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Isolation and Genotypic Characterization of New Emerging Avian Reovirus Genetic Variants in Egypt

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Abstract: Avian reovirus (ARV) strains cause a variety of symptoms in chickens, including viral arthritis/tenosynovitis, a disease that has emerged as a significant cause of economic losses in commercial chicken flocks in recent years in various countries, including Egypt. Furthermore, ARV strains are frequently isolated from birds suffering from malabsorption. In the actual study, seventy-five samples were collected in 2021 and 2022 from broiler and vaccinated broiler breeder flocks at different farms in Giza Province, Egypt, with reovirus-like symptoms such as significant weight fluctuation and arthritis/malabsorption. ARV was screened using real-time PCR, and fifteen positive samples were detected (20%), which were then subjected to embryonated chicken egg (ECE) isolation and molecular characterization (11/15 sample) of a partial segment of the sigma (σ)C gene (S1-gene). Phylogenetically, nine strains were found to belong to genotypic cluster IV, with 82–89% identity with Israeli ARV 2018, and two strains belong to genotypic cluster V with a 78% nucleotide identity with Japan ARV 2021. No correlation between lesions and genotype was found. The strains under study had a low sequence identity (43–55%) when compared with various commercial vaccines belonging to genotypic cluster I (e.g., strain S1133). These findings imply that novel ARV genotypes representing clusters IV and V have recently been introduced to Egyptian poultry farms. A homologous vaccine is suggested; because this variation raises the possibility that commercial vaccines may not offer protection against circulating ARVs.

Keywords: avian reovirus; broiler and broiler breeder; sigma (σ)C; virus real-time PCR; phylogenetic analysis

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1. Introduction

Reovirus infections are common in chickens, and it is assumed that nearly all commercial chicken flocks will get infected at some time during their lives [1]. It is estimated that 85–90% of isolated reoviruses are nonpathogenic [2]. Although the virus is not always the cause of a disease, pathogenic viral strains do exist and have been connected to a number of disease syndromes. Viral arthritis/tenosynovitis is an exception to this rule. Reoviruses have also been connected with malnutrition and stunting, but intensified research disconfirms this [1]. Avian reovirus (ARV) is a member of the *Reoviridae* family, *Spinareovirinae* subfamily, genus *Orthoreovirus*. Virus particles are non-enveloped and have icosahedral symmetry and double-stranded RNA (dRNA) separated into 10 segments [3]. Large (L1, L2, and L3); medium (M1, M2, and M3); and Small (S1, S2, S3, and S4) genomic segments

reflect the three size classes [4]. Furthermore, the proteins encoded by genome segments are divided into three categories: lambda (λ), mu (m), and sigma (σ). The genomic segments together encode at least eight structural proteins (σA, σB, σC, μA, μB, λA, λB, and λC) and four non-structural proteins (σNS, μNS, p10, and p17). Due to its great heterogeneity, the σA-encoding gene of the S1 segment was chosen as a target to detect and discriminate a wide spectrum of reoviruses from chickens, ducks, and geese [5–7]. Furthermore, molecular and phylogenetic studies are sufficient for understanding the origin, epidemiology, and evolution of novel ARV strains. The S1 region encodes the immunologically dominant structural sigma C protein (σC) [5]. Since the sigma C protein is essential in cell attachment, it is vulnerable to antibody neutralization and a key component to induce cellular death in vitro [6,7]. The current researches on the variety of ARV isolates associated with disease outbreaks has focused on the genetic characterization of a portion of the S1 gene. Due to the high discrimination power of σC, the S1 gene has become the basis of ARV genotyping [8]. Initially, Kant et al. (2003) [9], described five genotypes, a scheme recently refined with the suggestion of six genotypes [10]. Numerous categorization strategies based on serotyping [11], partial S1 genotyping using roman numerals [12], and letters are in use [13]. A variety of clinical issues can arise from ARV infections, predominantly in meat-type chickens, due to several diseases, such as viral arthritis/tenosynovitis and runting-stunting syndrome (RSS) [1,14–17].

