Archives of Razi Institute, Vol. 78, No. 3 (2023) 1095-1105

DOI: 10.22092/ARI.2022.359899.2511

Original Article

Study on Propagation and Adaptation of EDS-76 Avian Adenovirus in Duck and SPF Primary Embryonic Chicken Cell Culture Comparison to Duck and SPF Embryonated Chicken Eggs

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Received 3 September 2022; Accepted 26 September 2022
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Abstract

Egg drop syndrome (EDS) is a major viral infectious poultry disease with severe economic losses in laying hens. The disease is caused by an adenovirus and can be transmitted horizontally and vertically. This study investigated the EDS virus (EDSV) infection in duck embryo fibroblasts (DEF), specific pathogen-free (SPF) embryo fibroblasts, and SPF egg embryos using different methods. The results were compared to the virus culture in duck and SPF chicken eggs. Duck and chicken fibroblast cells were used as the primary cell culture in Dulbecco's Modified Eagle Medium, and the low-pathogenic duck adenovirus was used to infect the ducks and SPF fibroblasts primary cell cultures, as well as the duck and SPF eggs. The titer of the virus was measured by hemagglutination assay, $ECID_{50}$, plaque-forming unit, and $TCID_{50}$ methods. The results revealed that EDSV could proliferate in the chorioallantoic membrane of DEF cells and duck eggs, compared to the chorioallantoic membrane of chicken embryo fibroblasts (CEF) and SPF chicken eggs. The findings showed that duck egg embryos and primary DEF cell lines are more appropriate for EDSV replication, compared to CEF and SPF chicken eggs. This suggests that the use of DEF culture for producing avian adenovirus EDS-76 is a suitable alternative for the embryonic egg culture.

Keywords: EDS virus; Adenovirus; Duck Embryo Fibroblast; Cell Culture

1. Introduction

Egg drop syndrome (EDS) is one of the most important diseases of poultry with severe economic damage. EDS is an infectious disease of laying hens caused by an adenovirus that collects the poultry's red blood cells, reduces egg production, and leads to the production of eggs without shells or with thin shells without causing any specific clinical symptoms in the herd (1). Adenoviruses are DNA viruses with no envelope and a needle-like structure called Fiber on the capsid. EDS can be transmitted horizontally and vertically. The EDS virus (EDSV) multiplies in the cell nucleus and produces intracorporeal inclusion bodies that can be detected

in histopathology or observed by an immunofluorescence microscope.

EDSV cultivates easily in the cell culture media of the duck, goose, and embryonic chicken eggs, including the liver and kidney cells, whereas does not proliferate in embryonic poultry eggs (2).

The first clinical symptoms are reduced shell thickness and decreased pigmentation in colored eggs, and there have been reports of the presence of thin-shelled, soft-shelled, or shell-less eggs in severe infections. Egg shells may also show signs of mineral deposits, or the eggs might become deformed with abnormal shapes (3).

The first embryological research on bird eggs was conducted 2000 years ago in the time of Aristotle, which was based on the shape of the origin of animals and has retained its value for centuries after the use of fertilized bird eggs for vaccine production (4).

In the 1930s, the well-known pathologist, William Ernest, a pioneer in the science of virology, investigated a suitable carrier that could deliver the virus in non-excreted conditions, generate the virus, or change its structure on a large scale. He showed that fertilized eggs are a good carrier for the production of viruses and proved his findings by producing a vaccine, followed by the production of different types of influenza vaccines (5). Through this system, other researchers succeeded in producing and supplying other viral vaccines in embryonic eggs (2).

There have been many successful attempts in the production of biological products in eggs because of their accessibility and easy incubation. Nevertheless, using egg embryos to cultivate viruses has some disadvantages, including the need for a large volume of fertilized eggs, the possibility of allergies due to chicken embryo proteins, the possibility of the transmission of some pathogens, and the need for extensive manpower (6).

These restrictions could weaken or diminish the role of fertilized eggs as an important medium of virus replication and encourage researchers to look for more suitable alternatives for virus replication (7).

One of the best candidates is the use of different cell lines in a cell culture, which can compensate for the limitations of the embryonic egg culture system. Various systems have been considered by researchers based on cell lines derived from different birds, each of which, with its advantages and characteristics, can be a suitable candidate for vaccine production (8). In addition to their high reproducibility, they are genetically stable over higher passages and can accept a wide range of viruses with high production volumes without changing their structure. This study aimed to isolate and replicate duck egg embryonic fibroblast (DEF) cells and specific pathogen-free (SPF) egg embryonic fibroblast cells using the cell culture method and compare the cultivation of EDSV in these cell cultures to embryonated eggs. Another objective was to compare the replication of EDSV in duck and chicken fibroblast primary cell culture to its replication in duck and chicken embryonated eggs.

