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Detection and Molecular Characterization of Avian Reovirus (ARV) Variants in Egypt with studying the effect of Selenium Nanoparticles on Growth Performance after Experimental ARV challenge in broilers

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ABSTRACT

This study aimed to identify and characterize emerging ARV variants that originated from field outbreaks in commercial broiler flocks between 2021 and 2022 in El-Beheira and Qena governorates, Egypt. In addition, studying whether selenium nanoparticles (Se-NPs) treatment influenced the growth rate after the variant ARV challenge in broilers. First, for virus isolation, the collected samples were propagated into embryonated chicken eggs via the yolk sac route. ARV presence was verified by reverse transcriptase PCR (RT-PCR) and characterized by sequencing of seven representative samples targeting the sigma C gene (σC). Based on the amino acid sequences of the σC -encoding gene phylogenetic analysis revealed that the obtained seven variants of ARV isolates were grouped in the genetic cluster (GC) 4 and genetically distant from the available commercial vaccines and vaccine-related field strains. Depending on the σC gene's 256 amino acid sequence identity, the emerging ARV variants in this study shared only 39.5%-45.7% with available commercial vaccine strains, including S1133, 1733, and 2408. Second, Se-NPs were studied for their impact on the growth rate, gross, and microscopic lesions. The results revealed that the birds who received Se-NPs without ARV challenge had a mild, non-significant improvement in mean body weights when compared to the control, while the bird groups challenged with ARV either treated/ not treated with Se-NPs and Se revealed a significant difference ($P < 0.05$) in the mean

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body weights among each other and with the control group. The challenged birds showed mild and moderate gross and microscopic lesions in groups treated with Se-NPs and Se, respectively. In addition to stimulating the activity of innate immune system specially macrophages in group challenged with ARV and treated with Se-NPs. So, thorough genetic characterization of presently circulating ARV variants is critical for disease prevention and control planning and the usage of Se-NPs has an effective protection of poultry against ARV and any other pathogen.

INTRODUCTION

ARVs are widespread and cause major economic losses in the poultry industry (Jones, 2000), which emphasizes the significance of maintaining continuous research on their genetic features, pathogenicity, and evolution of new emerging ARV variants (**Dandár et al. 2013; Tang and Lu, 2016**). Avian reoviruses (ARVs) belong to the Orthoreovirus genus and the Spinareovirinae subfamily of the Reoviridae family. ARVs are non-enveloped viruses with a segmented double-stranded ribonucleic acid (dsRNA) genome which is comprised of based on their migration pattern on polyacrylamide gel electrophoresis, 10 distinct genomic segments were categorized as 3 large (L1, L2, L3), 3 medium (M1, M2, M3), and 4 small (S1, S2, S3, S4). (**Benavente and Martínez-Costas, 2007**). The S1 segment (length 1643 bp) is tricistronic containing three open reading frames for two non-structural p10 and p17 proteins, as well as one structural sigma σ C protein (**Bodelón et al. 2001; Shmulevitz et al. 2002**) σ C protein is a small outer capsid that has a crucial role in the early stages of infection, in viral cell attachment, and produces neutralizing antibodies specific to reovirus types (**Martínez-Costas et al. 1997; Kant et al. 2003**). Sequencing study of the region of the S1 gene that produces the σ C protein has been widely employed as a genetic marker for the characterization and categorization of circulating ARV into distinct clusters and genotypes. (**Liu et al. 2003; Goldenberg et al. 2010; Lu et al. 2015; Ayalew et al. 2017; Kovács et al. 2022; Sellers, 2022**).

ARVs are common in nature and have been linked to some diseases affecting various bird species. ARVs in meat-type chickens are asso-

ciated with up to 40% morbidity and up to 10% mortality as well as clinical symptoms such as viral arthritis-tenosynovitis, runting-stunting syndrome, pericarditis, malabsorption syndrome, hepatitis, neurological signs and immunosuppression (**Kant et al. 2003; Troxler et al. 2013**) and even concurrent infection with other microorganisms leading to several economically important implications including increase mortality, a general lack of productivity, lower weight gain, and poor feed conversion (**Van de Zande and Kuhn, 2007**).

Selenium (Se) is an essential mineral that is necessary for optimal growth and production, internal organs health, and disease prevention in birds (**Baylan et al. 2010**), It has a vital function in lowering the prevalence and severity of viral infections. (**Avery and Hoffmann, 2018**). It protects the host by regulating both oxidative stress and immune response (**Beck et al. 2004**). Antioxidant defense mechanisms protect tissues from tissue damage produced by reactive oxygen species (ROS) during infection, inflammation and stress (**Valko et al. 2007**) are subdivided into enzymatic and non-enzymatic constituents.

ARV infection was first diagnosed in Egypt in 1983 (**Tantawi et al. 1984**). It was confirmed using RT-PCR in many chicken flocks that had one or more ARV-associated symptoms. (**Kutkat et al. 2010; Ramzy et al. 2016; Mansour et al. 2018; Amer et al. 2019; Mohamed et al. 2019**). The use of molecular characterization and phylogenetic analysis to study the genesis, epidemiology, and development of new ARV strains is a successful strategy. (**Hellal Kort et al. 2013**).

Recently, (**Al-Ebshahy et al. 2020**) isolated

a strain from broilers through a partial nucleotides sequence of σA encoded protein in the S2 segment, Genomic sequence and phylogenetic analyses indicated that the isolated strain named EGY1 clustered in the S1113-like cluster of avian reovirus with the S1133 vaccinal strain. Kovács and his collaborators identified new strains of ARVs isolated from diagnostic samples collected from commercial broilers, layers, and breeders from 34 countries worldwide including Egypt. Based on the sequence of σC nucleotides and amino acids, genetic evolution revealed that the isolated strains from Egypt were dispersed in 1-5 clusters. (Kovács et al. 2022).

The current research aimed to investigate and characterize the emergent ARVs in broiler chicken flocks located in two different governorates: El-Beheira (in the north) and Qena (in the south), Egypt. In addition, the effect of SENPs on growth performance and degenerative alterations in the internal organs in broiler chickens following an ARV challenge is being investigated.

MATERIAL AND METHODS

2.1 Ethical approval:

The study protocol was reviewed and authorized by the Animal Care and Biosafety Committee of the Animal Health Research Institute (AHRI) and the College of Veterinary Medicine's Ethics Committee at South Valley University in Egypt (Certificate of approval #: VM/SVU/23(2)-20) None of the authors of this paper conducted any experiments with human subjects.

2.2 Sampling:

Twenty (n=20) field tissue samples, including tendon, joints, proventriculus, intestine, liver, spleen, and pancreas, were collected from necropsied birds showing clinical signs of ARV infection from various broiler chicken flocks in the Egyptian governorates of El-Beheira and Qena between the years of 2021 and 2022. These flocks had ARV symptoms such as stunting growth, ruffled feathers, and lameness. The collected samples were labeled with all data and stored at -80°C for further

analysis.

