



## Research article

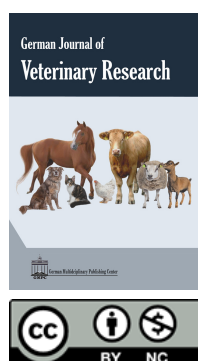
## Identification and genetic correlation of avian reoviruses to the currently used vaccines in Egypt

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## Abstract

A variety of illnesses, including arthritis, tenosynovitis, stunted growth, and malabsorption syndrome, are caused by Avian Reoviruses (ARVs), which have become more prevalent in Egypt during recent years and resulted in significant economic losses. This study investigated 27 suspected samples collected from 14 broiler breeders and 13 broilers suffering from immunosuppression, decreased body weight, and diarrhea. Fourteen samples tested positive based on RT-PCR, and the virus could be isolated from ten samples in Specific Pathogen Free (SPF) embryonated chicken eggs. Ten isolates were subjected to molecular and genetic analysis of the S1 gene (sigma C) and S2 gene (sigma A). The amino acid identity of the S1 gene revealed that these viruses are closely related to the viruses that were identified in Israel during 2020 (91.8%-97.2% identity) and belonged to the genetic cluster 5 (genotype 5), which also includes some viruses that are circulating in the United States and Canada. They also showed weak similarity (48.9%-50.2%) with the available vaccine strains in the Egyptian field that belong to cluster 1, genotype 1. The S2 gene showed amino acid homology of 91.7%-98.2% with the current vaccine used in Egypt. However, the Egy-Reo-7-2021 virus had the lowest similarity (84.2%-87.6%) to the available vaccine. It is hypothesized that the difference between field and vaccine strains may have contributed to the failure of current vaccinations to produce protective immunity against current ARV strains circulated in Egypt, which made the disease a problem to the poultry industry. Developing homologous vaccines and evaluating their potency and efficacy are required in Egypt.

**Keywords:** Avian reovirus, Genetic characterization, Surveillance, Sigma A, Sigma C

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## Introduction

Avian Reoviruses (ARVs) are *Reoviridae* family members that belong to the genus *Orthoreovirus*. Chickens, turkeys, and other bird animals frequently contract reovirus infections worldwide (Jones, 2003). ARVs have been found in chickens with various illnesses, primarily viral arthritis/tenosynovitis, stunting syndrome, gastrointestinal sickness, malabsorption syndrome, and immunosuppression (McFerran and McNulty, 1993; Jones, 2003). When breeder hens contract the virus horizontally via contaminated fomites and farms, the virus is transmitted vertically to the chicks (Jones and Georgiou, 1984; Ni and Kemp, 1995). ARVs infections result in financial losses due to direct mortality, lameness, increased slaughterhouse condemnation rates, and poor performance, such as decreased weight gains and poor growth. ARVs could suppress chickens' immune systems, increasing their susceptibility to other infectious agents and the frequency of secondary bacterial infections with (Kibenge et al., 1982).

The non-enveloped ARV's double-stranded RNA (dsRNA) genome is segmented (Benavente and Martínez-Costas, 2007). According to molecular size, the ARVs genome segments (n = 10) are divided into three groups: small (S1-S2), medium (M1-M3), and large (L1-L3) segments. At least 12 viral proteins, including eight structural and four non-structural proteins, are encoded in the genome of ARVs (Bodelón et al., 2001). The sigma C protein (encoded by S1) is a minor viral-cell attachment capsid protein used as a base for categorizing ARV into five lineages (Kant et al., 2003; Liu et al., 2003). It contains the

most hypervariable regions of ARV proteins that elicit specific ARV-neutralizing antibodies (Wickramasinghe et al., 1993; Liu et al., 2003; Goldenberg et al., 2010). The S1 gene region that encodes the C protein has frequently been employed as a genetic marker for the characterization and classification of ARV isolates through amplification and sequencing analysis (Schnitzer, 1985; Sellers et al., 2013; Lu et al., 2015; Gallardo et al., 2017). Recent research using S1 recategorized ARV into six lineages, from I through VI (Lu et al., 2015; Ayalew et al., 2017).