Arthritis/tenosynovitis appears mainly after 14 to 47 days of age in broilers and broiler breeder chickens, with clinical reports of lameness related to anterior or lateral limb deviation, stunting and a lack of homogeneity being recorded [18].These disease symptoms cause economic losses of up to 10% because of compromised feed conversion ratios and increased carcass condemnation [14]. There are commonly available live attenuated and inactivated vaccinations to prevent ARV infections and diseases [19–21]. They are frequently applied and considered safe, although the negative impact of live vaccination is reported [22].The attenuated ARV vaccine strain is grown in embryonated eggs and administered during rearing as a live vaccine, followed by oil-based inactivated vaccination [23,24]. Based on this scheme, viral arthritis/tenosynovitis in broilers is frequently prevented based on antibodies passing from breeders to the progeny following vaccination of the hen [24]; however, the full benefit is only apparent when the progeny is challenged with a homologous serotype [23,25]. Classical vaccine strains used for commercial flock vaccination, especially S1133, 1733, and 2408, have not been altered since the 1970s [18], despite the appearance of new strains in the field. RNA viruses are particularly subject to mutation/recombination, leading to strain variations with consequences on protection by antibodies generated by traditional vaccine strains, underlining the need for rapid diagnosis, typing, and updated homologous vaccine formulation [9,26].

The first stage of reovirus disease control and prevention is to define the strains causing disease in the field and the selection of the correct strain for autogenous or homologous vaccine formulation [18]. In Egypt, avian reoviruses were first detected in 1983 [27]. Afterwards, a high prevalence of ARV infections were observed in many governorates using a fluorescent antibody technique and an agar gel precipitation test [28]. Few studies have been published since then on Egyptian avian reoviruses [29–33]. RT-PCR was also utilized to identify ARV in broiler flocks with proventriculitis, tenosynovitis, and malabsorption syndrome [34–36]. Despite the significant occurrence, there is a lack of knowledge regarding the genetic profiles of ARVs in Egypt. According to the recent phylogenetic study on the σA-gene, the circulating strains of ARV that expanded in Egypt until 2020 belong to the S1113-like (GC1) of ARVs [36].The purpose of this study was to identify the molecular characteristics of the currently circulating ARVs isolated from broiler and broiler breeder chickens in Egypt (2021–2022), as well as to investigate the phylogenetic relationships between various ARV clusters and different ARV vaccine strains commonly used in Egypt based on S1 gene sequences (σC).

2. Materials and Methods

2.1. Case History and Sample Collection

Between May 2021 and July 2022, commercial broiler and broiler breeder flocks housed on different farms, with chickens of different ages in the Giza governorate, were sampled. The broiler chickens were derived from vaccinated breeders that were immunized against ARV with a live vaccine (S1133), representing cluster I (GCI), at two weeks of age subcutaneously (S.C), boostered by killed oil-based inactivated vaccine (S1133–GCI) at 7 weeks of age, followed by additional applications of TRI-REO® (Zoetis, Parsippany, NJ, USA) intramuscularly (I.M) at 12 and 19 weeks of age (one live and 3 inactivated vaccine doses). The sampled seventy-five flocks displayed clinical symptoms such as growth retardation/arthritis (40%) and increased mortality of 5–7% on average (Table 1). The postmortem examination revealed synovial membranes with excess mucus of clear fluid in the capsule, petechial synovial membranes with mild articular cartilage erosions, and intestinal distension from gas and poorly digested meals. The most likely diagnosis was viral arthritis/tenosynovitis. A total of 75 samples from different flocks located in different farms, including the trachea, lung, liver, pancreas, intestine, spleen, hock articular cartilage, synovial membrane and intestinal contents, were collected from sick birds, each sample representing a single flock. The samples were stored at −80 °C in 50% buffered glycerin until required [37].

Table 1. Clinical findings connected with avian reovirus in broiler chickens, Giza Province, Egypt, during 2021–2022.

	Isolates	Age/Day	Year	Mortality [a]	Clinical Symptoms	Type of Samples	Genotype [b]	Accession Number
1	Reo/Egypt/Broiler/GIZA-1-2021	14	2021	4%	Irregular wing feather development	Trachea-intestine-synovial membrane	Cluster IV	OP609778
2	Reo/Egypt/Broiler/GIZA-2-2021	17		5%	Splay legs			OP609779
3	Reo/Egypt/Broiler/GIZA-3-2021	20		6%	substantial weight variability			OP609780
4	Reo/Egypt/Broiler/GIZA-4-2021	34		6%	substantial weight variability	Hock articular cartilage-intestine		OP609781
5	Reo/Egypt/Broiler/GIZA-5-2022	22	2022	7%	Splay legs	Trachea-intestine-synovial membrane		OP609782
6	Reo/Egypt/Broiler/GIZA-6-2022	21		7%	Swollen hocks			OP609783
7	Reo/Egypt/Broiler/GIZA-7-2022	21		5%	Swollen hocks			OP609784
8	Reo/Egypt/Broiler/GIZA-8-2022	14		6%	Splay legs	Hock articular cartilage		OP609785
9	Reo/Egypt/Broiler/GIZA-9-2022	35		5%	substantial weight variability	Intestine		OP609786
10	Reo/Egypt/Broiler/D6366/2/23/2022	25		5%	substantial weight variability+ Splay legs	Lung	Cluster V	OP609787
11	Reo/Egypt/Broiler/D6366/2/15/2022	25		6%		Trachea		OP609788