2. Materials and Methods

2.1. Materials

The subsequent materials were used in the study: SinaPure ™ Viral Kit, SinaPure ™ DNA, trypan blue, DNA safe stain, loading buffer, agarose (Sinaclon Company, Iran), DNA ladder (Bioneer Company, South Korea), flasks, pipets, falcons, cell strainer, cell scarper (SPL Company, South Korea), fetal calf serum (Gibco, Germany), penicillin and streptomycin, amphotericin B, trypsin EDTA (Kalazist Company, Iran), high-glucose Dulbecco's Modified Eagle Medium (DMEM, KBC Company, Iran), and SPF chicken eggs (Venkateshwara Hatcheries, India).

2.1.1. Duck Eggs

The duck flock was confirmed free of chicken anemia virus (CAV), Reo virus (ReV), infectious bursal disease (IBD), infectious bronchitis virus (IBV), avian influenza H_9 (AIH_9), avian influenza H_5 (AIH_5), EDSV, Newcastle disease virus (NDV), avian encephalomalacia virus (AEV), *Mycoplasma gallisepticum*, *Mycoplasma synoviae*, and *Salmonella pullorum* by standard serological and molecular tests.

2.1.2. Viruses

In this study, a stock of low pathogenic avian adenovirus EDS-76 kindly was provided by Dr. Mansour Banani, Associate Professor of Razi Vaccine and Serum Research Institute.

2.1.3. Specific Pathogen-Free Eggs

In this study, SPF eggs were obtained from Razi Vaccine and Serum Research Institute.

2.2. Methods

2.2.1. Enzyme-Linked Immunosorbent Assay

Enzyme-linked immunosorbent assay (ELISA) was used to detect specific viral duck yolk antibodies against some important poultry viral diseases, such as CAV, ReV, IBD, IBV, AIH_9, AIH_5, EDSV, NDV, and AEV. It was conducted on yolk serum samples to establish the health and safety of duck flocks.

2.2.2. Serum Plate Agglutination Test

The serum plate agglutination (SPA) test was used to detect specific antibodies against *Mycoplasma gallisepticum*, *Mycoplasma synoviae*, and *Salmonella Pullorum*, which attach to an antigen and cause visible clumping and agglutination. The test is used to detect specific antibodies added to the dye to improve the reaction visibility. The approved amount of antigen is placed on a solid support, such as a glass, plate, or mirror, keeping each drop of the antigen separate, and equal amounts of the test serum are placed next to the antigen and then blended. After a short incubation, the mixture is observed for any evidence of agglutination, which appears as separated colored particles with a clear background. If no antibodies are detected, the mixture remains cloudy.

2.3. Cell Culture

Chicken embryo fibroblast (CEF) cells and DEF cells were obtained from 10-day-old SPF chicken embryos and 11-day-old Pekin duck embryos, respectively (Razi Vaccine and Serum Research Institute, northwest branch). The fetal head, neck, legs, wings, and internal organs were completely omitted, and only the body remained. It was washed several times with sterile phosphate-buffered saline (PBS) solution at pH 7.4 and high-glucose DMEM. It was then cut into 1 mm pieces using sterile scissors. The tissue pieces were treated at 37°C with trypsin (2.5% W/V) using a cell strainer and a magnet apparatus. The trypsin was inactivated by adding fetal bovine serum (FBS). This procedure was repeated several times until the embryo was completely digested. Finally, the suspension containing the digested cells was filtered by a sterile nylon strainer. The collected liquid was then centrifuged at 1400 rpm until the cells were settled. The cells were washed twice with sterile PBS and high-glucose DMEM and were then resuspended in 100 mL of the high-glucose DMEM cell culture. Afterward, they were supplemented with 10% FBS, 100 IU/mL penicillin, and 100 µg/mL streptomycin and amphotericin B. Cell suspensions were cultured in sterile 25 mL cell culture flasks and incubated at 37°C with 5% CO_2 to produce approximately 80-90% confluent monolayer fibroblast cells. Once the cells were cultivated into a confluent monolayer, they were sub-cultured by trypsinization, and 7×10^4 cells per mL were added in the high-glucose DMEM to each six-well plate and were gently shaken until they were distributed evenly. After the cells were seeded, they were allowed to grow 24 to 48 h. Afterward, they were checked under a light microscope to confirm their even distribution. The confluency of the cells reached over 90%.

