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STUDIES ON THE VACCINAL STRAIN OF REOVIRUS

(With 6 Tables)

By

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دراسات على لقاح الربو

ناديه حسن ، محمد ابو الخير ، فكرية البرديني ، سلوى الاصيلي
انصاف خشبة ، صلاح وهبه ، سامي صابر

اجريت هذه الدراسة لمعرفة انسب الظروف لحفظ ونمو لقاح الربو . واثبتت النتائج أن احسن طريقة لحقن الفيروس على الغشاء الالنتوسي، والحويف الامنيوسي وكان احسن تخفيف للفيروس لحقن الاجنة هو ٣٠ . واتضح ان اليوم الرابع هو انسب يوم لتجميع الفيروس. وقد اعطى الخليط المكون من الجنين والغشاء الالنتوسي والسائل الالنتوسى اعلى قوة عيارية للفيروس. ومن الدراسة اتضح أن اللبن الخالي من الدسم هو افضل مادة حافظة للفيروس. وقد لوحظ أن الفيروس يقاوم درجة حرارة ٥٦ م لمدة ٨ ساعات.

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STUDIES ON THE VACCINAL STRAIN OF REOVIRUS

SUMMARY

The present study was concerned with the detection of the most suitable condition for propagation and preservation of vaccinal strain of Reovirus. The most suitable route of inoculation for embryonated chicken eggs (ECE) was chorio-allantoic membrane (CAM) and allantoic cavity (AC) giving an infective titer $10^{7.5}$ and $10^{7.4}$/ ml EID_{50} respectively. The best dilution of Reovirus used for egg inoculation was 10^{-3}. Determination of the best time for harvesting the virus was the fourth day post inoculation. The suitable organ for virus multiplication was embryo and mixture of embryo plus CAM and AAF giving a titer of $10^{7.82}$ and EID_{50}/ml respectively. Studies on the effect of using different stabilizers on the EID_{50} of lyophilized virus showed that skimmed milk gave the highest EID_{50} ($10^{7.4}$/ml). Stability of Reovirus at 56 °C for 8 hours.

INTRODUCTION

Avian reoviruses are prevalent worldwide being found in most commercially reared poultry populations and propably in many other avian species. Reovirus comes from the abbreviation for Respiratory-Enteric-Orphan virus.

In Egypt, the disease was reported by *KHEIR EL-DIN and EL-SANOUSI (1987) and BEKHITE* in *(1988)*. The disease was first recognized as the cause of viral arthritis (VA) by *OLSON* et al. *(1957)*. Malabsorption syndrome was linked to avianreo viruses in *(1981)* by *VAN DER HEIDE* et al. Viral Arthritis is often refered to as teno synovitis because it affects the synovial membrane around the tendon sheath.

The clinical signs in birds vary from gross lameness to drop in egg production and hatchability. Birds may become lame and die from dehydration and starvation.

Conditions other than classical VA can be caused by reo viruses as degeneration of the femur known Femoral head necrosis, a feathering problem known as "helicopter chick", encaphalomalacia known as creasy chick disease, and presence of poorly digested food in the intestine and red brick coloured diarrhea, this syndrome led to the term of malabsorption syndrome *(VAN DER HEID, 1981)*.

Recently during last 4-5 years reovirus associated disease have been changed in severity for broiler breeding males from 25-35 weeks of age resulting in lameness in 50-75 percent of the males, thereby drastically reducing successful mating, fertility and hatchability. In addition, there is also a gradual increase in the incidence of malabsorption syndrome in young pullets between 15 and 25 weeks of age resulting in a reducing body weight and delay the onset of lay, smaller eggs and poor egg production peak.

Breeder vaccination with reovirus vaccine prevent reovirus infection in such breeder chickens during production and resulted in subsequent egg transmission of reovirus, leading to decrease hatchability, young chick mortality and incidence of VA at 7 to 14 days of age in progony broiler.

