gathered and examined for antibodies against the reticuloendotheliosis virus. Samples were taken between January 2019 and March 2021. (Table 3). In all 11 of the investigated provinces, 22 flocks tested positive for the REV antibody, with seroprevalence rates of 91.7% (22/24) (Al Sharqiyah, Al Monofiya, Al Daqahlia, Al Gharbia, Al Qualiobia, Al Beheira, Giza, Damietta, Kafr El Sheikh, Bani Suief and El Minya).

The geometric mean (GMT) ranged from (392 to 15524), the coefficient variation (CV) ranged from (14-156), and the positive proportion inside the farm ranged from 40 to 100 percent.

The 18-layer flocks ranged in age from 10 to 44 weeks. All the flocks had seroprevalence rates of 100% for the REV antibody (18/18). (Table 3).

The six broiler breeders' flocks were between 29 to 61 weeks old, and only 4 flocks showed REV antibody seroprevalence (66.7 %) (Table 3).

All 236 sera samples were tested for antibodies against avian leukosis virus subgroups A and B (ALV) using a commercial ELISA assay for differential diagnosis. 24 farms tested were negative, except three broiler breeder farms from the Al Beheira governorates (3/24) that tested positive for antibodies to the ALV viruses with seroprevalence rates of 12.5%. The farm's positive ALV antibody ranged from 20 to 100%, the GMT ranged from (464-2848), and the CV ranged from (18-188).

Table 3 Antibody detection by ELISA test from January 2019 to March 2021.

Year	REV		ALV (A, B)	
	Type of production			
	Layer (Positive/Total)	Breeder (Positive/ Total)	Layer (Positive/Total)	Breeder (Positive/ Total)
2019	1/1	1/1	0/1	0/1
2020	15/15	2/4	0/15	2/4
2021	2/2	1/1	0/2	1/1
Total	18/18 (100%)	4/6 (66.7%)	0/18	3/6 (50%)
	22/24 (91.7%)		3/24 (12.5%)	

Molecular detection using conventional PCR

A PCR assay was used to check 39 flocks of chickens from various production types for the molecular presence of REV (27 layers and 12 broiler breeders). The samples were taken from January 2019 to March 2021 (Table 4), and 15.4% (6/39) of the flocks tested positive for the REV virus. The age of the layer flocks ranged from 7 to 64 weeks; four flocks were positive 14.8 % for the REV virus (Table 4). The broiler breeder's flocks' ages ranged from 29 to 61 weeks; 2 flocks were positive (16.7%) for REV virus detection (Table 4).

Table 4. Molecular detection of REV from January 2019 till March 2021 by PCR test.

Year	Type of production	
	Layer (Positive/Total)	Breeder (Positive/Total)
2019	0/5	0/2
2020	3/20	2/8
2021	1/2	0/2
	4/27 (14.8%)	2/12 (16.7%)
Total	6/39 (15.4%)	

The current study's molecular detection of REV distinguished it from two additional oncogenic viruses, MDV and ALV (subtype A, B, C, D, and J). 39 farms were tested, of which 6 were positive for REV (2 were seropositive for REV), 7 were positive for MDV (5 were seropositive for REV), 1 was positive for ALV.J (the farm was seropositive for REV), and every farm tested was negative for ALV (subtype A, B, C, and D) (Table 5).

Table 5. Molecular detection of MD and ALV (A, B, C, D, and J) from January 2019 to March 2021 by PCR test.

Year	MD		ALV (A, B, C, D and J)	
	Type of production			
	Layer (Positive/Total)	Breeder (Positive/ Total)	Layer (Positive/Total)	Breeder (Positive/ Total)
2019	0/5	½	0/5	0/2
2020	2/20	3/8	*1/20	0/8
2021	0/2	½	0/2	0/2
Total	2/27 (7.4%)	5/12 (41.7%)	1/27 (3.7%)	0/12
	7/39 (17.9%)		1/39 (2.6%)	