2.3.1. Compatibility of EDSV in CEF and DEF

Monolayer CEF and DEF primary cells were cultivated in high-glucose DMEM, and the medium of all plates was removed after the confluency of the attached cells was 70-80%. The cells were gently washed with 5-7 mL of sterile PBS. Afterward, 0.2 mL of 10-fold serial dilution of 10% viral suspension was added to CEF and DEF monolayer cells, and they were incubated at 37°C for 60 min. After the virus was absorbed, the unabsorbed virus was removed by adding the DMEM growth medium, which contained 0.1% antibiotics and 5% FBS, and discarding it.

2.4. Cell Viability Assay

The cell viability of fibroblast cultures was determined after 24 and 48 h. The adhering cells to the bottom of the plates were isolated using the trypsin solution, and the survival rate of the cells was counted using trypan blue staining and a hemocytometer under a light microscope.

2.5. Virus Titration

The titer of the EDSV was determined using hemagglutination assay (HA), $ECID_{50}$, plaque-forming unit (PFU), and tissue culture infectious dose ($TCID_{50}$) methods. The amount of virus multiplication was monitored daily. The virus growth curves are shown in figure 1.

2.6. Hemagglutination Assay

The HA activity of all harvested virus fluids was calculated using the standard microtiter process. Serial double-fold dilutions of the antigens diluted in PBS were blended with a similar volume of 1% SPF chicken erythrocyte suspension and incubated at 25°C for 30 min. The joint of the highest antigen dilution showing hemagglutination was taken as the titer (HAU/0.025 mL) (9).

2.7. $ECID_{50}$

The $ECID_{50}$ is a valuable test for ascertaining the infectious dose in eggs. For $ECID_{50}$ calculation, EDSV was inoculated in SPF and duck embryonated egg groups (n=6), and a virus-harvesting fluid was serially diluted until the dilution was without virus (endpoint dilution). The last three dilutions that showed agglutination were selected, and 0.2 mL of the cultivated virus was used to inoculate six 10-12-day-old embryonated eggs for each group. Mortality within 24 h of the inoculation was not considered. The calculation of $ECID_{50}$ was based on the method by Karakus, Crameri (10), (11), by which the dilution of the inoculum-producing infection in 50% of the eggs was determined.

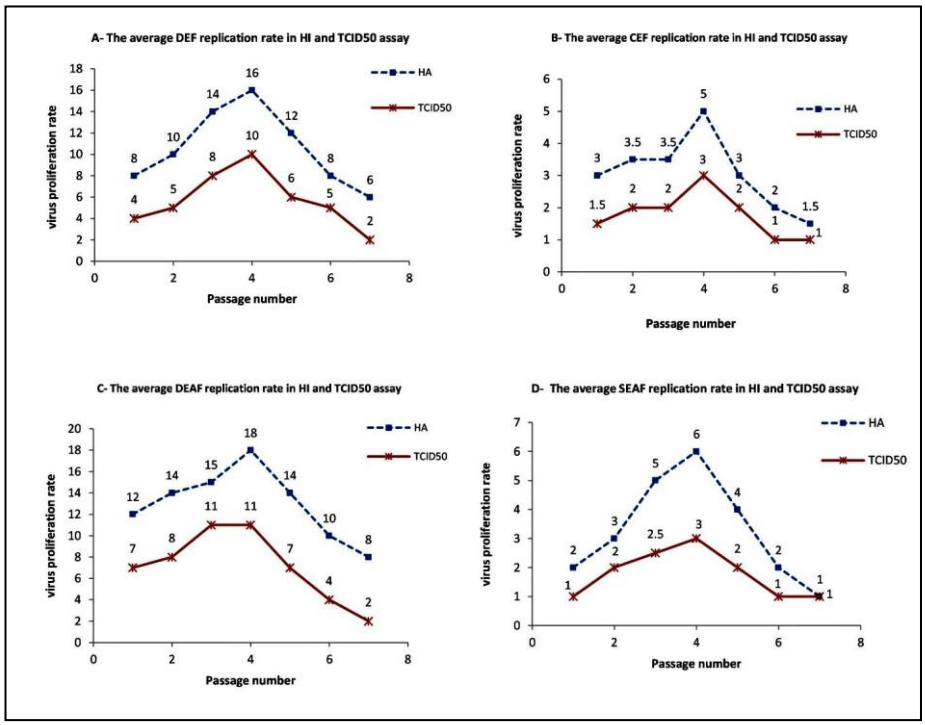

Figure 1. A: The average DEF replication rate used HI and $TCID_{50}$, **B:** The average CEF replication rate used HI and $TCID_{50}$, **C:** The average DEAF replication rate in HI and $TCID_{50}$ assay, **D:** The average SEAF replication rate in HI and $TCID_{50}$ assay