ARVs were initially discovered in Egypt in 1984 (Tantawi et al., 1984). Embryonic chicken eggs (ECEs) and chicks with clinical symptoms were used to isolate avian reoviruses (Madbouly et al., 1997; Madbouly and El-Sawah, 1999; Madbouly et al., 2001, 2009; Zaher and Mohamed, 2009; Abd El-Samie, 2014; Mansour et al., 2018). Seroprevalence (38.9% based on RT-PCR) and high nucleotide correlation (98-100%) of the  $\sigma$ A-encoding gene of ARV infections in Alexandria, Al-Behera, Giza, Kafr El-Sheikh, and Al Gharbia governorates, in Egypt during the period 2017-2018 was conducted by Al-Ebshahy et al. (2020) compared to S1133 vaccine strain. Furthermore, seroprevalence and ARV detection were confirmed by Safwat et al. (2019) in Al Behera governorates during 2015-2018.

Generally, reoviruses are highly resistant to different environmental factors. Therefore, well-carried-out disinfection programs could significantly reduce the viral pressure load to subsequent production cycles. Vaccination of breeders can protect young broilers by transferring maternal antibodies, which protect off-

spring during the first three weeks of the chick's life (a period of high susceptibility). However, in cases of high viral pressure in the field, vaccination with live attenuated vaccine must be given as early as possible (1 and 14 days of age).

Under current field conditions, the traditional Reovirus vaccine strains like S1133, S1733, S2408, S3005, and SS412 immunized in commercial flocks were shown to be ineffective. This study was conducted to provide documentation of the recent genetic characterization of ARV in Egypt and to advance our knowledge of the genetic variance concerning the different classical, traditional vaccines used in Egypt, focusing on both sigma C (S1) and sigma A (S2) genes.

## Materials and methods

### Flock history and clinical samples

A total of 27 chicken flocks (14 broiler breeders and 13 broilers) from four different governorates in Egypt (Giza, Al Menofia, Al Qalyoubia, and Al Beheira) were examined clinically, and all signs of lameness and/or poor growth were noted. The age of the broiler flocks ranged from 2 to 5 weeks, whereas that of the broiler breeder flocks was 30 to 46 weeks (Table S1). Tendons and synovial tissues from diseased birds with lameness were collected for the isolation and molecular identification of ARVs.

All broiler flocks were non-vaccinated against reoviruses while the breeder flocks were vaccinated at least two doses (once using S1133 live attenuated vaccine and boosted by avian reovirus inactivated vaccines); however, the precise broiler breeder vaccination programs were not available during the data collection sheets and study.

### Sample collection and preparation

A total of 135 clinically diseased birds were sampled, representing five birds from each flock. From each bird, the gizzard, pancreas, and intestinal swabs were collected, producing a total of 15 samples from each flock: intestinal swabs (n=5), gizzard (n=5), and pancreas (n=5). The pooled organ samples from each flock were septically homogenized using a sterile homogenizer and reconstructed using sterile phosphate buffer saline containing antibiotics (Penstrept, Lonza), and then frozen and thawed three times and centrifuged at 3000 rpm for 15 min. Then, the supernatant was used for RT-PCR and virus isolation. Moreover, the five intestinal swab samples from each flock were pooled and centrifuged at 3000 rpm for 15 min, the supernatants were used for RT-PCR and virus isolation.

## Molecular detection of ARV

### Extraction of viral nucleic acid

Viral nucleic acid was extracted following the manufacturer's instructions Patho Gene-spin™ DNA/RNA Extraction Kit (iNtRON, Jungwon-Gu, Korea). The total nucleic acid extracts were kept at -20°C for further analysis.

### Amplification of viral nucleic acid using conventional RT-PCR

The RT-PCR amplification has been done following the instruction of Maxime RT-PCR premix (iNtRON, Korea), as follows: Reverse transcription step at 45°C for 30 min, inactivation of RTase at 94°C for 5 min, 40 cycles as denaturation 94°C for 45sec, annealing at 55°C for 25 sec. and extension at 72°C for 1 min, then the final extension at 72°C for 10 min. The amplified products were subjected to gel electrophoresis. The results have been documented by a gel documentation system (Alpha Innotech, Biometra, Kasendorf, Germany), and the data was analyzed through computer software. Oligonucleotide primers: Supplied from (Metabion, Planegg, Germany) are as follows: S1 (sigma C) (Kant et al., 2003), S2 (sigma A) (Kant et al., 2003; Bruhn et al., 2005). The predicted molecular sizes for the S1 gene and S2 gene were 950 bp and 399 bp, respectively.