[a] Cumulative mortality for 14 days from onset of the disease. [b] Based upon the sequence of the sigma (σ)C gene (S1-gene), according to [12].

2.2. Sample Preparation

Using a sterile pestle and mortar, the frozen field samples were thawed and macerated into a 10–20 (*w*/*v*) solution in sterile PBS. To guarantee clarity, the suspension was centrifuged for 30 min at 3000 rpm. Supernatant fluid was treated with broad-spectrum antibiotics and antifungals (gentamycin 50 g/mL, penicillin 2000 units/mL, streptomycin 2 mg/mL, and mycostatin 1000 units/mL) for an hour at room temperature. Blood agar was used to test the sterility of the inoculum [38].

2.3. RNA Extraction and Screening of ARV

Total RNA was extracted directly from the supernatant using the MagMAX™ CORE Nucleic Acid Purification Kit (Applied Biosystems, Thermo Fisher, Foster City, CA, USA) following the manufacturer's instructions. The RT (real-time) PCR closed kit (Kylt® Avian

Reo virus RT-qPCR, AniCon-Germany) was used to test ARV in 75 samples. Subsequently, Kylt® Avian Reovirus S1133 DIVA (AniCon-Germany) was used to determine if the current strain belongs to genotype cluster I (vaccine strain), depending on the manufacturer's instructions. The reaction was carried out using a thermal cycler from the Applied Biosystems (Thermo Fisher, USA) QuantStudio 5 Real-Time PCR System.

2.4. Virus Isolation

The supernatant of positive RT (real-time) PCR samples was filtered through a sterile syringe membrane filter with a 0.45 µm pore size. Afterwards, 200 µL of the filtrate were administered via the yolk sac method to 5–7-day-old embryonated chicken eggs (ECE). The infected ECEs were then kept for up to 10 days at 37 °C with daily candling, deaths within the first 24 h was considered non-specific. At 6 days after infection, embryos or CAMs were examined for abnormalities described as characteristic for reovirus infection [39]. ECEs classified as positive at 14–16 days after inoculation displayed pathognomonic ARV lesions [1]. After being isolated from particular pathogen-free embryonated chicken eggs (ECE), the yolk fluid was extracted and utilized for RT-PCR [9].

2.5. Nucleotide Sequencing of the Segment 1 Gene (σC)

Positive samples of low Ct value (<29) by RT (real-time) PCR were genetically characterized based on partial S1 gene sequences (σC). For this, the AgPath-ID™ One-Step RT-PCR kit (Applied Biosystems, Foster City, CA, USA) was used to perform the reverse transcriptase-polymerase chain reaction on the obtained RNA, using specific primers targeting the gene encoding the σC gene [9]. Positive PCR fragments of about 790 bp were purified using the QIAquick Gel Extraction Kit (QIAGEN, LLC- Germantown, MD, USA) in accordance with the manufacturer's instructions. With the use of the Big Dye Terminator V3.1 cycle sequencing kit (Perkin-Elmer, Foster City, CA, USA), purified PCR products were sequenced.