2.8. Plaque-Forming Assay

The PFU method was also used to measure the titer of the infectious virus and show the cytopathic effects (CPE) of definite viruses on the cell culture medium. The number of plaques created in plates was counted, and viral dilutions from 10^{-1} to 10^{-6} were inoculated on monolayer CEF and DEF cells. After 1-2 h, the virus was removed, and the cells were covered by a medium containing 5% FBS and 1% agarose, whose temperature could not exceed 42°C to prevent cell damage and could not be less than 39°C to avoid gel solidification. On the third day, crystal violet dye was added, and the number of plaques was counted (12).

The cells were seeded in eight-well dishes with DMEM containing 5% FBS, penicillin, streptomycin, and 5% CO_2 and were incubated for 24 to 48 h. The culture medium was then discarded, and different dilutions of EDSV (0.2 mL) were added to each well and incubated at 37°C for 60 min with gentle shaking every 15 min. Afterward, the remaining virus was drained, and the culture medium was added to each well and incubated for 24 to 48 hours. The culture medium was then drained, and DMEM with 1% agarose was added to each well. After an hour, the DMEM and agarose were washed, and the crystal violet and formaldehyde were added. After 3-5 min, the wells were washed with water, and crystal violet-stained plaques were observed (13).

2.9. Tissue Culture Infectious Dose

Poultry adenovirus titration was performed on fibroblast cells, which were sensitive to the CPE of the EDSV, according to the method described by Karakus, Crameri (10). The cells were cultured in a 96-well microplate with a complete culture medium (containing 5% FBS and antibiotics). The microplate was then transferred to a 37°C room without CO_2 to form a single cell layer. After that, logarithmic dilutions of the EDSV suspension were prepared with intervals of one log (10^{-1} to 10^{-6}) in an FBS-free culture medium. Next, each EDSV dilution was inoculated into three wells of different rows inside the microplate (96 wells). In each microplate, the wells without virus inoculation were considered negative controls, and those with undiluted virus inoculation were considered positive controls. The microplate containing virus-inoculated cells was examined daily for the effects of cell damage. The reading of the results continued until 52 h after virus inoculation. A dilution of the virus suspension that infected 50% of the healthy cells was considered the endpoint of the $TCID_{50}$ test, and Karber's formula was used to determine the EDSV titer.

2.10. PCR

In this study, samples were taken from 10-day-old duck embryos for the PCR test. The genomic extraction was performed using standard extraction kits (Pure Sinaclon, Iran). The PCR test was performed using primers for EDSV. The EDSVs obtained from Razi Vaccine and Serum Research Institute were used as the positive control in PCR experiments. Forward and reverse oligonucleotide primers 5´-TTC TGT CAC CGA TAA AGG T-3´ and 5´-AGT TAT TCC AAA TGG GCA T-3´ were respectively used to amplify a 1901-bp fragment of the hexon gene of the EDSV (14).

3. Results

The ELISA and SPA results detected specific duck yolk antibodies against important poultry viral diseases (Table 1). The results revealed that no specific duck yolk antibodies were found against CAV, ReV, IBD, IBV, AIH_9, AIH_5, and AEV. On the other hand, specific antibodies were observed against EDSV and NDV. In addition, no specific antibodies were detected against *Mycoplasma gallisepticum*, *Mycoplasma synoviae*, and *Salmonella pullorum* in the SPA of duck yolk antibodies.