## Virus isolation

The supernatant filtrate (using 0.22 µL syringe filter) of each RT-PCR-positive sample was injected via the yolk sac route using 5-7-day-old specific pathogen-free embryonated chicken eggs (SPF-ECE) obtained from the Nile SPF farm (Qom Oshime, Alfayoum, Egypt). Two hundred µL of the supernatant filtrate was inoculated into 10 SPF-ECEs for each sample. The inoculated SPF-ECEs were incubated for ten days at 37°C and underwent daily candling. Positive samples were reported if clear pathognomonic ARVs lesions were observed (Jones, 2000) and confirmed by PCR.

### Nucleotide sequence of S1 and S2 genes

The appropriate size amplified PCR products were purified using a QIAquick gel extraction kit (QIAGEN, Hilden, Germany). The purified PCR products were subjected to sequencing reactions using a Big Dye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, CA, USA) according to the manufacturer's specifications, and the reaction product was purified by exclusion chromatography using a DyeEX 2.0 SpinKit (QIAGEN, Hilden, Germany). The recovered materials were sequenced using a 3500 XL DNA Analyzer (Applied Biosystems, CA, USA).

### Molecular analysis of the sequenced genes (S1 and S2 genes)

Multiple nucleotide sequence alignment was performed using BioEdit software version 7.0 using the ClustalW alignment algorithm, and the percentage identity matrices between different virus sequences were determined. Phylogenetic trees were constructed with the maximum likelihood analysis with bootstrap iteration 1000 replicates using MEGA11 software.

### Antibody epitope prediction of S1 gene (sigma C protein)

The Immune Epitope Database (IEDB) Analysis Resource (<http://tools.iedb.org/bcell/>) has predicted linear epitopes, and the selected predicted epitopes were tested for accessibility, flexibility, antigenicity, and hydrophilicity. The prediction of the conserved exposed epitopes by BepiPred-2.0: Sequential B-Cell Epitope Predictor (<https://services.healthtech.dtu.dk/service.php?BepiPred-2.0>), the conserved area has been detected by clustal omega multiple alignment (<https://www.ebi.ac.uk/Tools/msa/clustalo/>), then viewed by Jalview software

## Results

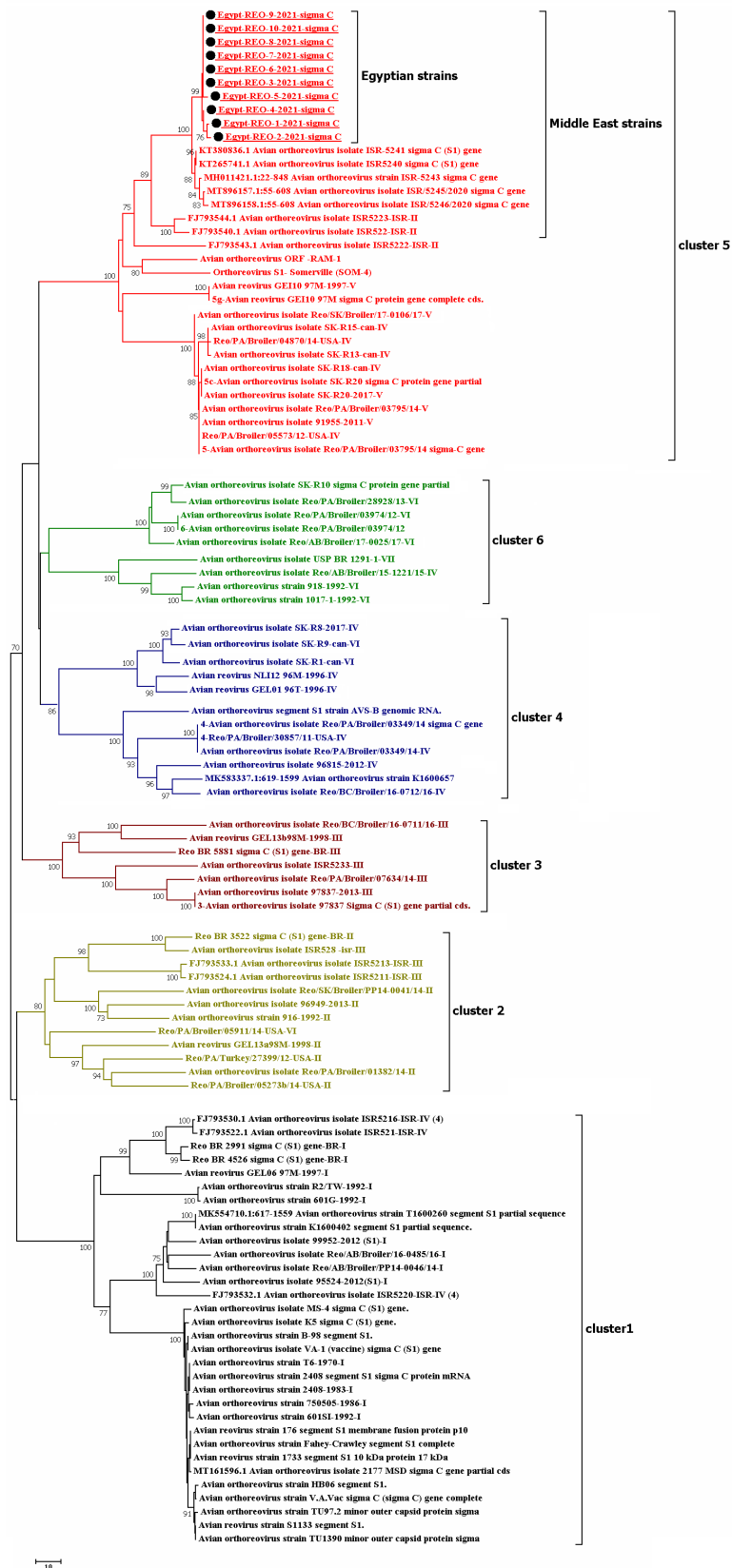
### Clinical signs and post-mortem findings