*The positive ALV farm was positive for ALV-j

Sequencing and phylogenetic analysis of REV

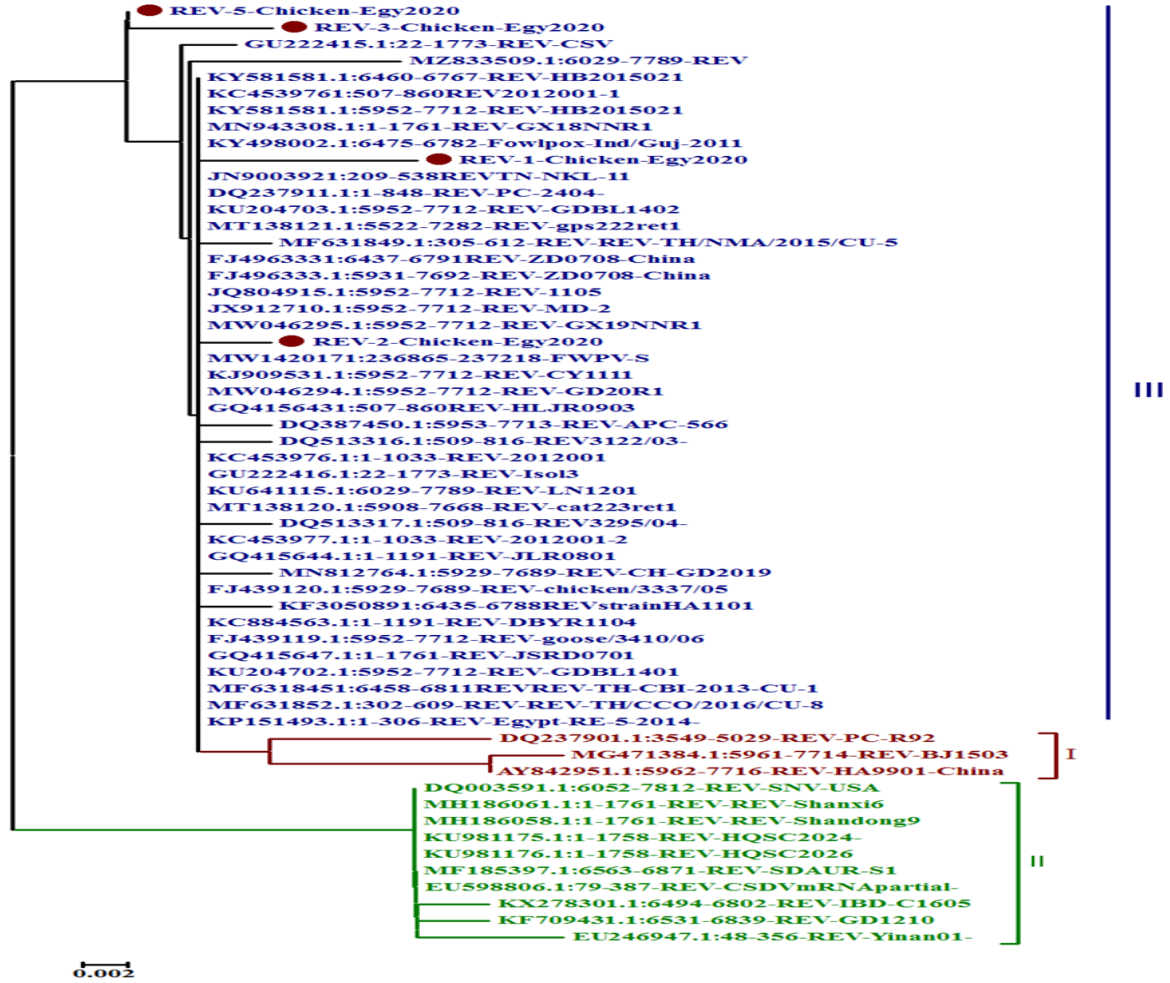
4 out of 6 samples were successfully sequenced for the envelope gene, and the nucleotide sequence of each product was deposited to GenBank under the accession numbers are shown in Table 6. Using BLAST (https://blast.ncbi.nlm.Nih.gov/Blast.cgi), 4 out of the six virus sequences generated in the present study had from 97 to 99% sequence identity with strains isolated in Thailand, China, Taiwan, and USA, which belonged to REV and were grouped with a prototype strain of REV subtype III (CSV; 98.4% nucleotide identity). Our results indicated that all four sequenced REVs were classified as subtype III, the most common REV subtype circulating in different avian species worldwide (Figure 1). Results also showed that all REVs were closely related to each other (99.7 to 100% nucleotide identity), although they were collected from different regions and times (Figure 2).