Table 1. Specific duck yolk antibodies detection by ELISA and SPA assay

No.	Collected samples	ELISA	SPA
1	CAV	Neg	-
2	ReV	Neg	-
3	IBD	Neg	-
4	IBV	Neg	-
5	AIH9	Neg	-
6	AIH5	Neg	-
7	EDSV	Pos wk	-
8	NDV	Pos	-
9	AEV	Neg	-
10	MG	-	Neg
11	MS	-	Neg
12	SP	-	Neg

The results of the PCR test using EDS-specific primers that amplified the 1901 bp on all duck egg embryos before the EDSV inoculation were negative, and only the positive control related to the EDSV vaccine strain showed positive results (Figure 2). The results of primary DEF and CEF cells before and after the EDSV inoculation are shown in figures 3-6. All samples of duck egg embryos were positive after the EDSV inoculation, and the electrophoresis of PCR products after the EDSV inoculation showed 1901 bp clear and specific bands. Only the negative control, which received sterile PBS, was negative (Figure 7).

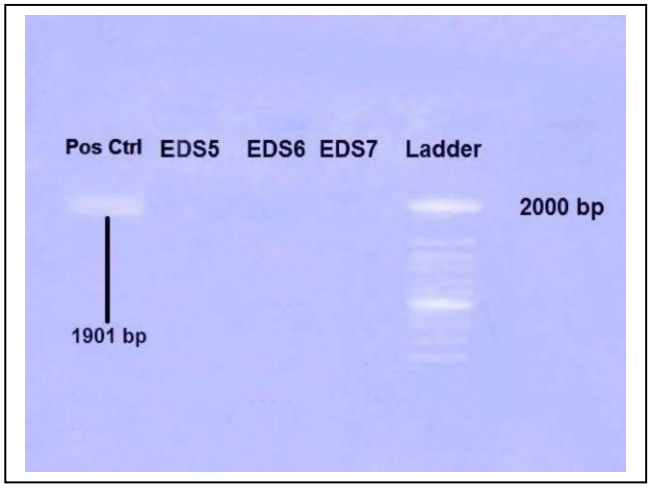

Figure 2. Results of PCR assay before inoculating the EDS virus
Pos Ctrl: Positive control, EDS5: Duck egg embryo, EDS6: Duck embryo fibroblast cell culture fluid, and EDS7: SPF embryo fibroblast cell culture fluid: Ladder: Marker (100 bp DNA Ladder)