Sequence purification was done using a Centrisep® kit spins column (Applied Biosystems, Foster City, CA, USA) following the manufacturer's instructions. Using the 3500XL genetic analyzer, we acquired the sequence chromatograms (Applied Bio-Systems, Foster City, CA, USA). To verify the sequence identity to GenBank published ARV, a BLAST® search (http://www.ncbi.nlm.nih.gov/blast accessed on 22 February 2023) was used. The BioEdit tool was used to analyze the nucleotide sequence data for the ARV-S1 gene [40]. Sequences of the selected strains were compared to other ARV strains from other genotype clusters and to the vaccine strain widely used in Egypt (S1133 acc.no. KP969039). The NCBI platform provided all the data. The phylogenetic analyses were conducted using MEGA-6 [41]. The best models were the General Time Reversible (GTR) substitution with Gamma distribution (G) and estimate of the proportion of invariable sites (I), a moderate-strength neighbor-joining approach, and 1000 bootstrap repeats [42].The pairwise nucleotide percent identity was calculated using BioEdit version 7.0 [40]. The accession numbers for the eleven sequences under study were published in the NCBI database, where they were subsequently deposited (Table 1). For the detection of recombination events, RDP v4.5 software was employed, and the presence of all recombination events was evaluated using RDP, GENECONV, BootScan, MaxChi, Chimaera, SiScan, and 3Seq tools. *p*-value B 0.05 was used to validate the findings of the recombination areas. Each recombination event's start and end breakpoints were determined. Moreover, the relationships between the identified parents in each recombination event were explored when the recombination areas were discovered [43].

3. Results

3.1. Clinical Signs and PM Lesions of Avian Reovirus Infections in Broiler Chickens

All the clinical investigated broiler flock cases showed unilateral lameness and swelling of the hock joints (Figure 1). Some broiler cases showed stunting growth and arthritis at 10 days of age. The postmortem findings showed unilateral arthritis, swelling of the hock

joints, minor erosions on the articular cartilage with marked hemorrhages in the tendon and tendon sheaths, and synovial membranes with excess mucus of clear fluid in the capsule with pale and dilated intestines with a markedly atrophied pancreas (Figure 1).

Figure 1. Clinical signs and PM lesions of avian reovirus infection in broiler and broiler breeder chickens. (**A**) Broiler cases with unilateral arthritis, (**B**) Swelling of hock joints (Arrows), (**C**) minor erosions on the articular cartilage, (**D**) Marked hemorrhages in the tendon and tendon sheaths (Arrow), (**E**) Synovial membranes with excess murky fluid in the capsule, (**F**) Pale and dilated intestine with markedly atrophied pancreas (Arrows), and (**G**) Broiler cases with stunting growth and arthritis at 14 days of age.

3.2. Screening of ARV

Using qRT-PCR, it was noticed that 15 out of 75 field samples (20%) tested positive for ARV. Positive samples also tested negative for the avian reovirus S1133 GC I by the qRT-PCR-DIVA strategy, clarifying that the strain responsible for clinical signs originated from a different genotype cluster rather than the S1133-like (GC I).

3.3. Reovirus Isolation

Following the success of viral detection and ECE isolation of 11 viruses out of 15 positive samples, the RT-PCR confirmed that the injected embryos contained ARV (isolation of four remaining positive broiler breeder samples failed which could be attributed to high Ct values (>29) in the RT-qPCR test). The embryos were slightly hemorrhagic, stunted, and their livers and spleens were enlarged (Figure 2). Necrotic foci in the liver and spleen were observed 14–16 days after injection, which is considered pathognomonic for ARV.

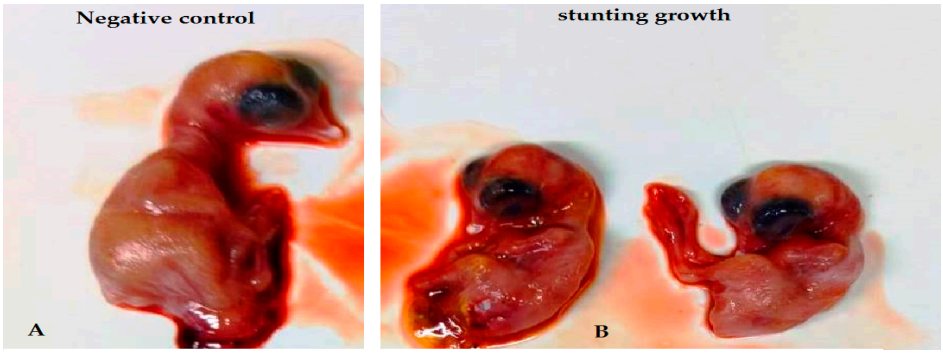

Figure 2. Evidence of ARV infection in ECEs. Yolk-inoculated chicken embryo 14 days post-inoculation. (**A**) Normal-sized embryo inoculated with sterile saline. (**B**) ARV-inoculated embryos showing dwarfed embryos (stunting growth).