The broiler flocks were aged between 2-5 weeks and showed lameness, difficulties in movement, lack of uniformity, and swollen hock joints. Furthermore, other flocks exhibited stunting, uneven growth, diarrhea, depression, and poor feathering. The mortality in the most severe cases was up to 10%-12%. Affected broiler breeders' flocks aged 30-46 weeks with routine vaccination programs against ARV suffered from lameness and joint affections.

A post-mortem examination showed enlargement in the proventriculus; the intestine was pale, dilated, and filled with undigested feed, and marked atrophy of the pancreas was observed in most affected broiler birds associated with atrophy of Bursa of Fabricius. Additionally, marked swelling in the hock joints, severe tenosynovitis represented by hemorrhages, and edema in the tendon and the tendon sheath in birds that suffered from lameness were observed (broiler and breeders).

### ARV isolation and molecular detection using RT-PCR

A total number of 27 chicken flocks (14 broiler breeders and 13 broilers) collected from different governorates of Egypt (Giza, Al Menofia, Al Qalyoubia, Al Beheira) that were clinically suspected have been investigated using RT-PCR and then virus isolation (Table 1). Only 14 cases were positive for ARV using RT-PCR and ARV isolation. The ARV detection rate was 57.1% (8/14) in broiler breeders' flocks and 46.2% (6/13) in broiler flocks, with a total detection rate of 51.9% (14/27). All the positive RT-PCR tested cases were successfully isolated, as shown in Table 1 and Table 2. The common cytopathic lesions recorded post-inoculation were embryonic hemorrhages, hepatic hemorrhages and discoloration, and embryo stunting and dwarfing. Most isolated viruses were able to cause embryo death after 7-9 days post inoculation at age 14-16 days of age.



**Figure 1:** The phylogenetic tree of the sigma C nucleotide sequence for the ten Egyptian strains are genetically related to cluster 5 (Genotype 5) "red color". The tree was constructed by MEGA11 software.

**Table 1:** Numbers of farms examined for avian reoviruses by PCR and isolation.

Governorates	Examined suspected flocks		
	Total No.	No. of positive	%
Giza	16	8	50
Al Menofia	5	5	100
Al Qualiobia	1	0	0
Al Behera	5	1	20
Total	27	14	51.9

### Sequencing and phylogenetic analysis

#### *Analysis of sigma C protein in S1 gene*

The accession numbers for the ten studies viruses were OR860432, OR860433, OR860434, OR860435, OR860436, OR860437, OR860438, OR860439, OR860440, and OR860441, respectively from 1-to-10. The phylogenetic analysis of the sigma C protein revealed that the detected viruses related to those that were seen in Israel in 2020 belong to the genetic cluster 5, including some viruses that circulate in the USA and Canada, besides the reference strains SOM-4 and RAM-1; as shown in [Figure 1](#). The detected viruses were closely similar to those in Israel, with a nucleotide identity range of 94.5-97.4% and an amino acid similarity range of 90.7-97.2%. Also, they have a slightly different similarity from other viruses belonging to genotype four and are isolated from other countries like the USA and Canada, with an identity range of 75.2%-79.3% and amino acid identity range of 77.7%-85.8%. They showed very low similarity with the vaccinal strains that belong to genotype 1 with a nucleotide homology range of 84.5%-50.7% and amino acid homology range of 42.1%-46.2%, as shown in ([Table S3](#)).