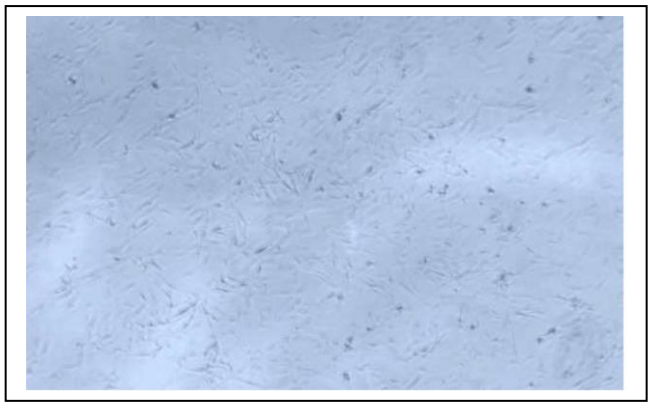

Figure 3. DEF second passages

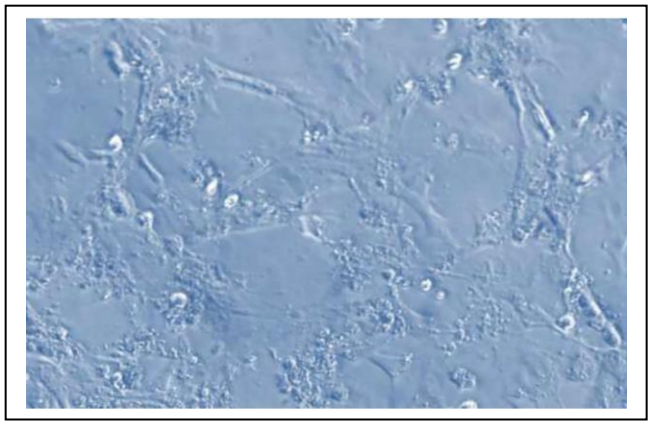

Figure 4. DEF infected by EDSV

Figure 5. CEF second passages

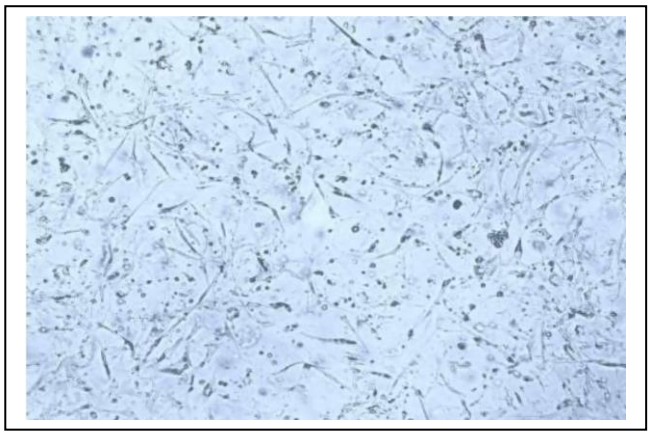

Figure 6. CEF infected by EDSV

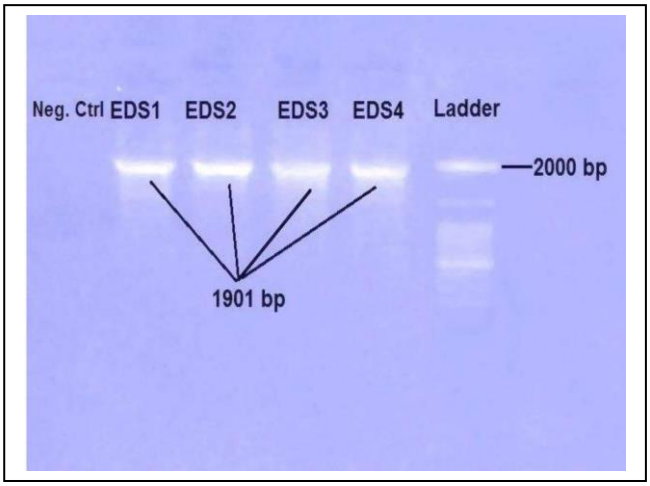

Figure 7. Results of PCR assay collected samples: Neg. Ctrl was negative control (PBS buffer), EDS1: Duck embryo fibroblast cell culture fluid; EDS2: SPF embryo fibroblast cell culture fluid of; EDS3: Duck eggs allantoic fluid; EDS4: SPF eggs allantoic fluid and Ladder: Marker (100 bp DNA Ladder)

The results of EDSV inoculation in 10-day-old embryonated duck eggs and primary DEF cells revealed that it is an extremely suitable environment for the cultivation of EDSV.

The EDSV inoculation did not replicate well in 10-day-old SPF embryonated eggs and primary SPF CEF cells. The infected duck embryos were found alive 74-86 h post-inoculation. The HA titer of the duck allantois-amniotic fluid, DEF cultured harvested fluid, SPF embryos, and the CEF cultured harvested fluid were "1:65536", "1:16384", "1:32", and "1:16", respectively, and the highest rate of Newcastle virus multiplication was in the fourth passage (Figure 1). The findings illustrated that EDSV could not replicate well in SPF chicken embryos and CEFs. This result could be related to the lack of receptors needed to absorb the virus in chicken embryos because adsorption is the most essential factor for virus replication.

Moreover, the negative control groups of duck allantois-amniotic fluid, DEF cultured fluid, SPF embryos, and CEF that received only sterile PBS did not have any titers in the HA tests. It should be noted that the existence of hemagglutinin on the surface of avian erythrocytes causes EDSV to create very clear agglutination by avian erythrocytes. The HA results are shown in figure 8 and table 2.

In addition, harvesting EDSV in DEF cells with DMEM revealed that this virus well replicated, whereas it did not grow well in SPF egg embryonic fibroblast cells.

The $ECID_{50}$ results showed that DEF cultured fluids were 10 and 14, and $ECID_{50}$ was a logarithmic expression using base 10. The results in the negative control groups were zero (Table 3 and Figure 9).

The DEF primary cell lines were assayed by EDSV PFU. The results showed that EDSV could be cultivated and produced well, and the PFU assay was 5×10^6. The plaques were formed within 84 h post-inoculation and caused extensive cellular lesions in duck primary cells (Table 3).