2.3 Egg inoculation and Virus isolation:

Tissue samples collected from suspected infected flocks with reovirus were each minced separately with a scalpel, homogenized, and diluted in Phosphate-buffered saline (PBS) to a weight/volume ratio of 1:10 with (5,000 IU /ml penicillin G, 5 $\mu\text{g}/\text{ml}$ amphotericin B, and 5 mg/ml streptomycin) (Sigma Chemical Co., St. Louis, MO). 200 μl of the clarified supernatant from each sample and fluid from joints centrifuged at 3000 rpm for 10 min at 4°C was inoculated into the yolk sac of 5-7 days old embryonated chicken eggs (ECEs) with no history of using reovirus vaccine. Each sample was inoculated into three eggs and 0.1 ml of phosphate buffer solution was inoculated into three eggs as a negative control. For seven days, the embryos were candled every day. The allantoic fluids were collected for a second passage or ARV detection using RT-PCR. At each passage, the embryos were examined for gross lesions. Also, the allantoic fluids were collected from negative control and tested for ARV.

2.4 RNA extraction, RT-PCR:

QIAmp® Viral RNA Mini Kit (Qiagen, Valencia, CA, USA) was used for the extraction of viral RNA of Twenty samples from the infected allantoic fluid according to the manufacturer's instructions. One-step RT-PCR was carried out using Superscript™ III One-step RT-PCR kit with Platinum Taq DNA polymerase (Invitrogen, Carlsbad, CA, USA) using the previously published Oligonucleotide primers P1 (Fw) 5'-AGTATTTGTGAGTACGATTG-3 and P4 (Rev) 5'GGCGCCACACCTTAGGT-3 (Kant et al. 2003) to amplify 1088 bp out of 1643 bp of the σC gene of S1 segment. The RT-PCR was carried out under the following thermal cycling conditions: 50°C for 30 min, 94°C /2 min, 40 cycles ($94^{\circ}\text{C}/15$ sec, $54^{\circ}\text{C}/30$ sec, $68^{\circ}\text{C}/90$ sec), $68^{\circ}\text{C}/5$ min resulting in amplification of 1088 bp of the S1 gene. The PCR product was examined using an ultraviolet transilluminator and gel electrophoresis on 1% agarose gel stained with SYBR Green.

2.5 Sequencing and Phylogenetic Analysis:

A commercial service provider supplied positive RT-PCR samples for sequencing. (MacroGen, Inc. South Korea) where the PCR products were purified by using EnzSAP PCR Clean-up Reagent (Edge bio, Netherlands) and then sequenced in both forward and reverse directions using the same primers. Forward and reverse sequences of ARVs were aligned together, edited, and assembled by Mega 11 (Tamura et al. 2021) and DNA Baser Sequence Assembler v5.15.0 software to obtain a σ C sequence of length 978 nucleotides. Based on the partial sequence of the σ C gene, a phylogenetic analysis was performed on the sequences acquired in this study as well as the other typical ARV sequences collected from NCBI GenBank (n=120) (768 nt and 256 aa long) to include all the previously submitted Egyptian ARV σ C sequences in the Genbank and to include other sequences representing different countries especially the area of the Middle East and European countries as most of the published sequences from these countries are of short lengths. The one hundred twenty sequences (n=120) were aligned based on the nucleotide sequence by the clustal W method using MEGA 11 software (Tamura et al. 2021) then translated into aa and realigned before protein phylogenetic analyses, With MEGA 11 software, the best-fit replacement model was chosen based on the lowest Bayesian Information Criterion. We used Jones-Taylor-Thornton (JTT) substitution model (Jones et al. 1992) using a discrete Gamma distribution (+G) with 5 rate categories. The Maximum Likelihood approach was used to create phylogenetic trees from aligned aa sequences. and tree topologies were validated by 1000 bootstrap replicate values (Felsenstein, 1985) as implemented in MEGA 11 (Tamura et al. 2021). Evolutionary distances between the sequences under consideration, the references, and vaccinal strains were inferred based on 256 aa of the σ C protein dataset and were computed using the tool BioEdit 7.0.5.3 (Hall, 1999).

2.6 Selenium sources:

Se-NPs were synthesized in accordance with (Shahabadi et al. 2021) by reduction of sodium selenite using 300 mL of vitamin C (0.1 M)

dropped with 600 mL of $\text{Na}_2\text{SeO}_3 \cdot 5\text{H}_2\text{O}$ (5 mM). By gently stirring for 24 h at room temperature. The color of this combination changed from colorless to yellowish orange with the addition of ascorbic acid, and then to reddish-orange after 24 hours. After centrifuging the product for 15 minutes at 8000 rpm, the residue was removed by washing multiple times with deionized water, and the recovered particles were vacuum-dried overnight. The pellet was suspended in sterile double distilled water for subsequent experiments. For the *in vivo* experiments, the original final concentrations were adjusted to 50 mg/L.

The sodium selenite (Se): was obtained from Sigma-Aldrich Chemical Co., St. Louis, MO, USA.

2.7 The virus was used for the Se-NPs experiment:

Egyptian reovirus isolate designated "EG/Beheira-5/22 (accession no. OP465138) isolated from broiler chickens presenting clinical signs of malabsorption syndrome and used at a dose of $10^{3.5}$ ELD₅₀/bird via oral and subcutaneous routes. This dose and route of inoculation were chosen according to (Rosenberger et al., 1989). Virus titer embryo lethal dose (ELD₅₀) was calculated in accordance with (Reed and Muench, 1938).

2.8 Experimental Bird Grouping and Design:

The effect of Se-NPs and Se in reovirus pathogenicity *in vivo* was assessed in 3-day-old commercial broiler chicks as reflected by weight gain, clinical signs, gross and pathological lesions. Healthy 1-day-old Cobb chickens (n = 50) obtained from a non-vaccinated breeder with comparable initial B (44.60 ± 1.32 gram) were used in the current study and were reared in an ecologically controlled isolation facility and allowed diets and water *ad libitum*. Birds were sub-grouped into five groups of 10 birds each as shown in (Table 1).

Table 1. Experimental bird grouping and design:

Groups	Treatment	Challenging with the ARV isolate
G1	Not treated	Control (-ve) inoculated with PBS
G2	Treated by Se-NPs	Inoculated with PBS
G3	Not treated	Control (+ve) challenged with ARV
G4	Treated by Se	Challenged with ARV
G5	Treated by Se-NPs	Challenged with ARV

The chicks in G3, G4, and G5 were challenged orally and subcutaneously with a suspension containing $1 \times 10^{3.5}$ (ELD₅₀) / 0.1ml per bird of the Egyptian isolate in this study designated “EG/Beheira-5/22” (accession no. OP465138) at the 3rd day of age. The dose of Se and Se-NPs was 0.5 mg/liter in drinking water daily until the end of the experiment (37 days). This dose was chosen according to the European Union (2004) (Ahmadi et al. 2018) recommendation to ensure feed safety.

All birds were weighed at 7, 14-, 21-, 30-, and 37 days post-infection (PI). At the end of the experiment, the obtained values of body weights, weight gain, and feed consumption per group were used to calculate the feed conversion ratio (FCR) for each group as indicators for broiler performance according to (Wagner et al. 1983). Clinical signs were recorded daily. At 7- and 30-day PI three birds were randomly humanely euthanized and attracted for necropsy evaluation and pieces from proventriculus, intestine, liver, and pancreas were collected and placed in 10% neutral buffered formalin, sectioned, stained with hematoxylin, and eosin (H&E) stain and evaluated for histopathological lesions (Layton et

al. 2019). At 7 days PI, cloacal swabs, and pieces of the proventriculus, intestine, liver, and pancreas were collected from each bird of different inoculated groups (G3-5). Swabs and tissues taken from birds within each group were pooled and processed for virus re-isolations and ARV detection using Rt-PCR.

