TRIALS FOR PROPAGATION AND ADAPTATION OF EGG DROP SYNDROME VIRUS EDS-76 ON CHICKEN EMBRYO FIBROBLAST CELL CULTURE AND SPF EMBRYONATED CHICKEN EGGS

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Abstract

Duck embryo adapted strain of EDS-76 (EID_{50} 10^6/ml) was propagated and adapted on chicken embryo fibroblast. Thirty serial passages were prepared and titrated. After every 5 passages, the virus was inoculated into chickens 21 days old and observed for 21 days post-infection, then, challenged with a virulent EDS-76 virus. The 24th passage induced complete loss of virus pathogenicity and gave 100% protection, and used for the preparation of a live attenuated and inactivated oil emulsion chicken embryo fibroblast (CEF) cell culture (EDSV) vaccines. The evaluation of the prepared vaccines was carried out for sterility, safety and potency. The potency test was performed by measuring the cellular and humoral immune response, as well as, the protection percentage after challenge. The efficiency of the prepared vaccine was estimated up to six months. Passage number 15 and passage number 30 of EDS virus adapted on CEF cell culture were inoculated on SPF embryonated chicken eggs propagation and titration for successive seven passages. Haemagglutination (HA) was estimated to collected materials (allantoic fluids-chorioallantoic membrane-embryonic extract) by rapid haemagglutinating EDS virus and slow quantitative Haemagglutination reaction.

INTRODUCTION

EDS virus is the sole member of the subgroup III avian adenoviruses. It is not related serologically to the subgroup I and subgroup II viruses. Only one serotype of EDS virus has been recognized.

EDS-76 is a disease of laying hens characterized by a sudden and frequent drop in egg production with lying of soft shelled eggs (Van Eck *et al.*, 1976), and the effect on egg production persists for 4-10 weeks. In Egypt, Ahmed (1995) succeeded for the first time to isolate EDS virus from chicken farms.

EDS-76 virus grew well on chicken embryo liver cells, chicken kidney cells and chicken embryo fibroblast cells (Isak and Kiasry, 1981). Vaccines were evaluated in broiler with neutralizing antibody response and challenge reaction (Kaur *et al.*, 1997).

EDS-76 virus adapted on chicken embryo fibroblast cells was inoculated on SPF embryonated chicken eggs and propagated EDS-76 virus on SPF embryonated chicken eggs for 10 successive times (Wo, 1995) .

The aim of the present study is to prepare and evaluate an attenuated and inactivated EDS vaccine on chicken embryo fibroblast cells and trying to cultivate and propagate the adapted EDS virus on CEF cells on SPF (ECE).

MATERIALS AND METHODS

1. **Chicks**: four hundred susceptible 21-days old Hubbard chicks were used for vaccine evaluation.
2. **Virus strain:** EDS-76 virus strain was supplied by the Central Veterinary Laboratory, Weybridge, England with a titer of 106.

3. Embryos

a. Embryonated chicken specific pathogen free (SPF) eggs were obtained from Ministry of Agriculture, Koum Osheim, Fayoum, Egypt. Nine to 10 days old SPF-ECE were used for preparing chicken embryo fibroblast cell culture according to Plowright and Ferris (1959), and also, used for propagation of EDS-76 virus.

b. Embryonated duck eggs: they were obtained from United Company for Poultry Production, and used for propagation and titration of EDS-76 virus.

4. Cell culture media, reagents and solutions

a. Minimum Essential Medium (MEM): it was used as growth medium with 10% newborn calf serum and as a maintenance medium with 2-3% newborn calf serum in pH 7.2. It was supplied by Sigma.

b. Hank's balanced salt solution (HBSS): it was used for virus titration and was prepared according to Hank and Wallace (1949).

c. Trypsin (1:250): it was used in primary cell culture preparations at a concentration of 0.25% according to Lennette (1964).