3.4. Molecular Analysis and Clustering of Isolated Reoviruses

All partial S1 genes retrieved from eleven ARV isolates were submitted to the GenBank database and assigned the following accession numbers: OP609778–OP609788 (Table 1). The 790 bp reovirus sigma-C gene was used to create a neighbor-joining phylogenetic tree by using representative strains for each VI genotypic cluster of reovirus σC. Based on phylogenetic analyses, the obtained strains could be allocated into two different clusters. Nine of the Egyptian strains belonged to cluster IV, with the highest similarity with Israeli ARV 2018 (GC IV). The other two samples showed the highest similarity with cluster V (GC V), with a close similarity to China ARV 2022, and Japan ARV 2021, assigned as cluster V (Figure 3).

Figure 3. Phylogenetic tree of ARV isolates based on the nucleotide sequences of the σC-encoding gene. The phylogenetic analysis was performed using MEGA6. Consensus neighbor-joining trees were obtained by using general time reversible (GTR) from 1000 bootstrap replicates. The red rhombi strains indicate avian REO strains under study, and the blue color represents the authorized vaccine of GC I.

The cluster IV classified strains were found to show 81–89% identity to Israeli ARV 2018 (ARV-Israel-5242-2018) and 66–80% identity to Canadian AVR strain 2020 (ARV-CANADA-D12-2020),while the two strains belong to genotypic cluster V showed a 78% nucleotide identity with Japan ARV 2021 (ARV-Japan-CS-108-2021) (Table 2). The isolated and sequenced reovirus from GC IV and V exhibited considerably greater divergence with the vaccination strains, as revealed by sequence comparisons. The σC-encoding gene of eleven isolates shared only 43–55% identity with commercial vaccination isolates (S1133, 2408, and 1733 GC I) (Table 2).

A recombination event was observed between the strains under study namely Reo-Egypt-Broiler-Giza-2-2021, Reo-Egypt-Broiler-Giza-7-2022, and ARV-Israel-5242-2018, which related to cluster IV (GC IV) (Figure 4), while no recombination event was detected in the strains of genotype cluster V.

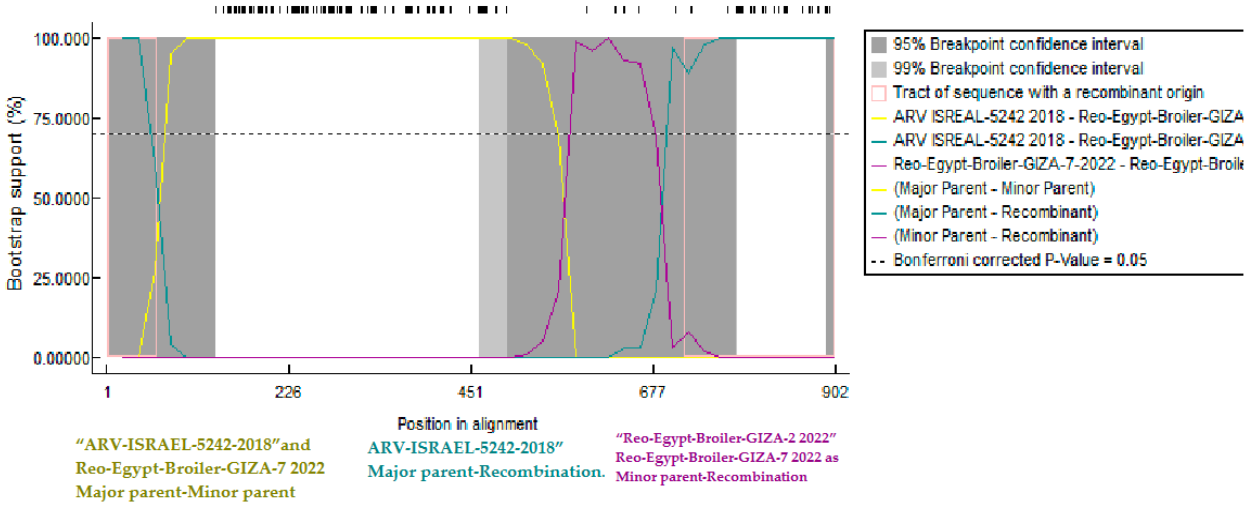

Figure 4. Recombination analysis displaying possible recombination events predicted to have occurred in the S1 segment of "Reo-Egypt-Broiler-GIZA-7 2022" as Minor parent-Recombination and "ARV-ISRAEL-5242-2018" Major parent-Recombination.