2.9 Statistical analysis:

Statistical analyses of body weight results obtained from the experiments at different intervals, and feed conversion ratio (37days PI) were reported as means \pm SE and were assessed by Independent-Samples T-Test using the SPSS Statistics for Windows, version 23.0 (IBM Corp: Armonk, NY, U.S.A.). Differences were judged significant when (P<0.05).

RESULTS

3.1 Clinical and necropsy data:

In this study, flocks with an age ranging from 15 to 22 days (Table 2) were characterized by non-uniform growth, increased culling rates, distended abdomen, retarded and defective feathering unilateral (hockey stick), and bilateral lameness (hock sitting posture) with subcutaneous hemorrhages. At necropsy, the proventriculus was enlarged and mottled, the

pancreas appeared atrophied, and the small intestine was distended with gases and undigested food (Fig.3, D).

3.2 virus isolation and RT-PCR:

One or two passages of tissue homogenates from suspected infected flocks with ARVs into embryonated chicken eggs (ECEs) were sufficient for virus propagation. In the 1st passage the embryos showed petechial hemorrhage, while in there were the 2nd passage congestion and hemorrhage in the whole body and head, associated with submandibular edema and ne-

crotic foci in the congested liver (Fig. 1). The allantoic fluids of all inoculated samples were tested for ARV by application of RT-PCR assay that targeted the σ C sequence of the S1 gene, whereas 12 (60 %) out of the 20 allantoic fluid samples after egg inoculation showed positive results for ARV with effective amplification of 1,088 bp. Meanwhile, the allantoic fluid sample collected from the negative control was free from ARV.

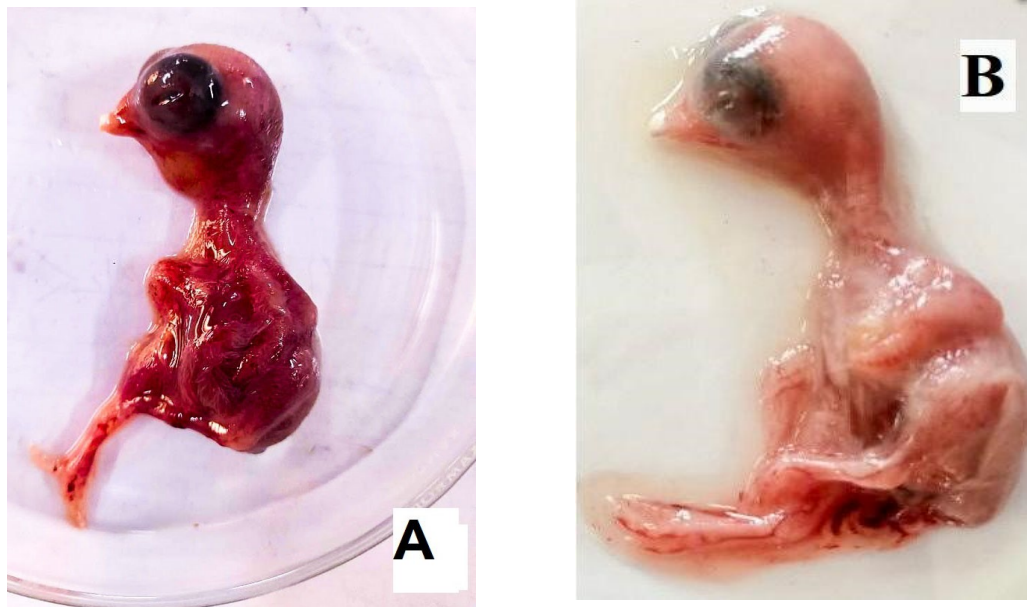


Fig 1. Effect of ARV infection in ECEs. (A) Inoculated chicken embryos after the 2nd passage showing diffuse congestion and hemorrhage in the whole body with submandibular edema. (B) Normal-sized embryo inoculated with sterile saline.

3.3 Phylogenetic analysis and sequence alignment of the σ C gene:

Seven samples (n=7) out of twelve (n=12) positive samples with RT-PCR were sent for sequencing of the σ C gene. The acquired sequences were aligned and then utilized to build a phylogenetic tree using 256 deduced aa sequences from the σ C-encoding gene, including 120 sequences representing the different genetic clusters (GCs) 1 to 5, retrieved from the GenBank. The topology of the partial σ C

gene phylogenetic tree indicated that the seven Egyptian ARV isolates isolated from broiler chickens during 2021–2022 clustered together in the GC 4 with other chicken isolates from Germany, Egypt, Netherlands, Canada, Switzerland, Hungary (Fig 2). These seven isolates were found to be related among themselves with (82.8 % - 99.6 %) aa sequence identity, (Table 3) and sub-clustered in a single monophyletic clade (Fig. 2). The two sequences isolated from Qena Governorate are more divergent than the remaining five isolates from El-

Beheira Governorate with genetic aa sequence identity ranged from (82.8%- 89.8%). Furthermore, The σ C gene homology of the seven isolated strains in GC 4 in comparison to Egyptian strains D2572/3/2/14EG VAR1 and D2095/1/4/12EG was 76.6 %- 86.3 % and 54.7 %- 63.3%, respectively (Kovács et al. 2022). The aa sequence identity between the Egyptian

isolates and vaccinal strains, including, 2408, S1133, and 1733 was ranging from (39.5%- 45.7%) (Table 3). The nucleotide sequences obtained in this study were assigned in the NCBI GenBank under accession numbers OP465133- OP465139 (Table 2)

Table 2. Details of ARV isolates selected for genetic characterization in this study:

No	ID	Locality	Collection date	Sample	Chicken breed	Type	Accession No.
1	EG/Qena-Q1/21	Qena. Abu Tisht	Aug. 2021	Indian River		Tissues	OP465133
2	EG/Qena-Q2/21	Qena, Nag Hammadi	Sept. 2021	Indian River		Tissues	OP465134
3	EG/Beheira-B1/21	Beheira, Kom Hamada	Mar. 2021	Arber-Acres		Tissues	OP465135
4	EG/Beheira-B2/21	Beheira, Abu El Matai	Jul. 2021	Cobb		Tissues	OP465136
5	EG/Beheira-B3/21	Beheira, Hosh Issa	Sept. 2021	Ross		Tissues	OP465137
6	EG/Beheira-B5/22	Beheira, Abu Homs	Feb.2022	Cobb		Tissues	OP465138
7	EG/Beheira-B11/22	Beheira, Rashid	Apr. 2022	Hubbard		Tissues	OP465139