Methods

1. Adaptation and propagation of EDS-76 on chicken embryo fibroblast for thirty passages: Titration on tissue culture (Pedro and Graham, 1980) and experimental infection in susceptible chicken were carried out on different passages.
2. Preparation of different types of vaccines was carried out as follows

a. A live attenuated EDS-76 on CEF (the 30th passage according to Isak and Kiarsy (1981).

b. Inactivated oil emulsion EDS-76 on CEF according to Rozhdest Venskii (1984).

c. Inactivated oil emulsion EDS-76 on embryonated duck eggs according to Rozhdest Venskii (1984).

The previous vaccines were tested for sterility according to OIE and for immunological effect with serum neutralization test (Rossiter *et al.*, 1985), haemagglutination inhibition (Anon, 1971), lymphocyte blastogenesis assay (Lee, 1984), protection percent and keeping quality (Lee and Hopkins, 1982).

RESULTS

Table 1. Propagation and titration of EDS-76 virus in chicken embryo fibroblast cells (CEF).

No. of passages	Time of harvesting post-inoculation (day)	Log_{10} $TCID_{50}$/ml
1	4	4
5	3	9
10	2	12
15	2	12
20	2	12
25	2	12
30	2	12

- Infectivity titer of EDS-76 virus propagated in embryonated duck eggs for three passages EID_{50} (10^5-10^5-10^6).
- Original (EDS) virus 10^6 EID_{50} in embryonated duck egg.
- The virus titre was calculated according to Reed and Muench (1938).

Table 2. Experimental infection on 21 days old chicks with EDS-76 virus propagated on chicken embryo fibroblast cells (CEF).

No. of passages	No. of chicks used	No. of dead chicks	Mortality percent	No. of contact control not challenged	No. of dead contact control
1	10	10	100	3	3
5	10	10	100	3	3
10	10	2	20	3	1
15	10	4	40	3	1
20	10	0	0	3	0
22	10	0	0	3	0
23	10	2	20	3	0
24	10	0	0	3	0
25	10	0	0	3	0
30	10	0	0	3	0

Table 3. The result of the challenge test of inoculated chickens, 21 days post-inoculation with propagated egg drop syndrome (EDS-76) virus on chicken embryo fibroblast cells (CEF).

No. of passages	No. of challenged chicks	No. of dead chicks	Mortality percent	No. of challenged control	No. of dead challenged
10	8	2	20	3	3
15	6	2	30	3	3
20	10	0	0	3	3
22	10	0	0	3	3
23	8	0	0	3	3
24	10	0	0	3	3
25	10	0	0	3	3
30	10	0	0	3	3

Table 4. Sterility of the prepared EDS vaccine.

Media	Living attenuated	Inactivated oil emulsion	
	CEF cells propagated vaccine	CEF cells propagated vaccine	Embryonated duck eggs vaccine
Nutrient agar medium	NC	NC	NC
Tioglycollate broth	NT	NT	NT
Sabauroud's glucose agar	NC	NC	NC
Grey media	NC	NC	NC

NC = no colonies NT = no turbidity (CEF) = chicken embryo fibroblast

Table 5. Lymphocyte blastogenesis of chicks vaccinated with the prepared vaccines.

Groups	Type of vaccine used	Weeks post vaccination		
		1	2	3
1	Live attenuated EDS-76 propagated on CEF cells	0.172	0.476	0.273
2	Inactivated oil emulsion EDS-76 propagated on CEF cells	0.408	0.672	0.245
3	Inactivated oil emulsion EDS-76 propagated on duck eggs	1.040	0.480	0.268
4	Control	0.10	0.10	0.10

Table 6. Log_2 mean neutralizing antibody titers of sera from vaccinated chicks with different prepared vaccines.

Groups	Types of used vaccines	Weeks post-vaccination																	
		1	2	3	4	5	6	7	8	9	10	11	12	14	16	18	20	22	24
1	Live attenuated EDS-76 on CEF cells	10	11	11	12	12	12	12	12	12	12	12	12	12	12	12	12	12	12
2	Inactivated oil emulsion EDS-76 on CEF cells	5	8	10	12	12	12	12	12	12	12	12	12	12	12	11	10	10	10
3	Inactivated oil emulsion EDS-76 on duck eggs	4	4	8	6	11	12	12	12	12	12	12	12	12	12	10	10	10	10
4	Control non-vaccinated	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Table 7. Mean HI antibody titers (log_2) of sera from chickens vaccinated with different prepared EDS-76 virus vaccines.