#### *Analysis of sigma A protein in S2 gene*

The partial nucleotide sequences segment 2 (S2) of the sigma A of seven Egyptian strains are closely related in the phylogenetic analysis ([Figure 3](#)). The Egyptian strains showed high homology, as shown in [Table S4](#) contained nucleotide similarity percent ranging from 93.1%-100%, while amino acid homology ranged from 85.9%-100%. However, the vaccinal strains showed

low homology with the Egyptian strains, with nucleotide identity ranging from 75.4%-79.3% and amino acid homology ranging from 83.4%-95%. Although the Egyptian strain Egy-Reo-7-2021 is more related to the other Egyptian strains, it showed the lowest similarity with all the Egyptian and vaccinal strains. The amino acid mutations compared to the vaccinal strains have been recorded in [Table 3](#) including M64L, S81N, F91L, L93F, A118V, and V159I.

#### *In-silico B-cells immunogenic epitopes prediction in the sigma C protein*

The *in-silico* prediction for the immunogenic epitopes of B-cells on the surface of the sigma C protein revealed a partial homology in the structure of the vaccinal strains and the Egyptian strains. As shown in [Figure 2](#), seven mutual predicted linear epitopes (A-G) were observed, which are distributed on the globular head of the sigma C protein; most of those epitopes are exposed with high accessibility, antigenicity, flexibility, and hydrophilicity ([Figure 2](#)).

### Discussion

Recently, ARVs have generated multiple problems in broiler and vaccinated breeder flocks, severely hurting the poultry industry's performance. Multiple studies have been adopted on these significant viruses to raise awareness of their current effects in the world and the Egyptian field ([Madbouly et al., 2009](#); [Zaher and Mohamed, 2009](#); [Abd El-Samie, 2014](#); [Mansour et al., 2018](#); [Safwat et al., 2019](#); [Al-Ebshahy et al., 2020](#)).

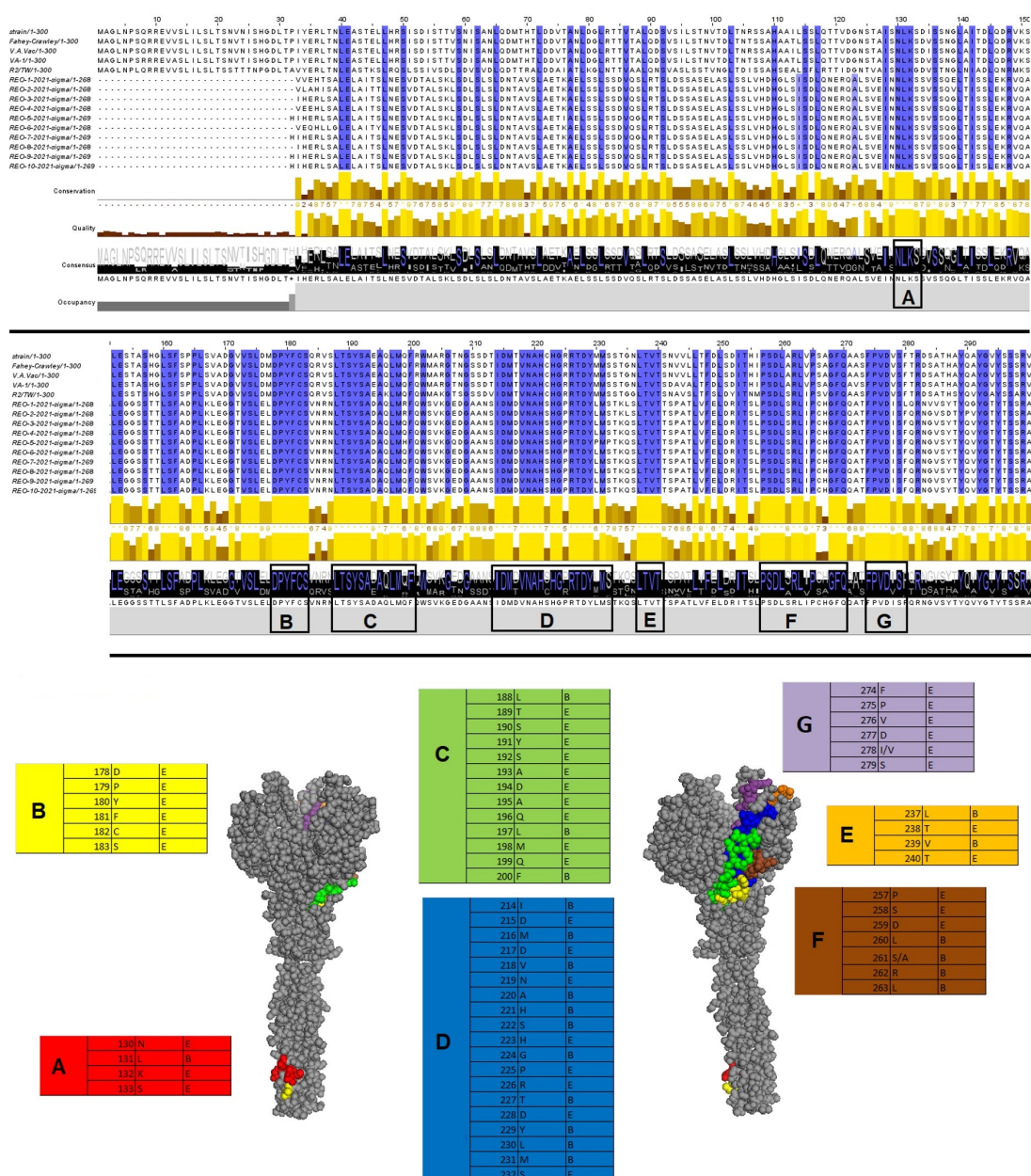
**Table 2:** Virus detection rate of avian reoviruses/production sector in the current study.