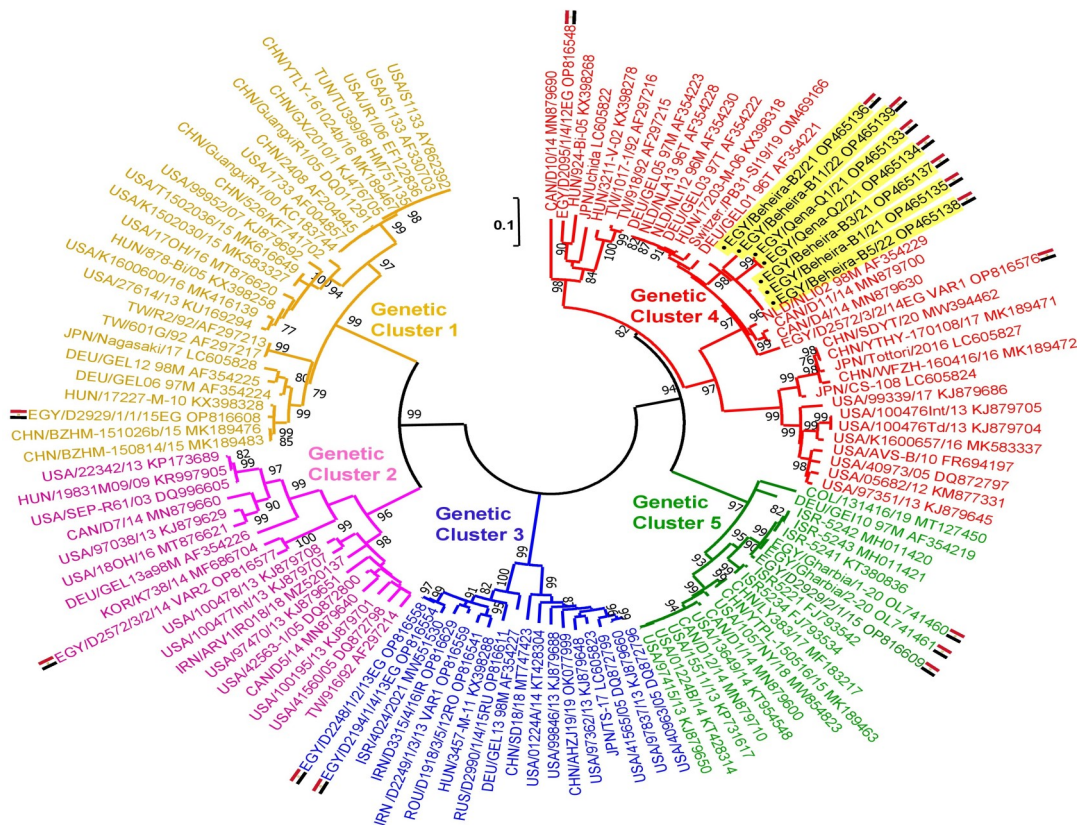


Fig 2. Phylogenetic tree shows the five GCs of ARV isolates. The analysis was based on 256 aa sequences of the σ C protein encoded in the S1 gene of ARVs. The phylogenetic tree is constructed using the Maximum Likelihood method for 120 ARVs aa sequences. Branch lengths are proportional to the evolutionary distances between aa sequences. The scale bar represents amino acid substitutions per site. Colors are used to differentiate between GCs. The Egyptian ARVs are marked with the Egyptian flag. Our isolates are highlighted with yellow color and a black circular symbol.

Table 3. Amino acid percent identity (upper right) and distance (lower left) of σC protein (256 aa) of selected ARV isolates representing different 5 GCs. The isolates in the study with bold font and marked with an asterisk.

Isolate name	Cluster	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	
S1133 (Vaccine)	(GC1)	1	98	97.7	73	51.2	49.6	46.9	47.3	45.3	45.3	45.7	45.7	43	39.5	39.5	45.7	45.3	43	43.4	42.6	1
1733 (Vaccine)	(GC1)	2	2	99.6	74.2	52	50.4	47.3	47.6	45.3	45.3	45.7	45.7	43	39.5	39.5	45.7	45.7	43.7	44.1	43.4	2
2408 (Vaccine)	(GC1)	3	2.3	0.4	74.2	52	50.4	47.3	47.6	45.3	45.3	45.7	45.7	43	39.5	39.5	45.7	45.7	43.7	44.1	43.4	3
EG/D2929	(GC1)	4	27	25.8	25.8	50.4	50	44.1	44.1	45.7	45.7	46.1	46.1	44.5	40.2	40.2	45.3	46.9	46.9	47.3	46.4	4
EG/D2572 VAR2	(GC2)	5	48.8	48	48	49.6	89.1	44.9	45.3	49.2	49.2	48.4	49.6	46.9	44.1	43.7	49.6	46.5	48.8	49.2	48.4	5
KOR/K738	(GC2)	6	50.4	49.6	49.6	50	10.9	43.4	43.7	48.4	48.4	48.4	48.8	46.9	43	42.6	49.2	44.9	46.5	46.9	46.1	6
EG/D2194	(GC3)	7	53.1	52.7	52.7	55.9	55.1	56.6	98.4	52.7	52.7	52.3	53.1	50	46.9	47.3	52	51.6	46.1	46.9	46.5	7
EG/D2248	(GC3)	8	52.7	52.3	52.3	55.9	54.7	56.3	1.6	53.1	53.1	52.7	53.5	50.4	47.3	47.7	52.3	52	46.5	47.3	47.3	8
EG/Beheira-B1/21*	(GC4)	9	54.7	54.7	54.7	54.3	50.8	51.6	47.3	46.9	98.4	97.7	99.6	92.2	88.7	89.5	85.9	62.9	54.7	55.5	55.1	9
EG/Beheira-B2/21*	(GC4)	10	54.7	54.7	54.7	54.3	50.8	51.6	47.3	46.9	1.6	96.9	98.8	91.8	87.9	88.7	86.3	62.9	54.7	55.4	55.1	10
EG/Beheira-B3/21*	(GC4)	11	54.3	54.3	54.3	53.9	51.6	51.6	47.7	47.3	2.3	3.1	98	91.8	87.1	87.9	85.2	62.9	54.3	55.1	54.7	11
EG/Beheira-B5/22*	(GC4)	12	54.3	54.3	54.3	53.9	50.4	51.2	46.9	46.5	0.4	1.2	2	92.6	89.1	89.8	86.3	63.3	55.1	55.9	55.5	12
EG/Beheira-B11/22*	(GC4)	13	57	57	57	55.5	53.1	53.1	50	49.6	7.8	8.2	8.2	7.4	82.8	83.6	80.1	60.9	53.1	53.9	54.3	13
EG/Qena-Q1/21*	(GC4)	14	60.5	60.5	60.5	59.8	55.9	57	53.1	52.7	11.3	12.1	12.9	10.9	17.2	98	76.6	55.1	50	50.8	50.4	14
EG/Qena-Q2/21*	(GC4)	15	60.5	60.5	60.5	59.8	56.3	57.4	52.7	52.3	10.5	11.3	12.1	10.2	16.4	2	77	54.7	50.4	51.2	50.8	15
EG/D2572 VAR1	(GC4)	16	54.3	54.3	54.3	54.7	50.4	50.8	48	47.7	14.1	13.7	14.8	13.7	19.1	23.4	23	60.5	55.5	56.2	55.9	16
EG/D2095/1/4/12	(GC4)	17	54.7	54.3	54.3	53.1	53.5	55.1	48.4	48	37.1	37.1	37.1	36.7	39.1	44.9	45.3	39.5	41.8	41	42.2	20
EG/Gharbia/1-20	(GC5)	18	57	56.3	56.3	53.1	51.2	53.5	53.9	53.5	45.3	45.3	45.7	44.9	46.9	50	49.6	44.5	58.2	99.2	95.7	17
EG/Gharbia/2-20	(GC5)	19	56.6	55.9	55.9	52.7	50.8	53.1	53.1	52.7	44.5	44.5	44.9	44.1	46.1	49.2	48.8	43.8	59	0.8	96.5	18
EG/D2929/2/1/15	(GC5)	20	57.4	56.6	56.6	53.5	51.6	53.9	53.5	52.7	44.9	44.9	45.3	44.5	45.7	49.6	49.2	44.1	57.8	4.3	3.5	19
			1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20