Groups	Types of used vaccines	Weeks post-vaccination																	
		1	2	3	4	5	6	7	8	9	10	11	12	14	16	18	20	22	24
1	Live attenuated EDS-76 on CEF cells	5	6	8	8	9	9	10	8	8	7	7	7	7	7	6	6	5	5
2	Inactivated oil emulsion EDS-76 on CEF cells	5	5	7	9	11	8	8	7	7	7	6	6	6	6	6	5	5	5
3	Inactivated oil emulsion EDS-76 on duck eggs	9	10	9	8	8	6	7	7	7	7	7	7	7	7	6	6	5	5
4	Control non-vaccinated	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Table 8. Rate of protection for the prepared EDS-76 virus vaccines.

Group	Types of used vaccine	1st month		2nd month		3rd month		4th month		5th month	
		No. of challenged chicks	Protection %	No. of challenged chicks	Protection %	No. of challenged chicks	Protection %	No. of challenged chicks	Protection %	No. of challenged chicks	Protection %
1	Live attenuated EDS-76 on CEF cells	5	100	5	100	5	100	5	100	5	100
2	Inactivated oil emulsion EDS-76 on CEF cells	5	100	5	100	5	100	5	100	5	100
3	Inactivated oil emulsion EDS-76 on duck eggs	5	100	5	100	5	100	5	100	5	100
4	Control non-vaccinated	5	0	5	0	5	0	5	0	5	0

Table 9. Keeping quality of prepared EDS-76 virus vaccines (months).

Group	Types of used vaccine	Temperature of storage	Protection percentage											
			1 m		2 m		3 m		4 m		5 m		6 m	
			S	%	S	%	S	%	S	%	S	%	S	%
1	Live attenuated EDS-76 on CEF cells	-20 °C	5/5	100	5/5	100	5/5	100	5/5	100	5/5	100	5/5	100
2	Inactivated oil emulsion EDS-76 on CEF cells	+ 4 °C	5/5	100	5/5	100	5/5	100	5/5	100	5/5	100	5/5	100
3	Inactivated oil emulsion EDS-76 on duck eggs	+ 4 °C	5/5	100	5/5	100	5/5	100	5/5	100	5/5	100	5/5	100
4	Control non-vaccinated	-	0/2	0	0/2	0	0/2	0	0/2	0	0/2	0	0/2	0

S = survived chickens m = month

Table 10. Propagation, infectivity and haemagglutination activity of [adapted EDS virus on CEF cells] on SPF embryonated chicken eggs.

Passage 15 on CEF cells									
Passage No.	No.	+ve	HA slide			HA plate			EID_{50}/ml
			F	E	M	F	E	M	
1st	10	10	10	-	-	3	2	2	3.80
2 day									
2nd	10	10							
2 day		3	3/3	1/3	1/3	3	2	2	
3 day		3	2/3	2/3	2/3	3	2	2	
4 day		3	2/2	2/2	2/2	5	4	4	
5 day		-	--	--	--	--	--	--	
6 day		2	2/2	2/2	2/2	3	1	1	3.80
3rd	10	10							
2 day		2	2/2	1/2	1/2	2	1	1	
3 day		3	3/3	2/3	2/3	4	3	3	
4 day		2	2/2	2/2	2/2	5	3	3	
5 day		1	1/1	1/1	1/1	2	5	3	
6 day		2	2/2	2/2	2/2	2	4	3	3.60
4th	10	10							
2 day		2	2/2	1/2	1/2	2	2	2	
3 day		2	2/2	2/2	2/2	2	2	2	
4 day		--	--	--	--	--	--	--	
5 day		--	--	--	--	--	--	--	
6 day		6	6/6	4/6	4/6	3	2	3	5.70
5th	10	10							
2 day		--	--	--	--	--	--	--	
3 day		--	--	--	--	--	--	--	
4 day		2	2/2	2/2	2/2	11	6	7	
5 day		2	2/2	2/2	2/2	11	6	7	
6 day		--	--	--	--	--	--	--	
7 day		6	2/6	2/6	2/6	7	3	3	8.30
6th	10	10							
2 day		3	3/3	1/3	1/3	5	2	2	
3 day		3	3/3	2/3	2/3	7	3	3	
4 day		1	1/1	1/1	1/1	9	3	2	
5 day		1	1/1	1/1	1/1	10	3	2	
6 day		2	2/2	1/2	1/2	6	3	2	7.50
7th	10	10							
2 day		--	--	--	--	--	--		
3 day		3	1/3	2/3	2	7	5		
4 day		4	3/4	3/4	4	6	7		
5 day		--	--	--	--	--	--		
6 day		3	1/3	1/3	5	4	5		7.80