Table 2. Nucleotide identities of partial σC gene sequences (790 bp) compared to other selected field and vaccine strains available on GenBank.

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26		
	56%	49%	53%	100%	52%	53%	98%	53%	42%	54%	53%	44%	43%	43%	55%	55%	52%	43%	43%	43%	45%	44%	44%	45%	45%	1	ARV-Vaccin-strain-S1133-2017-CI
		49%	55%	56%	55%	54%	56%	53%	47%	54%	53%	48%	47%	48%	54%	54%	54%	48%	50%	49%	51%	50%	50%	51%	52%	2	ARV-SK Canada-BROLIER-R38-2017-C-II
			50%	49%	54%	52%	49%	52%	51%	52%	51%	52%	51%	52%	52%	52%	51%	50%	50%	52%	53%	52%	54%	55%	55%	3	ARV-isolate-ISREAL-5233-2010-C-III
				53%	62%	81%	53%	64%	69%	65%	65%	53%	52%	52%	64%	64%	80%	66%	68%	67%	69%	67%	71%	72%	73%	4	ARV-CANADA-D12-2020-S1C-IV
					52%	53%	98%	53%	42%	54%	53%	44%	43%	43%	55%	55%	52%	43%	44%	43%	45%	45%	44%	45%	45%	5	ARV-isolate-Reo-Broiler-YTLY-161024b
						59%	52%	90%	52%	65%	65%	59%	60%	58%	66%	66%	59%	51%	52%	51%	54%	53%	56%	56%	57%	6	ARV-SK Canada-BROLIER-R33-2017-C-VI
							53%	62%	66%	65%	64%	50%	50%	51%	62%	62%	77%	64%	64%	64%	66%	64%	68%	69%	70%	7	ARV-Netherlands-GEI10-97M-2004
								53%	43%	54%	53%	44%	43%	43%	55%	55%	53%	43%	44%	43%	45%	45%	44%	46%	46%	8	ARV-INDIA-VA-1-vaccine-2008
									51%	68%	69%	57%	57%	56%	69%	69%	62%	51%	51%	50%	53%	52%	55%	55%	56%	9	ARV-Reo-Canada-AB-Broiler-2012
										52%	52%	58%	57%	58%	52%	52%	82%	82%	83%	81%	83%	82%	87%	89%	89%	10	ARV-ISRAEL-5242-2018
											89%	66%	64%	65%	78%	78%	62%	51%	52%	52%	53%	52%	54%	55%	55%	11	ARV-Japan-CS-108-2021
												63%	62%	63%	76%	76%	62%	51%	52%	52%	52%	53%	54%	55%	55%	12	ARV-China-SDYT2020-2022
													88%	88%	77%	77%	51%	53%	54%	54%	55%	54%	55%	56%	56%	13	ARV-Lebanon-D2291-1-3-13LB-2023
														93%	74%	74%	51%	52%	53%	54%	54%	54%	55%	55%	55%	14	ARV-OMAN-D3122-1-15OM-2023
															74%	74%	52%	53%	54%	54%	55%	54%	56%	56%	56%	15	ARV-AUE-D1897-12AE-2023
																100%	62%	50%	51%	51%	52%	51%	55%	55%	55%	16	Reo-Egypt-Broiler-D6366-2-23-2022
																	62%	50%	51%	51%	52%	51%	55%	55%	55%	17	Reo-Egypt-Broiler-D6366-2-15-2022
																		78%	79%	78%	80%	79%	88%	89%	90%	18	Reo-Egypt-Broiler-GIZA-1-2021
																			91%	85%	89%	95%	83%	85%	85%	19	Reo-Egypt-Broiler-GIZA-2-2021
																				88%	92%	91%	84%	86%	86%	20	Reo-Egypt-Broiler-GIZA-3-2021
																					89%	86%	82%	84%	85%	21	Reo-Egypt-Broiler-GIZA-4-2021
																						90%	85%	87%	87%	22	Reo-Egypt-Broiler-GIZA-5-2022
																							84%	86%	86%	23	Reo-Egypt-Broiler-GIZA-6-2022
																								97%	97%	24	Reo-Egypt-Broiler-GIZA-7-2022
																									99%	25	Reo-Egypt-Broiler-GIZA-8-2022
																										26	Reo-Egypt-Broiler-GIZA-9-2022