3.4 Clinical Signs and gross lesions after experimental challenge:

Clinically after infection with the ARV strain, the infected groups showed depression, ruffled feathers, increased water intake, reduced appetite, diarrhea with undigested food, and a decrease in body weight (Fig. 3. B) comparing with the control G1 (Fig. 3. A). The clinical signs comparing between the different groups were listed in (Table 4). Concerning the statistical analysis of body weight (Table 5) revealed a significant difference ($P < 0.05$) in the mean body weights of the three infected groups (G3, G4, and G5) compared to the control group G1. Birds in G2 showed no significant

difference in body weight when compared with G1. In addition to the decreased weight, a large variation in individual body weights was seen in the birds of G3 followed by G4. Also, the statistical analysis of FCR shown in (Table 5) revealed a significant difference ($P < 0.05$) in FCR occurred among infected groups (G3, G4, and G5) compared with control group G1 at 37 days PI. Birds in G2 showed no significant difference in FCR when compared with G1. The main gross lesions were an enlarged proventriculus (Fig. 3. C) and a distention of the intestine which was filled with gases and undigested food (Fig. 3. D).

Table 4. Clinical score lesions:

Clinical lesions	G1	G2	G3	G4	G5
Decrease in body weight	-	-	+++	++	+
Depression	-	-	+++	++	+
Ruffled feather	-	-	+++	++	+
Reduced appetite	-	-	+++	++	+
Diarrhea with undigested food	-	-	+++	++	+

+++ severe, ++ moderate, + mild, - no lesion

Table 5. Body weight in grams for each group at x intervals and feed conversion ratio at 37 dpi:

Groups	7 days PI	14 days PI	21 days PI	30 days PI	37 days PI	FCR at37dpi
G1	255.00±3.28 ^a	595.00±15.16 ^a	950.20±13.52 ^a	1758.00±31.36 ^a	2215.00±103.94 ^a	1.83±0.08 ^d
G2	261.60±2.46 ^a	617.50±9.68 ^a	1020.00±34.20 ^a	1836.00±40.10 ^a	2383.00±69.16 ^a	1.80±0.06 ^d
G3	217.20±2.99 ^d	348.25±43.87 ^d	470.00±55.45 ^d	1089.00±138.82 ^d	1368.00±195.34 ^d	2.57±0.16 ^a
G4	236.20±1.77 ^c	433.00±32.47 ^c	543.00±36.41 ^c	1261.00±122.56 ^c	1500.00±135.07 ^c	2.3±0.14 ^b
G5	244.20±1.46 ^b	510.40±12.90 ^b	748.75±26.72 ^b	1463.00±106.43 ^b	1772.00±98.92 ^b	2.0±0.11 ^c

Means within the same column under the same category carry different superscripts significantly different ($P<0.05$).

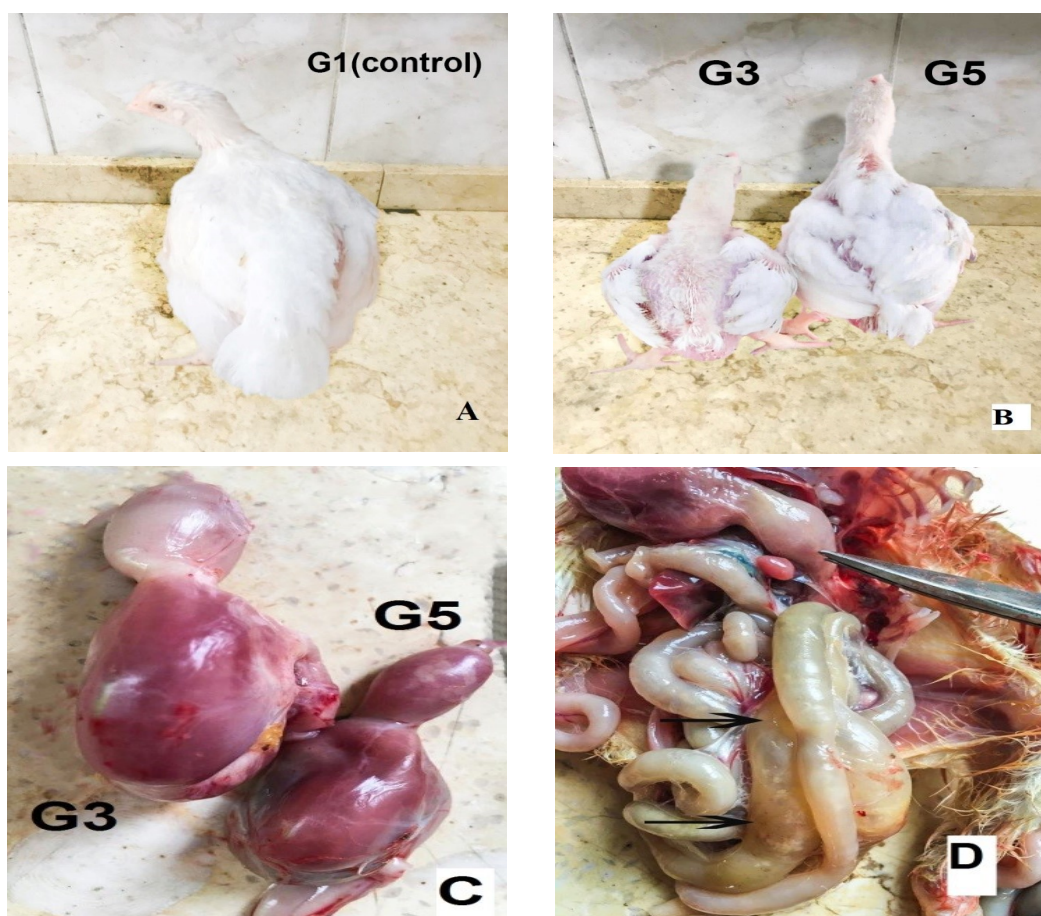


Fig 3. (A) Showing normal body weight of control group G1 (B) Comparing between body weight between G5 and G3. (C) showing large size proventriculus of G3 to that of G5. (D) Showing gas intestinal distention (arrow) and the proventriculus was enlarged and mottled.