Type	Examined farm		
	Total	No. of Positive	%
Broiler Breeders	14	8	57.1
Broilers	13	6	46.2
Total	27	14	51.9

**Table 3:** The amino acid substitutions in the sigma A protein of the Egyptian strains compared to the vaccinal strains.

Type viruses	GB accession #	Amino acid differences					
		64	81	91	93	118	159
Vaccine strains							
1733	AF293773	M	S	F	L	A	V
2408	AF247724	M	S	F	L	A	V
OS161	AF294770	M	S	F	L	A	V
601SI	AF294769	M	N	L	L	V	V
T6	AF294768	M	S	F	L	A	V
750505	AF294767	M	S	F	L	A	V
919	AF294763	M	S	F	L	A	V
R2/TW	AF294765	M	N	L	L	V	V
918	AF294766	M	S	F	L	A	V
916	AF294764	M	N	F	L	V	V
1017-1	AF294762	M	N	F	L	V	V
Egyptian viruses under study							
Egypt-REO-1-2021-A	OR860442	L	N	L	F	V	I
Egypt-REO-2-2021-A	OR860443	L	N	L	F	V	I
Egypt-REO-3-2021-A	OR860444	L	N	L	F	V	I
Egypt-REO-4-2021-A	OR860445	L	N	L	F	V	I
Egypt-REO-5-2021-A	OR860446	L	N	L	F	V	I
Egypt-REO-6-2021-A	OR860447	L	N	L	F	V	I
Egypt-REO-7-2021-A	OR860448	L	N	L	F	V	I





**Figure 2:** *In-silico* prediction and the 3D molecular structure simulation of the mutual immunogenic B-cell epitopes of the sigma C protein in the Egyptian and vaccinal strains showing seven mutual predicted epitopes (A-G) on the globular head of the sigma C monomer. The letter "E" means that the mutual predicted epitope is exposed, while the letter "B" means buried or unexposed epitope.

In this work, ARVs were isolated and molecularly characterized from broilers and breeder hens suffering from arthritis and retarded growth rates in four different Egyptian governorates: Giza, Al Menofia, Al Qalyoubia, and Al Beheira. Typical symptoms were reported, including diarrhea, lameness, enlarged proventriculus, swollen intestinal tracts, poor feathering, limited growth, and pancreatic atrophy. Similar symptoms and lesions were also recorded by (Page et al., 1982; Hieronymus et al., 1983; Jones, 2013).