4. Discussion

The impact of an ARV infection can be characterized by economic losses due to poor productive parameters, condemnations at processing, and compromised welfare in meat-type poultry [44]. Avian reoviruses are well known for their genetic variability, with the emergence of pathogenic variant strains causing negative impacts to the poultry industry worldwide [10,24,28]. Recently, there is an increase in the rate of tenosynovitis and malabsorption syndrome in chicken flocks in Egypt. The obvious clinical lesions observed in the broiler and broiler breeder flocks sampled in this study were analogous to those triggered by ARV, as reported in literature [10]. The detection of different viruses not associated with a certain chicken breed indicates that the virus is not associated with a certain chicken breed [45]. Broiler breeder flocks in the actual study were vaccinated against ARV with a commercial vaccines (GC I), although genotype cluster I (GC I) live attenuated ARV vaccines such as S1133 have not been updated since the 1970s [46]. Based on previous reports, these findings corroborate the lack of efficacy of the applied vaccinations in protecting birds against strains that are actually circulating and raise the possibility that the causal genotype cluster may differ from the genotype used in the commercial vaccine [10,18,24,47].

Out of seventy-five tested samples, fifteen were positive for ARV (20%), all positive cases differentiated by DIVA strategy as non-vaccine seeds (S1133-like virus).The egg inoculation confirmed the suspected virus is pathogenic, as it developed lesions in the ECE following yolk sac inoculation, as previously described [48]. Possible genetic variants of ARV in Egypt have been hypothesized by the isolation of reoviruses causing tenosynovitis in broiler and broiler breeder chickens acquired from vaccinated farms with a standard vaccine strain (S1133). The genome of the reovirus isolates sequenced in this investigation were highly diverse [49].

For characterization and categorization of ARV isolates, a portion of the S1 gene sequence has frequently been employed, although other genome regions have also been targeted, with comparable aims [50,51]. Five genotypic clusters have historically been used to categorize ARV strains using partial S1 gene characterization methods, but more recent research has attempted to add additional genetic clusters (GC VI) and sub-clusters [10,12,18,52–55]. Unfortunately, new typing nomenclature was proposed when more strain diversity became clearly evident. However, these new typing nomenclature was frequently contested, because various sequence data and analysis tools were applied and cluster names differed, which precluded the introduction of a uniform σC-based classification scheme for ARVs [12]. We utilized the original categorization previously schemed because of these flaws [9,12]. Nevertheless, our data also showed variety outside of the existing system, which further supports the necessity to develop a molecular ARV-type method that is widely acknowledged, standardized, and reliable and that most likely includes several genomic regions.

In an attempt to classify the Egyptian ARVs field isolates, the constructed phylogenetic tree by the neighborhood method using Ayalew et al. (2017) classification scheme [12] and including the reference strains representing each previously identified cluster indicate that the isolated virus belong to clusters IV and V, and this is the first record for these emerging clusters in the poultry industry in Egypt. The phylogenetic analysis revealed that the investigated strains clustered with viruses isolated primarily from poultry, with a global distribution, including Canada, the United States, and China, but they were distinct from the representative ARVs from continental Europe. Based on phylogenetic analysis, the obtained strains could be allocated into two different clusters. Nine of the Egyptian strains belonged to cluster IV, as did the Canadian virus isolates D1, D3, D6, D8, and D12, which were all assigned to Genotype cluster IV (GC IV) [9,56]. The other two samples showed the highest similarity with cluster V (GC V) and a recent Japanese strain (ARV Japan CS-108 2021), as well as Canadian isolate D2, D10 [56,57]. None of the obtained sequences in this investigation were classified as GC I, GC II, GC III, or GC VI. Different reovirus genotypes multiply differentially in the tendons, hearts, and duodenum of infected

chickens, causing dissimilar pathologic lesions and lymphoid depletion degrees in the challenged chickens [58,59].