3.5 Histopathological examination

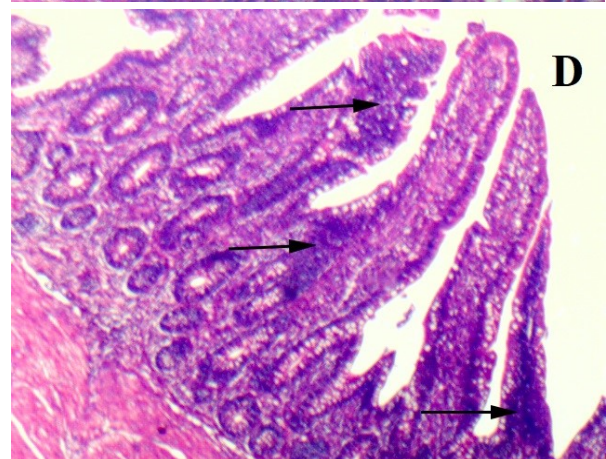
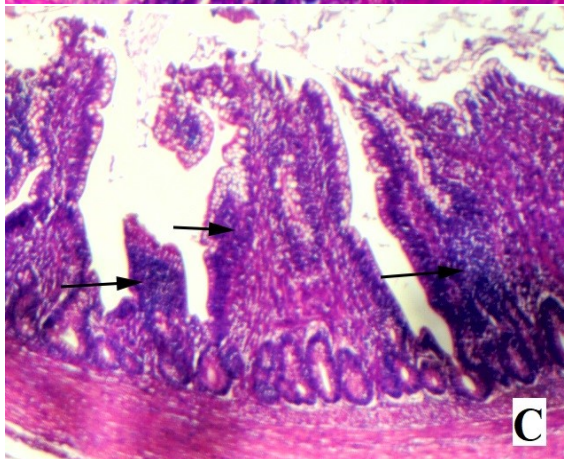
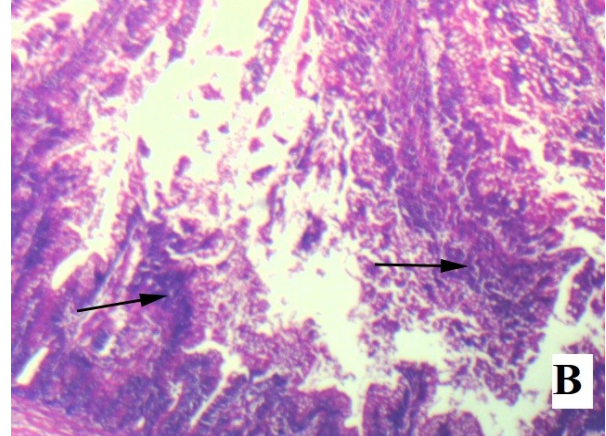
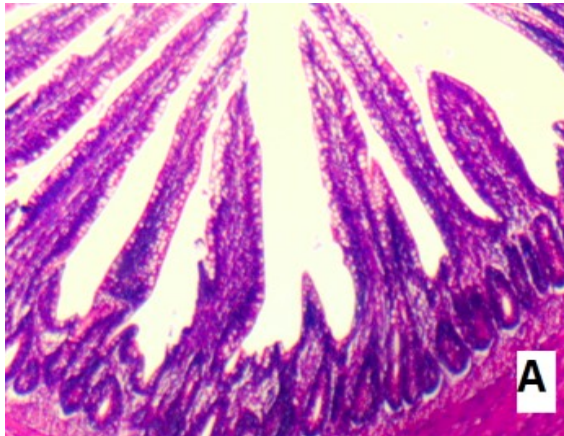
As shown in (table 6), All samples collected from G1 and G2 revealed no lesions at day 7 and 30 PI. Lesions were more extensive at day 7 PI than at day 30 PI in G3, G4, and G5. The most pronounced lesions were seen on the intestine; which revealed necrosis accompanied by round cell infiltration, atrophy, and sloughing of the villus epithelium (Fig. 4: B, C, D). The pancreas showed degeneration and vacuolation of acinar cells (Fig. 4: F) while, the proventriculus showed dilation of glandular acini, necrosis, and sloughing of the lining epitheli-

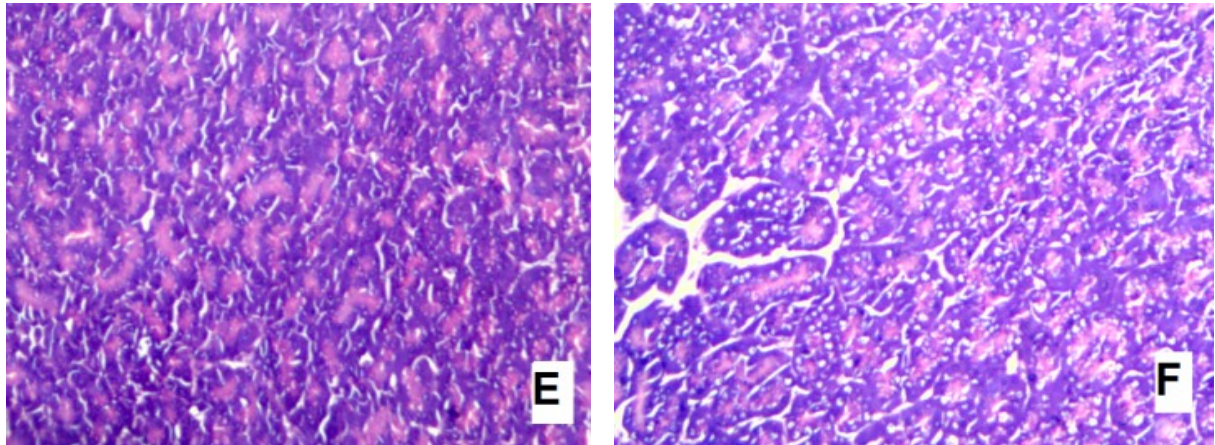
um with excessive infiltration with round cells (Fig. 5: B), there were focal collection of macrophages, epithelioid cells, lymphocyte and plasma cells (granulomatous reaction) in G5 appear at day 7 PI (Fig. 5: D), but delayed to appear to the day 30 PI in G4 (Fig. 5: C). The histopathological examination of liver revealed vacuolar degeneration and necrosis, the infiltrating inflammatory cells was round cells (Fig. 6: C, D)

Table 6. Histopathological score lesions of different groups:

Organ	Lesion	G 3	G 4	G 5
Intestine	Degeneration and necrosis of the villus epithelium associated with round cells infiltration	+++	++	+
	Atrophy and sloughing of the villus epithelium	+++	+	-
Pancreas	Degeneration and vacuolation of acinar cells	++	-	-
	necrosis, and destruction of acinar epithelium	+++	++	+
Proventriculus	granulomatous reaction	-	+	++
Liver	hepatocellular vacuolation accompanied by round cells infiltration	++	+	+