Table 6. Complete Descriptive Data for REV-positive farms

Flock ID	Year	Provence	Age (WK)	Type of production	REV ELISA	Other tumor viruses	GenBank Acc.no.
1	2020	Al Sharqiyah	30	Broiler breeders	*Nd	Neg	OQ137282
2			9	Layers	Nd	Neg	OQ137283
3			10	Layers	Pos	Neg	OQ137284
4			10	Layers	Nd	Neg	-
5	2020	Al Menya	29	Broiler breeders	Pos	Neg	OQ137285
6	2021	Al Behera	64	Layers	Nd	Neg	-

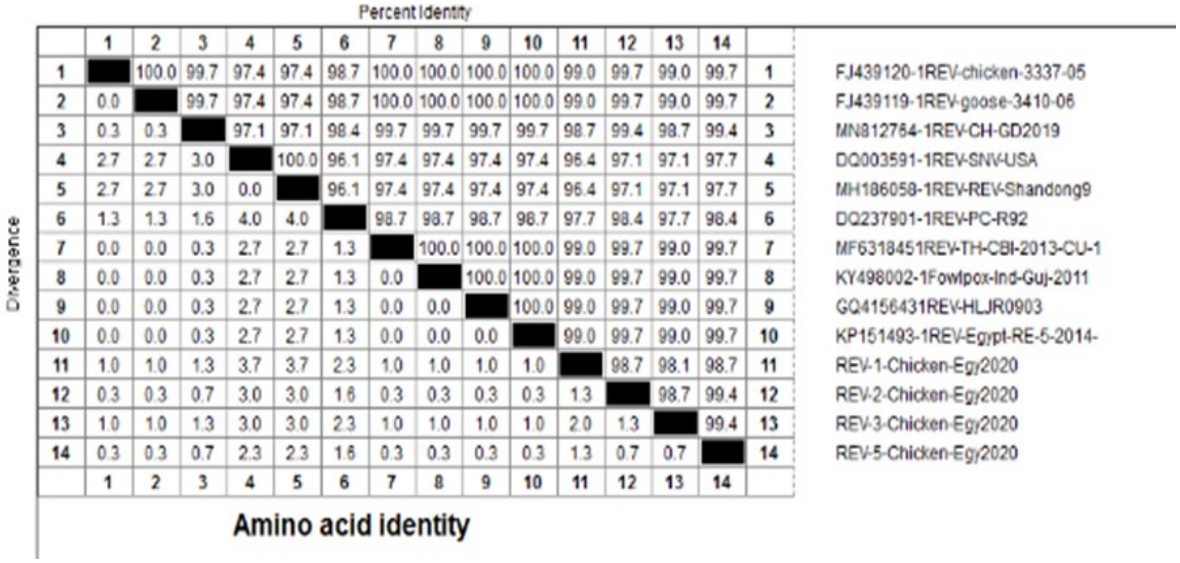
*Nd: Not done means blood samples were not collected. Pos; positive – Neg: negative

Figure 1: Phylogenetic tree of Amino acid sequence of Env gene



Phylogenetic analysis of the Amino acid sequences of the partial *env* gene sequence with representative REV reference strains. The phylogenetic trees were constructed in MEGA v.6.0 using the neighbor-joining algorithm (Tamura et al., 2013).
Strains in this study are grouped with a prototype strain of REV subtype III.

Figure 2:.Pairwise identity matrix of amino acid sequences: -



The identity % between strains under study ranged between 98.1 to 99.7 and between some reference strains ranged between 96,1 to 99.7.

DISCUSSION:

A typical immunosuppressive, runting, and carcinogenic virus in chickens called REV is significant economically for the poultry industry. The objective of the current study was to investigate the frequency and genetic makeup of REVs in hens in Egypt between January 2019 and March 2021.

The Delta region of Egypt is where intensive commercial chicken production takes place. As a result, AL Sharqiyah, AL Beheira, Al Monofiya, and Al Gharbia provided most of the samples and positive farms detected during this investigation (Table 1).

Affected chickens suffered from abnormal swelling in the head region, runting, and feathering abnormality. The gross lesions of infected flocks were creamy and friable tumors in the head, congested and enlarged liver, Spleen, proventriculus, and gizzard with nodular tumor infiltration. These findings agree with previous studies conducted in commercial broiler breeder flocks **(El-Gohary et al. 2000; Awad et al. 2004).** Tumor lesions are commonly observed in adult chickens while runting without tumor lesions has been frequently reported in young chickens (**Nair et al. 2013**).

Serological and molecular diagnosis used in the present study are essential tools for quick and accurate diagnosis (**Hafez 2001; Cao et al. 2013).** The REV in Egypt has been diagnosed in chickens by serology screening and PCR test **(Aly et al. 1993; Aly et al. 1998; Awad and Youssef 2008; Eid et al. 2019).**

Serological and molecular diagnosis were choices due to the difficult isolation of REV from seropositive chicken flocks (Witter et al. 1982; Witter and Johnson 1985) as well as the histopathological diagnosis of the neoplastic disease as MD, ALV, and REV is very difficult as the pathological picture became mixed and non-pathognomonic. Still, it is a very important technique for the preliminary diagnosis to decide if a neoplastic disease or not. There is no commercial vaccine to control REV; it is reasonable to reflect the REV infection status with serological methods. Maternal-derived antibodies (MDA) could interfere with the seroprevalence of REV under field conditions; hence, we have chosen all flocks in the study to be over 3 weeks of age to avoid false positive results derived passively from MDA

sources.