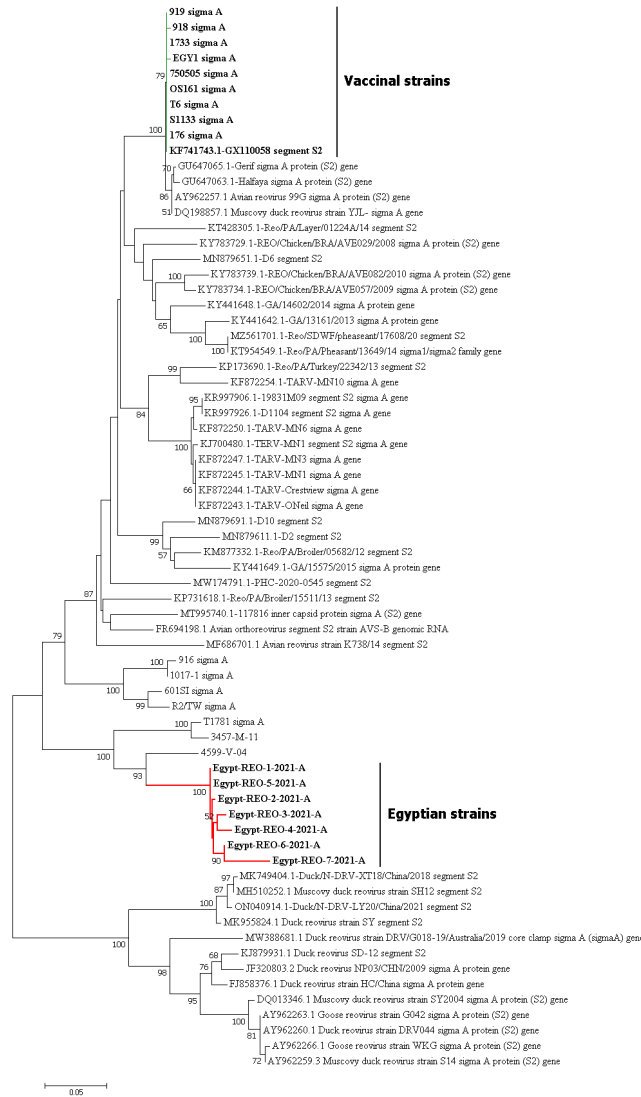
Typically, ARVs are seen in domestic broiler and broiler breeder chickens, which results in low weight gain and high mortality rates that have a significant negative economic impact on the poultry sector (Rosenberger et al., 1989). Our study involved twenty-seven suspected flocks (14 broiler breeders and 13 broilers). Of these, 57.1% (8/14) of the broiler breeders' flocks and 46.2% (6/13) of broiler flocks tested positive for the virus during the virus isolation process. This is consistent with the findings of (Mansour et al., 2018), who isolated (7/18) positive samples using RT-PCR on SPF-ECE from broiler flocks, with a rate of virus isolation positive percentage of 38.9%.

In Egypt, several studies were carried out to separate and

detect ARVs from chickens displaying symptoms or embryonic lesions (Tantawi et al., 1984; Madbouly et al., 1997; Madbouly and El-Sawah, 1999; Madbouly et al., 2001, 2009; Zaher and Mohamed, 2009; Abd El-Samie, 2014; Mansour et al., 2018). RT-PCR was used to measure seroprevalence (Safwat et al., 2019; Al-Ebshahy et al., 2020). For the  $\sigma$ A-encoding gene, the nucleotide sequences of ARV viruses were determined (Al-Ebshahy et al., 2020). The analysis of nucleotide diversity of the ARV  $\sigma$ C-encoding gene sequence among the viruses collected from Egypt was first described in our work.

The sigma C protein, the minor outer capsid protein, is encoded by the S1 gene's 3-proximal cistron. This protein can attach to host cells and start an infection because it is soluble in infected cells. A receptor-binding domain is present in the C-terminal region of the globular head C of the sigma C protein (residues 151–326). Furthermore, because its exposed surface contains antigenic epitopes, it stimulates the manufacture of specific neutralizing antibodies (Benavente and Martínez-Costas, 2007).