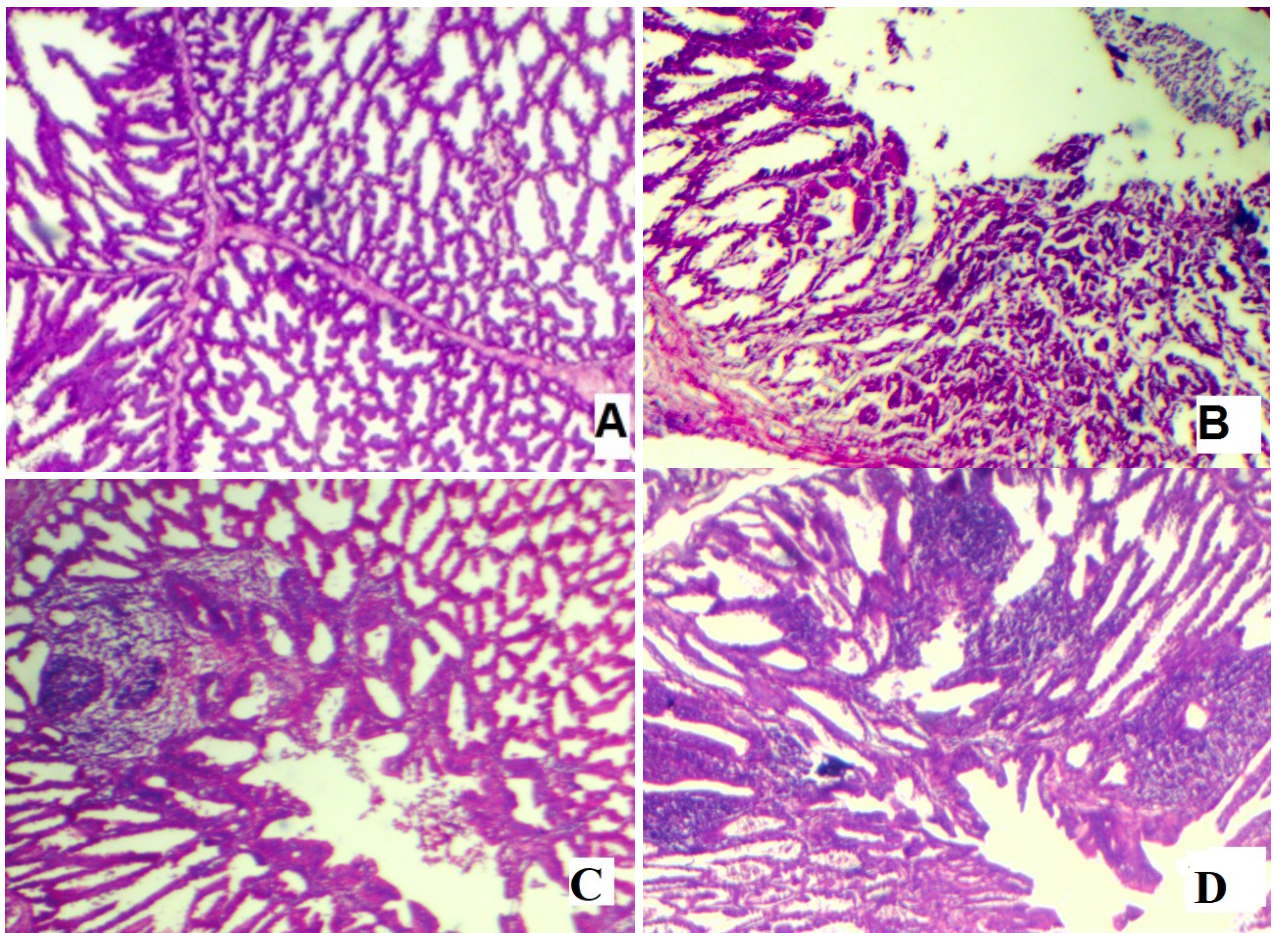
+++ severe, ++ moderate, + mild, - no lesion





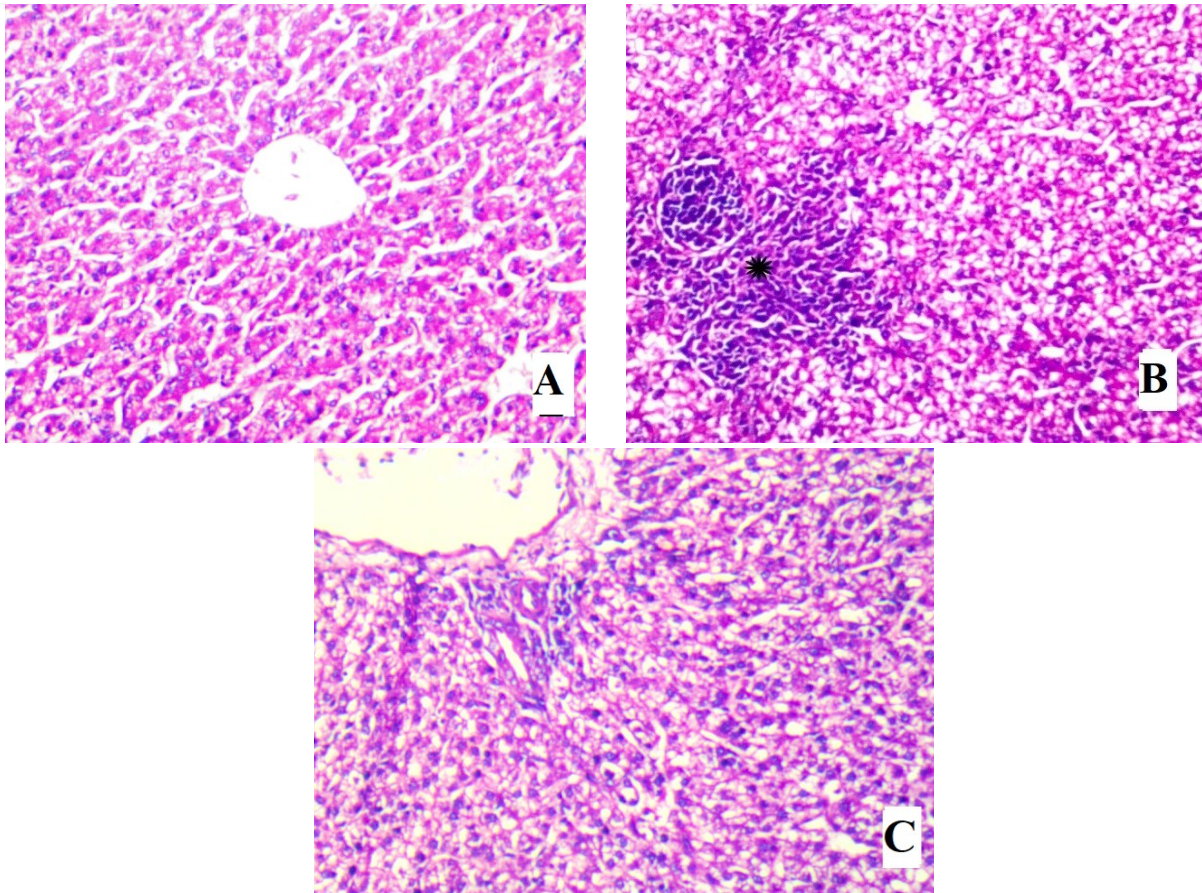
A: (G1&G2) – B: (G3) – C: (G4) – D: (G5) – E: (G1, G 2, G4&G5) – F: (G3)

Fig. 4: Intestine (A) showed normal intestinal villi (B) showed variable degrees of intestinal necrosis, atrophy, and sloughing of the villus epithelium especially upper parts with round cells infiltration (arrow) (C) showed moderate necrosis and infiltration of round cells (arrow) (D) showed mild necrosis and infiltration of round cells (arrow) (H&E stain x100). **Pancreas (E)** showing normal acinar cells (F) showed vacuolation of acinar cells (H&E stain x200).