To induce maternal immunity in chicks, a breeder vaccine was developed and modified by *VAN DER HEID et al. (1976).* This vaccine was recomended at 10-17 weeks of age, because breeder vaccines are not completely pathogenic for young chicks and when it was given at a very early age induce lesions of VA with the advent of inactivated reovirus vaccines, emphasis was placed for stimulating serum antibody titer in breeders and their progony *PAGE et al. (1982).*

The object of the present study was to investigate the physico-chemical and biological properties of reovirus modified vaccinal strain for further production of local reovirus vaccine.

MATERIAL and METHODS

MATERIAL:

1- Fertile chicken eggs: Fertile commercial chicken eggs were obtained from the United Company for Poultry Production (UCPP), they were inoculated through polk sac, chorio-allantoic membrane or allantoic sac routes.

2- Vaccinal strain of reovirus: A modified egg adapted live and lyophilized virus produced in specific pathogen free eggs (SPF) kindly supplied by Intervet Company., Batch No. 1004, 1000 doses strain 1133 its titer was 10^6/ml EID_{50}.

3- Stabilizer: Three kinds of stabilizers were used in this study:

(a) Skimed milk: Produced in Asketon Company Limerk Irland Wyeth SMA. It was used as 15% dilution in distilled water and sterilized by autoclaving, then added to the virus fluid as 10% dilution.

(b) Gelatine: Obtained from M.S.C. Lab. "Gelatin pure" and prepared as 20% in distilled water, sterilized by autoclaving and used as 10% with the vaccine.

(c) Lactalbumin: Lactalbumin consists of sucrose 20% and lactalbumin 10%. Disolved in distilled water in a water bath at 70-80°C then autoclaving at 121°C and 2 1b presure for half and hour. Kept in incubator at 37°C till used as 10% with the vaccine.

METHODS:

1- Virus titration: Serial ten fold dilutions of the virus from 10^{-1} through 10^{-8} were prepared in sterile saline containing 1000 I.U. crystalline penicillin plus 1000 mg streptomycine sulphate per/ml, five 9-10 days old embryonated chicken eggs were sealed and incubated at 37°C, candling was done daily for 6 days. Embryonic deaths within 24 hours post inoculation were considered non specific deaths and discarded. There after dead embryos were removed daily, recorded and kept at 4°C until the end of incubation period.

Both the surviving embryos and the dead ones were examined thoroughly for criteria taken as indicator for infection by avian reovirus according to *DESHUMKH and POMEROY (1969).*

EXPERIMENTS

Experiment No. 1:

1) Determination of the effect of routes of inoculation on EID_{50} of reovirus: Using chorio-allantoic membrane (CAM), yolk sac (Y.S.) and allantoic sac (A.S.) routes. The age of embryonated chicken eggs were 6 days yolk sac and 10 days old for both CAM and A.S.

For each route 20 embryos were inoculated, the virus was diluted 10^{-3} and each egg received 0.2 ml in case of CAM, 0.1 ml in both Y.S. and A.S. Eggs were incubated at 37°C, candling was done daily for six days post inoculation.

The eggs of each route were harvested separately by collecting CAM and embryos in sterile containers. The contents of each container were grinded, hemogenized in laboratory mixture and frozen at -20°C. After repeated freezing and thawing 3 times, the suspension is clarified by centrifugation for 20 minutes at 2000 r.p.m. The supernatant is harvested and stored at -20°C prior to lyophilization and titration. The results are shown in table (1).

Experiment No. 2:

Choice of the most suitable virus dilution for inoculation: One hundred 10 days old embryonated chicken eggs were divided into 5 groups of 20 eggs each. Serial ten fold dilution of reovirus in normal physiological saline from 10^{-1} to 10^{-4} was done.

Each dilution together with the concentrated virus was inoculated in one group via chorio-allantoic membrane route, each egg received 0.2 ml of inoculum. Eggs were incubated and candling was done daily for 6 days. The eggs of each group were harvested separately by collecting the CAM and embryos, weighted, grinded and add equal volume of physiological saline and centrifuge for 15 minutes at 1500 r.p.m.

The supernatent fluid of each group was titrated in embronated chicken eggs via CAM using dilution 10^{-3} to 10^{-8}, and the embryo infective dose 50 (EID_{50}) were calculated by *REED and MUENCH (1938)*. The results are shown in table (2).