F = allantoic fluid
E = embryonic extract
M = chorioallantoic membrane

Table 10. Contd.

Passage 30 on CEF cells									
Passage No.	No.	+ve	HA slide			HA plate			EID_{50}/ml
			F	E	M	F	E	M	
1st	10	10	10	--	--	4	3	3	3.50
2 day									
2nd	10	10							
2 day		3	3/3	1/3	1/3	3	2	2	
3 day		2	2/2	2/2	2/2	3	2	2	
4 day		2	2/2	2/2	2/2	3	3	3	
5 day		3	3/3	3/3	3/3	3	3	3	
6 day		--	--	--	--	--	--	--	4.10
3rd	10	10							
2 day		2	2/2	1/2	1/2	2	1	2	
3 day		3	3/3	2/3	2/3	4	3	2	
4 day		2	2/2	2/2	2/2	5	3	3	
5 day		1	1/1	1/1	1/1	2	5	3	
6 day		2	2/2	2/2	2/2	2	4	3	4.10
4th	10	10							
2 day		2	2/2	1/2	1/2	3	3	3	
3 day		2	2/2	2/2	2/2	3	5	3	
4 day		--	--	--	--	--	--	--	
5 day		--	--	--	--	--	--	--	
6 day		6	6/6	5/6	5/6	5	4	3	6.10
5th	10	10							
2 day		--	--	--	--	--	--	--	
3 day		4	4/4	2/4	4/4	7	4	5	
4 day		--	--	--	--	--	--	--	
5 day		1	1/1	1/1	1/1	11	7	7	
6 day		2	1/2	1/2	1/2	8	5	4	
7 day		3	1/3	1/3	1/3	6	3	3	8.50
6th	10	10							
2 day		2	2/2	1/2	1/2	5	2	2	
3 day		2	2/2	1/2	1/2	6	4	5	
4 day		4	3/4	2/4	3/4	8	3	5	
5 day		2	2/2	2/2	2/2	8	4	7	
6 day		--	--	--	--	--	--	--	
7 day		--	--	--	--	--	--	--	8.50
7th	10	10							
2 day		6	5/6	4/6	4/6	7	2	2	
3 day		6	2/2	2/2	2/2	9	3	2	
4 day		2	2/2	2/2	2/2	9	3	9	
5 day		--	--	--	--	--	--	--	
6 day		--	--	--	--	--	--	--	
7 day		--	--	--	--	--	--	--	7.80
8th	10	10							
2 day		3	3/3	2/3	3/3	8	7	7	
3 day		7	6/7	6/7	5/7	11	11	9	
4 day		--	--	--	--	--	--	--	
5 day		--	--	--	--	--	--	--	
6 day		--	--	--	--	--	--	--	
7 day		--	--	--	--	--	--	--	9
9th	10	10							
2 day		--	--	--	--	--	--	--	
3 day		2	2/2	1/2	1/2	7	5	6	
4 day		5	5/5	3/5	4/5	9	7	7	
5 day		3	3/3	3/3	3/3	10	8	9	
6 day		--	--	--	--	--	--	--	
7 day		--	--	--	--	--	--	--	9.30
10th	10	10							
2 day		--	--	--	--	--	--	--	
3 day		4	3/4	2/4	3/4	8	4	5	
4 day		3	3/3	2/3	3/3	10	5	7	
5 day		3	3/3	2/3	2/3	11	7	7	
6 day		--	--	--	--	--	--	--	
7 day		--	--	--	--	--	--	--	9.50

DISCUSSION

EDS is a disease of laying hens which causes great economic losses due to sudden drop of egg and inferior quality of egg shell (Van Eck *et al.,* 1976). The aim of the present study was to adapt and propagate EDS on chicken embryo fibroblast and, to prepare safe and protective EDS live and inactivated vaccines.