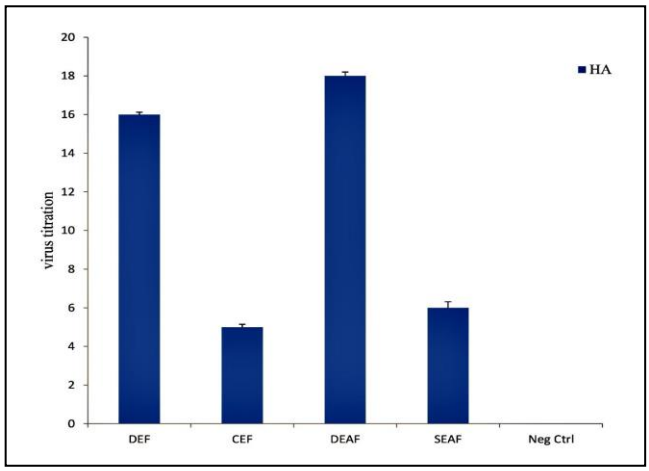

Figure 8. Comparison of HA and $TCID_{50}$ EDSV titration in DEF: Duck embryo fibroblast cell culture fluid, CEF: SPF embryo fibroblast cell culture fluid, and Neg. Ctrl: Nagative Ctrl

Table 2. EDS virus titration by $ECID_{50}$ assay, HA test and PCR results

Group No.	Collected samples	HA titer [I]	$ECID_{50}$ [II]	PCR [III]
1	Duck embryo fibroblast cell culture fluid	16±0.12†††	10±0.22***	Positive
2	SPF embryo fibroblast cell culture fluid	5±0.15	4±0.32	Positive
3	Duck eggs allantoic fluid	18±0.02†††	-	Positive
4	SPF eggs allantoic fluid	6±0.31	-	Positive
5	Negative Ctrl	0	-	Negative

Ctrl = control; Significant differences: ††† P<0.001compared to the 2, 4 and 5 groups, *** 001compared to the 2, 4 and 5 groups, all the experiments were performed in triplicate. I and II were logarithmic expression using base 2, 10 and also III was amplification of the 1901 bp is related to the EDS virus respectively

Table 3. HA, $TCID_{50}$ and PFU assay for EDS virus titration

Group No.	Collected samples	HA titer I	$TCID_{50}$/ml II	PAF/ml
1	Duck embryo fibroblast cell culture fluid	16±0.12†††	10±0.4***	8✕109 ▣▣▣
2	SPF embryo fibroblast cell culture fluid	5±0.15	3±0.2	3✕106
3	Negative Ctrl	0	0	0

Ctrl = control; Significant differences: ††† P<0.001compared to the 2, and 3 groups, *** 001 compared to the 2, and 3 groups and ▣▣▣ P<0.001 compared to the 2, and 3 groups, All the experiments were performed in triplicate. I and II were logarithmic expression using base 2, 10

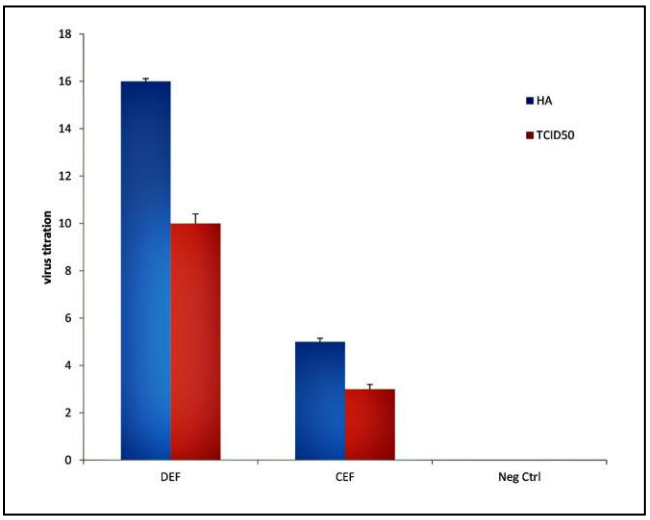

Figure 9. Comparison of HA and $TCID_{50}$ EDSV titration in DEF: Duck embryo fibroblast cell culture fluid, CEF: SPF embryo fibroblast cell culture fluid, and Neg. Ctrl: Nagative Ctrl

4. Discussion

EDSV is one of the most important infectious agents in poultry, which reduces the quality and quantity of egg production and causes respiratory diseases in chickens. EDSV antibodies have been reported in domestic ducks in different parts of the world, which could explain the global distribution of the disease. Furthermore, the virus has been reported in geese and other species of wild waterfowl (15). Although many investigations aimed to establish adenovirus infection in various human cell lines, there is limited knowledge about EDSV infection in cells derived from birds. The results of our study showed that EDSV could reproduce in DEF but not in CEF cells. The virus efficiency reached its peak in the fourth passage and then decreased in the following passages. In addition, EDSV

caused clear CPEs on primary DEF cells. We evaluated the proliferation of EDSV in this primary cell line by different quantitative virus titer methods, and the obtained results are supported by other studies (16, 17).