Experiment No. 3:

Determination of the best time for harvesting of reovirus:

In this experiment, the most suitable dilution evaluated from the previous experiment (10^{-3}) and the best route of inoculation (CAM) were used.

Sixty, 10 days old embryonated chicken eggs were inoculated with 10^{-3} reovirus vaccinal strain, via CAM. The eggs were incubated at 37°C and candled daily. 10 embryos were collected daily from incubator (5 alive and 5 dead if present).

The eggs were chilled immediately at 4°C, at the end of 6^{th} day post inoculation, every day, group was harvested separately collecting CAM, embryo, and fluids, grinded, centrifuged and titrated in 10 days old embryonated chicken eggs via CAM for virus content. The results are shown in table (3).

Experiment No. 4:

Choice of the best target organ for virus multiplication:

Ninty embryonated chicken eggs were used in this experiment. The eggs were divided into 2 groups, the first one was inoculated with reovirus vaccinal strain diluted 10^{-2} and divided into 2 subgroups which were inoculated via CAM and YS. The aforementioned procedure was performed with second group but using virus dilution 10^{-3}. The inoculated eggs were candled daily. Dead embryos during 24 hours P>I> were considered non specific death and discarded. Dead embryos were kept in refrigerator until the end of 6^{th} day incubation period.

Each group was harvested separately by collecting CAM, whole embryos, yolk and AAF. The CAM and embryos were washed by shaking in sterile saline solution to make them free from any virus contained in AAF. The separately harvested embryos and CAMs were separately grinded in blender with sterile sand and saline solution containing 1000 I.U. crystalline penicilline and 1.0 g of streptomycin sulphate/ 1 liter.

These suspension were centrifuged for 15 minutes at 2000 r.p.m. and the supernatent fluid was used as the material for titration. The AAF, yolk, CAM and whole embryos and mixture of CAM, AAF and whole embryos weretitrated in embryonated chicken eggs for calculation of EID_{50}. The results are shown in table (4).

Experiment No. 5:

Effect of using different stabilizer on the stability of lyophilized reovirus: The stabilizer used in this experiment were skimed milk, lactalbumin and gelatine. Each stabilizer was added to reovirus as 10% and lyophilized.

After lyophilization each stabilized vaccine and vaccine without stabilizer which used as control were titrated in ECE and the titers were calculated after *REED and MUENCH (1938).* The results are shown in table (5).

Experiment No. 6:

Effect of heat on EID_{50} of reovirus: Undiluted reovirus fluid was distributed in 2 ml amount in a number of ampoules and flame sealed. The ampoules were submerged in a water bath adjusted to 56°C with temperature deviation of not more than 0.5°C. The ampoules were removed at a selected intervals 15, 30, 60 and 90 minutes followed by one hour interval until the 17^{th} hour.

The ampoules were immediately chilled in ice or kept in freezer at -20°C until the titers were determined. Serial 10 fold dilutions from 10^{-1} to 10^{-8} of the heat treated fluid were inoculated in ECE for virus titration and at the same time non heated virus was titrated as a virus control. The EID_{50} were determined after *REED and MU ENCH (1938).* The results are showin in table (6).

RESULTS

Are presented in tables 1 - 6.

DISCUSSION

Egyptian field and laboratory data pointed clearly that reovirus infection is established as an evolving disease which requires well designed scientifically based on vaccination and control programmes.

The results of experiment (1) as shown in table (1) showed that, the route of inoculation influence clearly the development in chicken embryo. So, virus inoculated via CAM, showed small whitish pock lesions on CAM and sometimes embryonic odema and liver necrosis. While embryos inoculated

via YS at 6 days-old seems to be haemorrhagic and dwarfed. Embryonic mortalities ranged between 3-6 days post inoculation.

These results agreed with that results obtained by *OLSON and KERR (166)*. They use YS route for isolation of VA virus, using 5-6 days old embryonated chicken eggs. The embryo mortalities was 100% between 72 and 96 hours post inoculation, embryos were slightly dwarfed, purplish in colour with severe subcutaneous haemorrhage.