Despite this, it is crucial to link genetic alterations to the virulence and antigenicity of variant strains when selecting autogenous vaccines [58]. However, the clear assignment of certain strains to a specific disease picture is not possible [12]. Reovirus-related clinical signs could not be clearly connected to any particular virus strain belonging to a specific genetic group, implying the lack of strict association between disease forms of ARV infection and the investigated genetic features of ARVs [7]. Large-scale full-genome sequencing of ARV strains could be a useful approach to uncover the missing link between strain diversity and pathogenic features [7]. Prior studies noticed a majority of isolates linked to malabsorption syndrome belonging to clusters I and IV, with only a few belonging to clusters II and V [18]. The majority of isolates from arthritis/tenosynovitis belonged to cluster IV. In the same context, our findings show no link between the ARV lesions and the different genotypes under investigation, as all strains were obtained from birds with identical clinical symptoms.

The RNA virus lacks proofreading and is subject to mutation and recombination events, ending in genotypes that are neither partially or totally neutralized by antibodies produced by the standard vaccine strains [18]. By studying the identity percent between the two genotypes (GC IV and V) co-circulated in Egypt that are detected in this study, an identity of 50–62% was revealed. The strains under study that belonged to cluster IV (GC IV) shared an average identity of 78–99% to each other, whereas the strains belonging to cluster V (GC V) showed 100% identity to each other. The eleven isolates, on the other hand, exhibited poor resemblance to other clusters (GC II, III, and VI). The cluster IV classified strains were found to be identical (69–80%) to published Canadian strains belonging to GC IV, such as ARV-D12-Canada. Reo/Egypt/Broiler/GIZA1-2021 had the highest similarity to other strains of genotype IV. The isolated and sequenced reovirus from GC V showed nucleotide identities of 76% and 78% to ARV-China-SDYT 2020-2022 and ARV-Japan-CS-108-2021, respectively [49], and exhibited considerably greater divergence with the vaccination strains, as revealed by sequence comparisons. The σC-encoding gene of eleven isolates shared only 43–55% nucleotide identity, with high nucleotide and amino acid substitutions with commercial vaccine strains such as ARV-S1133-2017 belonging to GC I. These findings might explain why the used vaccinations failed to protect broiler breeders against circulating strains in Egyptian broiler flocks [18]. When at least two viral genomes co-infect the same host cell and exchange genetic segments, recombination occurs. Based on the crossing site's structure, many viral recombination processes may be identified [60,61]. In both parental strands, homologous recombination takes place at the same location [62], but non-homologous or illegitimate recombination takes place at different locations of the genetic segments involved, usually resulting in abnormal structures [63]. The recombinant virus most likely evolved from two Egyptian ARVs related to GC IV and ARV-ISRAEL-5242-2018 at the C-terminus protein (Figure 4). The unique recombinant virus (Reo-Egypt-Broiler-GIZA-7-2022) might open the door to additional types. Further research is needed to understand the pathobiological and clinical characteristics of this virus in a chicken model. Moreover, continuous sequence analysis for presently circulating ARV is strongly advised in order to examine the virus's spread and genotype.

In general, the disease is prevented by the use of live classical and inactivated (licensed and autogenous) vaccines; however, they have a limited impact against circulating genetically aberrant variants and must be updated on a regular basis [64]. The first step in developing control and preventive methods for reoviruses is the definition of strains causing illness in the field after which a viral strain might be selected for an autogenous vaccine [65]. The frequency of clinical cases of arthritis/tenosynovitis in commercial poultry has increased substantially in recent years in different parts of the world, with commercial vaccinations found inadequate to provide complete disease prevention [10,24,47,66]. The circulating field viruses discovered in this investigation were genetically dissimilar from the

current commercial vaccine strains based on a partial sigma (σ)C gene (S1-gene) analysis, which are members of genotype cluster I (GC I). Virus genotyping may not directly relate to the immunogenicity of vaccines; the antigenic changes of the reoviruses is unclear and not well understood in terms of protection [36]. However, in an earlier study in France, it was noticed that even infections with field strains from cluster I remained unprotected by commercial vaccines used in breeders [24]. It is challenging to identify and choose field isolates for consideration in vaccines, particularly when many reovirus clusters co-circulate within flocks. Additionally, the field data indicate that, in some situations, autogenously customized vaccines could offer considerable protection from disease [48].

5. Conclusions

The current investigation revealed the co-circulation of different ARV genotypes (GC IV and V) in Egyptian broilers and highlighted the prevalence of ARV pathogenic variants inducing the disease. The circulating strains differ from the vaccine strains that have been granted licenses in Egypt, despite limited genetic similarities to local strains. This favors the formulation of homologous autogenous vaccines involving multiple clusters and examining its protection against different variants.

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