A: (G1&G2) – B: (G3) – C: (G4) – D:(G5)

Fig.5: Proventriculus: (A) showing normal glandular epithelium, (B) showing necrosis, and destruction of the acinar epithelium. (C) showing focal collection of macrophages, epithelioid cells, lymphocyte and plasma cells (granulomatous reaction). (D) showing excessive focal collection of macrophages, epithelioid cells, lymphocyte and plasma cells (granulomatous reaction) (H&E stain x100)



A: (G1&G2) – B: (G3) – C: (G4, G5)

Fig.6: Liver (A) showing normal hepatic parenchyma (B) showing hepatocellular vacuolation accompanied by round cells infiltration (asterisk) (C) showing mild vacuolar degeneration and necrosis (H&E stain x200).

DISCUSSION

Our studies showed that the propagation of samples \in ECEs revealed hemorrhage with submandibular and subcutaneous edema in embryos as well as liver congestion, these lesions were similar to that observed by (Styś-Fijoł et al. 2017; Mansour et al. 2018; Al-Baroodi, 2020; Pitcovski and Goyal, 2020).

Our analysis for phylogenetic of the σ C protein showed that the seven Egyptian isolated strains of this study were clustered within GC4 with other Egyptian strains D2572/3/2/14EG VAR1 and D2095/1/4/12EG and these were reported previously by (Kovács et al. 2022). The aa sequence homology with these two strains was 76.6 %- 86.3 % and 54.7 %- 63.3%, respectively. The seven isolated strains in this study had aa identity ranging from (39.5%-45.7%) with the viruses that are used in commercial live and inactivated vaccines

(S1133, 1733 and 2408). This very far distant between our isolates and the commercial vaccinal strains might be explaining the lack of effectiveness of these vaccines in protecting commercial broilers. Due to the genetic and antigenic variety of the field strains and the development of novel ARV variations. These novel variant strains are capable of evading the immune responses induced by commercially available vaccines as mentioned by (Troxler et al. 2013; Lu et al. 2015; Ayalew et al. 2017; Chen et al. 2019; Mirbagheri et al. 2020).

In our experimental trial to evaluate the effect Se-NPs on the growth performance after ARV challenge, our studies revealed that there was a significant difference ($P < 0.05$) in the mean body weights of the three infected groups with ARV (G3, G4, and G5) compared to the control group (G1) because ARV is one of the most causes of stunting syndrome in the

broiler, these results are consistent with (Awandkar et al. 2017; Mansour et al. 2019). Birds in G2 showed no significant difference in body weight when compared with control (G1) when kept under normal conditions, and these results are agreeing with (Ahmadi et al. 2018) while (Liu et al. 2015) found no beneficial effect of Se-NPs on the growth performance of broilers.

According to the results of our experimental studies, the ARV targets the digestive system including the distal parts of the small intestine and the lesions were more extensive in day 7 PI than day 30 PI, the same changes were reported by (Tang et al. 1987), who explain that on day 20 PI the inflammatory reaction had been regressed.

Also, showed that there were more improvements in G4 and G5 than G3 due to the effect of Se and Se-NPs in reovirus pathogenicity leading to improvement in the growth performance and internal organs health after ARV challenge and that were consistent with studies after heat stress as reported by (El-Deep et al. 2016; Safdari-Rostamabad et al.,2017) and after challenge with *Staphylococcus aureus* bacteria in rabbits reported by (Ghfaar et al. 2020) but there are No available previous literature data on using Se-NPs with ARV challenge.

These results were due to the significant effect of Se on body health during infection or any stress factor as it integrated into selenocysteine, which is an essential element of enzymatic antioxidant defense systems since it is a precursor of the enzyme glutathione peroxidase and thioredoxin reductase activities (Labunskyy et al. 2014), They act as antioxidants and protect against free radicals (hydrogen peroxide and lipid peroxides) produced by the immune system in response to viral infection (Baylan et al. 2010; Rayman, 2012).

Selenium Nanoparticles (Se-NPs) have gained considerable attention because traditional Se supplements often had limited absorption and high toxicity. (Hosnedlova et al.

2018), While Nanoparticles are smaller in size, have a larger surface area, higher surface activity, enhanced intestine absorption and tissue deposition, greater mucosal permeability, and are less toxic than Se. (Wang et al. 2007; Zhang et al. 2008). So, Se-NPs are more effective than Se in improving the antioxidant defense systems and the health of internal organs after infection or stress factors (Radwan et al. 2015).

Microscopically, our studies revealed that infected G3 showed variable degrees of intestinal epithelial necrosis, villus atrophy, and sloughing of villus epithelium. The pancreas showed vacuolation of acinar epithelium similar changes were reported by (Davis et al. 2013; Abd El-Samie, 2015; Awandkar et al. 2017).

The proventriculus and as shown in our studies revealed dilatation and or necrosis of glandular epithelium and associated with round cell infiltration, the same results were obtained by (Abd El-Samie, 2015; Mansour et al. 2019), as mentioned by Rebel et al. 2006, these histopathological changes may explain the uneven patterns of growth, weight loss in the groups infected by the virus due to impairment of enzymatic digestion of pancreas and proventriculus and absorption of nutrients from the intestinal tract. These previous changes were less in severity in G4 and G5 and these may be attributed to that Se and Se-NPs decrease the severity of viral infections (Avery and Hoffmann, 2018) and protect the host by decreasing the oxidative stress, improving the health of internal organs and jejunal morphology (Safdari-Rostamabad et al. 2017).

Our histopathological examination of the proventriculus showed focal granulomatous reaction on G5 specially at day 7 PI and these changes were less in severity on G4 specially at day 30 PI. As reported by (Dietert et al.,1990) who owes these changes to increase the phagocytic activities of macrophages which is stimulated by Se. In our opinion, the beneficial effect of Se-NPs on innate immune system by increasing granulomatous reaction earlier than the usage of Se. these were sup-

ported by (Lee et al. 2013 and Roman et al. 2014) they declared that, as Se was found in the structure of lymphocytes, granulocytes, monocyte and macrophages, and also, selenocysteine is involved in cellular activation and differentiation hence being of importance for innate and adaptive immune responses.

CONCLUSION

Our results concluded, detection of seven new variants of ARV in two governorates in Egypt (two strains from Qena governorate and five strains from El-Beheira governorate) which are completely different from the vaccinal strains used. Se-NPs is more effective than Se in improving the antioxidant defense systems and the health of internal organs after ARV infection or stress factors.

Recommendation:

We recommend to use native isolate ARV variant in Egypt for preparation of effective vaccines against ARV.

More studies are needed for monitoring other areas in Egypt to obtain more data about other ARV strains in these areas, and this is very important for preparation of effective vaccines against ARV.

For effective protection of poultry against ARV and any other pathogen We should add Se-NPs to the drinking water.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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