Using a commercial ELISA test, 236 sera samples from 24 farms' of commercial chicken flocks, 18 broiler breeders, and 6 layers were obtained. 22 flocks were found to be positive, with seroprevalence rates of 91.7 percent (22/24). The age of the flocks that tested positive, which ranged in age from 10 to 61 weeks, shows the frequency of REV among the tested flocks.

The positive percentage inside the farm flocks ranged from 40-100%, GMT ranged from (392-15524) and CV ranged from (14-156), indicating different individuals infected by REV, which may be related to the REV incidence in different regions or susceptibility to REV of different breeds flocks. The serology results were agreed with Moshira et al. 2016, where the serological prevalence for the REV antibody ranged from 25-100 % at 12^{th} and 25^{th} weeks of age for crossbreed chicken farms, respectively. **Zhao et al. 2012**, where Serum samples analysis revealed 32.16% samples positive for REV-antibody. Alfaki et al. 2019, investigated the serological prevalence in Sudan from local and commercial chicken breeds, which was 74.6%.

Many serological studies on REV infection in commercial chicken flocks were conducted. They showed that the seroprevalence of REV infection in chicken flocks is relatively high **(Witter et al. 1982; Yang et al. 2017) in Egypt (Aly et al. 1998; Hafez 2001; Awad and Youssef 2008).**

All 236 sera samples were also examined against avian leukosis virus subgroups A and B (ALV) using a commercial ELISA test as a differential diagnosis. All the tested farms were negative except for three broiler breeders; farms were positive for antibody detection of avian leukosis virus. The three farms may indicate the coinfection with both ALV and REV, the same results were recorded by **(Sun and Cui 2007; Cui, et al. 2009).**

The PCR is an effective and sensitive tool for identifying REV infection (Aly et al. 1993). With prevalence rates of 15.4% (6/39), six of thirty-nine chicken flocks of various production types (27 layers and 12 broiler breeders) were positive for REV by PCR. Furthermore, MDV and ALV were negative in the six positive REV farms. Molecular detection and characterization of reticuloendotheliosis virus in broiler breeder chickens (30–40 weeks of age) with visceral tumors in Egypt were conducted **by El-Sebelgy et al. 2014.**

Molecular detection of REV in the current study was differentiated from other neoplastic viruses MD, ALV(subtype A, B, C, D, and J), which may be circulating undetected in Egypt; from 39 farms, 6 farms were positive for REV by PCR test (2 of them are seropositive for REV), seven farms positive for MDV (5 of them are seropositive for REV), one farm positive for ALV.J (the farm was seropositive for REV). Co-infection of REV with other avian oncogenic viruses has been detected in chickens, increasing disease severity and virus transmissibility in infected chickens **(Sun et al. 2010; Dong et al. 2014; Bao et al. 2015).** In addition, REV contamination in avian vaccines has been continuously reported, causing REV infection and reduced vaccine effectiveness in vaccinated chickens **(Wei et al. 2012; Li et al. 2016).** The high Sero-positivity of the examined chicken flocks, when compared with the detection of the REV virus by PCR, could be related to the latency of REV infection, which causes the presence of antibodies in the bird serum and the absence of the virus.

Sequence analysis of the amplified PCR products revealed genetic similarity to REV and submitted in NCBI GenBank with access numbers OQ137282, OQ137283, OQ137284, and OQ137285. Our results indicated that REV strains were classified as subtype III, the most common REV subtype circulating in different avian species worldwide **(Mays et al. 2010; Bao et al. 2015).** Our results also showed that all REV strains were closely related to each other (99.7 to 100% nucleotide identity), although they were collected from different regions and times. This indicated the low genetic variation of REV strains circulating in Egypt chicken flocks during the tested period.

Our study's findings confirmed the circula-

tion of REV among different commercial poultry flocks. We all know there are several ways that REV can spread among flocks of chickens, including horizontal, vertical, and vaccine -contamination routes **(Fadly et al. 1996),** so we can conclude the need for focused national surveillance among breeders, layers, and broilers flocks, to have accurate data about the prevalence and possible transmission routes of REV in Egypt to adopted correct prevention and control measures to minimize the impact of such immunosuppressive disease to the poultry value chain.

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