The duck adapted strain of EDS-76 was propagated on CEF for 30 passages. At the 24^{th} passage to 30^{th} passages, the virus lost its pathogenicity. The high passages on CEF cells over 24^{th} induced loss of pathogenicity. The diversity was from 100% protection with the 24^{th}, but, still being protective for susceptible chicks.

Table 1 showed that the titer of the propagated EDS virus on chicken embryo fibroblast cells (CEF) increased from passage 5^{th} P (10^{9}) and to reach its maximum (10^{12}) from 10^{th} passage to 30^{th} passage ,while, the harvesting time was declined by serial passages from 4 to 3 days, and fixed at 2 days from 10^{th} to 30^{th} passage. These results are in agreement with those obtained by Nancy *et al.* (2003).

Tables 2 and 3 showed that the EDS virus lost its virulence gradually from the 1^{st} to 15^{th} passage and completely from 20-30 passage, but still became immunogenic. These results are in agreement with those obtained by Nadia (2004).

EDS virus from passage number 22 to passage number 24 was used for determining the most safe passage that did not induce any mortality when inoculated in susceptible chicks. Passage number 22 did not induce any mortality, but passage number 23 induced 20% mortality, while passage 24-25 did not induce any mortality. Therefore, the best safe passage of choice was the passage number 24.

The prepared vaccine was tested for sterility, and proved to be free from any contaminants (Table 4). It was noticed in table 5 that the high value of lymphocyte blastogenesis for three weeks post-vaccination was shown with the prepared vaccines when compared with control (Table 5) (Umesh-Kumar *et al.*, 1989), where inactivated EDS-76 on CEF gave higher value in first, second weeks than inactivated EDS-76 on duck egg or live attenuated EDS-76 on CEF.

Tables 6 and 7 showed the evaluation of the humoral immune response of chicken vaccinated with tissue culture vaccines in comparison with other commercial vaccines. It was clear that the mean neutralizing antibody titer $\log_2$ in sera of vaccinated chicks with the live prepared vaccine was higher than the others from the 1^{st} two weeks post- vaccination till 24^{th} week. Then, the value of neutralizing antibody was increased in all groups with maximum value 10^{12} till the 24^{th} week, while the control group was zero.

Also, a high titer value of haemagglutination inhibition titer was noticed in vaccinated groups for 24^{th} weeks. These results agree with Adair *et al.* (1986).

Table 8 shows that chicks vaccinated with different prepared vaccines gave a high protection percentage reaching 100% when challenged with virulent EDS virus after 3 weeks. The challenged chick did not show any abnormalities, while the non-vaccinated group did not show any protection. This result agreed with Brugh *et al.* (1983).

By Discussing the keeping quality of the prepared live attenuated and inactivated EDS virus vaccines when stored at -20 °C and +4 °C (Table 9), it was clear that the vaccines were stable and potent for a period of 6 months when the protection reached 100%.

Table 10 showed the results of the propagation of adapted EDS virus on CEF cells passage 15 and passage 30 on SPF (ECE) for 10 passages, being each passage inoculated on 10 SPF (ECE) eggs. The First passage on SPF (ECE) of adapted passage 15 and passage 30 made complete death to all inoculated eggs within 2 days. The allantoic fluids gave positive HA, but, embryonic and membranes supernatants gave negative HA. In the 2^{nd} passage on SPF (ECE), some inoculated eggs died within the first 2 days and some within 3, 4, 5, 6 days in passages (15 and 30).