As we mentioned, there is a wide range of similarity (91.8%–97.2%) between the S1 gene (sigma C protein) sequences of our



**Figure 3:** The phylogenetic tree of the sigma A nucleotide sequence. The seven Egyptian strains are genetically distant from the vaccinal seeds, constructed by MEGA11 Software.

strains and the strains in the same genotype; that's because the substantial evolution and mutation of the avian reoviruses even within the same genotype (Egaña-Labrin et al., 2019). According to the S1 sequences, the seven genotypes have been documented internationally (Schnitzer, 1985; Lu et al., 2015; Zhong et al., 2016; Ayalew et al., 2017). Within a genotypic cluster, nucleotide and amino acid identity can range from less than 50% to 100%. Consequently, these ARV genotypic clusters are split into sub-genotypes (Sellers et al., 2013; Sellers, 2017; Gallardo et al., 2017), which makes it more difficult to identify and classify these viruses molecularly.

In Egypt, there are insufficient studies on the ARV; however, our study points out the predomination of genotype cluster 5 despite the currently implemented vaccination regimes using vaccines derived from the genotype cluster 1 viruses as S1133. Previous world studies admitted the ability of the genotype cluster 1 vaccines to evoke neutralizing antibodies that could produce partial protection against the other genotype strains (Meanger et al., 1997).

Several characteristics of polypeptide chains, including their consistency, polarity, turns, accessibility, hydrophilicity, flexibility, and antigenic propensity, have been connected to the site of continuous epitopes. This has led to a search for empirical recommendations that would allow continuous epitope sites to be predicted based on specific features of protein sequences. Prediction computations are based on propensity scales for each of the 20 amino acids. Each scale has 20 associated values, based on the relative probability that each amino acid residue exhibits the quality shown by the scale (Parker et al., 1986; Kolaskar and

Tongaonkar, 1990).

Furthermore, based on the *in-silico* molecular analysis for the amino acid sequences of the sigma C protein, we found that our strains share some mutual epitopes with the S1133 vaccine strain, which might provide the probability of cross-protection, which wasn't the case under field conditions. Therefore, it has been emphasized that the heterologous vaccine strains may play a crucial role in driving the genetic variability of the ARV, as the heterologous vaccine does not eliminate the field viruses leading to its long persistence in the environment (Egaña-Labrin et al., 2019).

However, in the same line with previous studies (Goldenberg et al., 2010), we alert to the conservative epitopes on the sigma C protein structure, which could be a solution key for producing a universal subunit vaccine that can induce general immunity for all the genotypes of avian reoviruses (Goldenberg et al., 2010). So, this observation could open a research era for vaccine evaluation using newly developed ARV homologous vaccines and re-evaluate the genotype-specific protection studies.

The inner core of the virion is the  $\sigma A$  protein, which is highly conserved and less immunogenic (Wellehan et al., 2009). The sequences of the viruses in this study were closely related to each other with a high similarity range (93-100%), in the same phylogenetic group as the Hungarian pathogenic viruses with an identity range between 82.3% and 90.9%. On the contrary, the examined Egyptian Reoviruses are quite far from the previously isolated Egyptian Reovirus in 2020, which belongs to the vaccinal strains (S1133, 1733, and T98) from the USA and China (Al-Ebshahy et al., 2020). Some amino acid substitutions have

been reported in our Egyptian strains compared to vaccinal ones. As mentioned in Table 3, the observed amino acid substitutions were M64L, S81N, F91L, L93F, A118V, and V159I; there was no evidence in any previous study proved the impact of these mutations on the function of the translated protein. However, there are no mutations in the amino acid residues R134, R135, and R155, which are crucial in the dsRNA binding activity of the  $\sigma$ A protein (Guardado-Calvo et al., 2008). These recorded mutations need further investigations and studies to be able to evaluate their impact on ARV evolution, ARV vaccine immunogenicity, and effectiveness.

In conclusion, avian reovirus is one of the most important causative agents that cause malnutrition and arthritis syndrome in chickens. This study revealed the spreading of genotype cluster 5 in broilers and broiler breeders' flocks in Giza, Menofia, Qaliobia, and Behera of Egypt. The *in-silico* prediction analysis of the sigma C protein pointed to mutual antigenic sites between the vaccinal and field strains that could support partial cross-immunity. However, the sigma A protein analysis revealed some substitutions of unknown impact. Furthermore, we recommend further studies to develop new homologous vaccines to evaluate their efficacy and potency compared to currently available vaccines.

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**Authors contribution.** Conceptualization and study design (WHK, MS, and MHH); data collection, field investigations, (MR, and MHH), laboratory investigation, and data analysis (MS, AA, WHK), preparation of the manuscript (MS, AA, and WHK). All the authors read and approved the final version.

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