EDS is one of the most important economic diseases in the poultry industry. Aghakhan et al. reported EDS in Iran and strongly emphasized the regular vaccination of herds of mother chickens and commercial layers against EDSV (18). More than 440 differentially expressed genes (DEGs) were recognized after EDSV contamination. These DEGs were related to numerous biological functions, such as signal transduction, host resistance, virus infection, cell apoptosis, cell proliferation, as well as pathogenicity-associated and other metabolic process signaling pathways (12).

The result revealed that EDSV can replicate in both primary DEF and CEF cells and that primary DEF cells are one of the most important cell lines for EDSV replication, which was confirmed by previous studies (16).

EDS cell culture-based vaccines can reduce concerns about restrictions on the use of eggs for virus replication. Cell-based viral vaccines have significant advantages over egg vaccines (19), including faster growth, larger production, reduction of allergic egg components, elimination of different stages of virus culture in embryonated eggs, such as incubation, candling, refrigeration, inoculation, and harvesting the virus from eggs, and a reasonable cost (20). Various viral vaccines, such as measles, mumps, rabies, tick-borne encephalitis, influenza, polio, and corona, which are based on cell culture, are currently available. Some of these vaccines, such as rubella, polio, and hepatitis A, are produced using human cell lines, some others, such as rotavirus and seasonal flu, are produced in mammalian cell lines, and some, such as measles and mumps, are made using bird cell lines. Therefore, most vaccines made from avian cell lines are propagated in avian fetal fibroblasts (21).

According to the results of HA and $ECID_{50}$, the proliferation of EDSV happened in the DEF cell line, and there was a significant difference between duck egg embryo and CEF cell culture, as well as SPF embryonated eggs (22). The application of the cell cycle and apoptosis induction are the main processes selected by many viruses to take superiority of the host cell and ensure their reproduction and spreading. Previous studies described that adenovirus provides a pattern to explain the probable mechanisms of apoptosis, which are possibly common to many virus-host relations, but the details are still unclear (23, 24).

HA, $ECID_{50,}$ and hemagglutination inhibition could be used effectively by avian diagnostic laboratories for monotonous monitoring of poultry farms, EDS vaccine controlling, and the investigation of EDS vaccination schedules in poultry production companies. The rate of virus yield of all EDSV groups was measured by standard HA and $ECID_{50}$, which could be used easily to measure the growth rate of EDSV in primary cell cultured systems.

The results of this study indicated that HA and $ECID_{50}$ are accurate, sensitive, specific, and inexpensive tests for the rapid titration of EDSV. PCR, HA, and $ECID_{50}$ were also convenient methods for the isolation and titration of EDSV, which could also be used in virus laboratories (25, 26).

All the harvest allantois-amniotic of the study groups were examined by HA. The results of the fluid harvested from the duck and SPF embryos were "1:65536" and "1:32", and the results of $ECID_{50}$ fluid harvested from the duck and SPF embryos were 10 and 3, respectively. These results revealed that DEF cells have a much higher capacity for EDSV replication.

The primary DEF cell culture system is consistently susceptible to EDSV proliferation and could be used to detect other viruses found in domestic and wild birds. It has several advantages over the egg embryo for virus proliferation. The most important one is that it is less expensive than preparing a large number of fertilized eggs. It is also faster, more cost-effective, and available, without the allergenic potential of egg proteins. In addition, different stages of virus culture in

embryonated eggs can be eliminated, such as incubation, candling, refrigeration, inoculation, and harvesting of the virus from eggs. The quantity and quality of EDSV harvested from duck egg embryos, as well as the primary DEF cell line, was much higher, compared to SPF eggs in HA and $ECID_{50}$. Therefore, it is suggested that the primary DEF cell line is more suitable for the replication of EDSV, compared to egg embryos.

Authors' Contribution

Study concept and design: N. R.
Acquisition of data: A. A. R.
Analysis and interpretation of data: R. A.
Drafting of the manuscript: J. Sh.
Critical revision of the manuscript for important intellectual content: A. A. R.
Statistical analysis: M. M.
Administrative, technical, and material support: N. R.

Ethics

This study was approved by the ethics committee of the Islamic Azad University, Shabestar, Shabestar, Iran.

Conflict of Interest

Due to the undeniable role of razi vaccine and serum research institute in launching and providing, all rights of utilizing low pathogenic avian adenovirus EDS-76 belongs to this institution.

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