Rapid HA (allantoic fluid, embryonic and membranous extract) gave positive result, and, also, quantitative HA gradually increased from the second till the fifth day, but the quantity decreased from the sixth day of inoculation.

From the third passage on SPF (ECE) till the tenth passage, there was gradual decrease in the number of dead eggs through 48 hours, but inoculated eggs still lived till six to seven days after incubation.

There was gradual increase in the level of HA quantity in allantoic fluid, embryonic membranes extract, but, after six to seven days of inoculation there was decrease in quantity HA.

Infectivity titer EID_{50}/ml gradually increased from passage one ($10^{3.50}$) to passage two ($10^{4.10}$) till reached to $10^{9.50}$ in passage ten.

It was noticed that the rate of dead inoculated embryos was high in the first two days, then, declined through 3, 4, 5 and still survived on 6^{th} and 7^{th} days. The HA activity was ranging from $2^2 - 2^4$ at the beginning, then, the value increased to its maximum (2^{11}). These results were parallel with the value of EID_{50} which ranged from 3.60/ml at the beginning till reached its maximum $10^{8.30}$/ml and $10^{9.50}$/ml.

Therefore, the suitable time for harvesting the inoculated eggs is 4-5 days after inoculation which provide high infectivity level of haemagglutination activity and high titer. This result agreed with Wo (1995).

We could conclude that the 24^{th} attenuation passage of EDS virus on CEF cells and the preparation of live attenuated and inactivated oil emulsion vaccines gave safe,

potent and immunogenic vaccines. So, we could use the cell culture system instead of the embryonated duck eggs.

We could conclude that the adapted EDS virus on CEF cells could propagate on SPF (ECE) and could give high infectivity titer and high haemagglutination activity. Therefore, we could use the SPF (ECE) system instead of embryonated duck eggs, and we recommend the preparation of EDS vaccine on CEF instead of embryonated duck eggs.

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محاولات لتمرير فيروس مرض تدني البيض في الدواجن (EDS-76) على خلايا أجنة الكتاكيت والبيض المخصب الخالي من المسببات المرضية

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تم تمرير فيروس مرض تدني البيض في الدجاج على خلايا أجنة الكتاكيت ٣٠ تمريرة مع عمل معايرة للفيروس-وقد تم حقن كل ٥ تمريرات في كتاكيت عمر ٢١ يوماً وقد تمت ملاحظتها لمدة(٢١ يوما") ثم حقنها بالفيروس الضاري . وقد وجد أن التمريرة رقم ٢٤ قد فقدت القدرة المرضية وأعطت حماية ١٠٠% للكتاكيت المحقونة واستخدمت لتحضير لقاح حي مستضعف وكذلك لقاح مثبط على خلايا أجنة الكتاكيت. تم تقييم اللقاح المحضر لقياس المناعة الخلوية لمدة ٦ أشهر وباستخدام تجربة التحدي بالفيروس الضاري. وقد تم حقن التمريرة رقم ١٥ ورقم ٣٠ على خلايا أجنة الكتاكيت في البيض الخالي من المسببات المرضية، التمريرة رقم ١٥ تم تمريرها في أجنة البيض ٧ تمريرات وتمريرة رقم ٣٠ على خلايا أجنة الكتاكيت ثم تمريرها ١٠ تمريرات في أجنة البيض وقد تم قياس القوة العيارية وقوة التلازن الدموي لكل تمريرة على حدة.

من النتائج السابقة وجد أنه يمكن استخدام خلايا الزرع النسيجي و خاصة خلايا أجنه الكتاكيت في تمرير فيروس مرض تدني البيض في الدواجن (EDS 76) و كذلك أمكن استخدام أجنه البيض الخالي من المسببات المرضية في تمرير الفيروس بدلاً من استخدام أجنة بيض البط.

كما أمكن تحضير لقاح حي مستضعف و لقاح مثبط علي خلايا أجنة الكتاكيت و قد أثبت اللقاح أنه آمن و ذوكفأة عالية ويعطي رد فعل مناعي يكفي لحماية الكتاكيت المحصنة مدة لا تقل عن ستة أشهر .