Allantoic sac inoculation route showed the same mortalities of the previous, 2 routes. In contrast to the results obtained by *DESHMUKH and POMEROY (1969)* who stated that allantoic cavity inoculation by reovirus did not results in embryonic death. The obtained EID_{50} were $10^{7.5}$, $10^{7.4}$, $10^{7.5}$ per 1 ml for CAM, YS respectively.

Although there was no difference in EID_{50} between the three routes, we prefere the use of CAM as recomended by *DESHMUKH and POMEROY (1969)* who stated that reoviruses could be passed serially on CAM of embryonated chicken eggs, where they produced plaques, multiplied to a high concentration, caused liver necrosis and death of the embryos.

Determination of the most suitable virus dilution for egg inoculation, and the results were recorded in table (2). The EID_{50}/ml were $10^{9.3}$, $10^{9.1}$, $10^{7.3}$, $10^{7.7}$ and $10^{7.6}$ for concentrated virus and dilution of 10^{-1}, 10^{-2}, 10^{-3} and 10^{-4} respectively. Although the concentrated virus and dilution 1:10 gave high virus titers which may reached 2 logs more than the other dilutions but due to the early high mortalities which reached to 80% and 73% of both concentrated and 10^{-1} respectively, beside the difficult handling of the dead embryos, decrease of the output quantity of virus yield and from the practical point, dilution 10^{-3} was the best dilution for the normal mortality rate and large quantities of the harvested virus with titer of $10^{7.7}$/ml EID_{50}.

Connerning the determination of the optimal time for harvesting the virus yield, table (3) showed that the infectivity titer continued to increase from the first day up to the fourth day post inoculation recording $10^{5.4}$, $10^{6.5}$, $10^{6.5}$ and $10^{6.8}$ respectively. The EID_{50} began to decrease from the 5th day to 7th day post inoculation. So, the best day for harvesting the virus yield was the 4th day post inoculation either for infectivity titer ($10^{6.8}$/ml) or for virus yield either from living or dead embryos which gave 13 and 10 ml allantoic fluid respectively.

Choice of the best target organ for virus multiplication as indicated in table (4) using dilution 10^{-2} and 10^{-3} and CAM & YS route of inoculation of the embryo and the mixture gave

$10^{7.8}$ and $10^{8.0}$ EID50/ml respectively, while in the groups of eggs inoculated via yolk sac gave 10^{7} EID50/ml. In micyutr in both 10^{-2} and 10^{-3} virus dilution and $10^{7.05}$ and $10^{7.6}$ EID50/ml for embryos also for both dilutions.

So, it is clear that the mixture of embryo, CAM and fluid was the best source giving 10^{8} EID50/ml when inoculated with 10^{-3} virus dilution.

Table (5) illustrates the effect of adding different common stabilizers used with reovirus and their effect on EID50 after lyophilization. The experiment showed that skimmed milk was the most suitable stabilizer used. These results were in agree with results obtained by *NARGIS* et al. *(1987)*, they made comparative experiment using the locally available gelatin beside the skimm milk. Both substances gave similar stabilizing effect on Newcastle disease virus (NDV). The calculated titers were $10^{7.4}$, $10^{6.9}$, $10^{7.0}$, $10^{6.2}$ EID50/ml for milk, lactalbumin, gelatin and vaccine without stabilizer respectively.

The physical stability of the reovirus vaccinal strain was measured through detection of EID50 after exposure for heating at 56°C for different period of times, table (6). It is clear that reovirus is stable at 56°C for 1 1/2 hour. The virus was heat resistant until the 8^{th} hours followed by detectable reduction of EID50 to $10^{4.5}$ EID50/ml. On the other hand, the virus was still surving for 17 hours giving a titer of 10^{-3} EID50 This result agreed with those results obtained by *OLSON and KERR (1966)* who used different degrees of temperature and different exposure times.

From the aforementioned results, it can be concluded that:

For preparation of reovirus vaccine it is recommended to use 9 to 11 days old embryonated chicken eggs inoculated either via Ac or CAM respectively. The seed virus ($10^{7.7}$ EID50/ml) used for inoculation is diluted to 10^{-3}. The inoculated eggs are harvested on 4^{th} post inoculation. Mixture of ECE, CAM and AAF have to be collected, homogenized and lyophilized with skimmed milk.

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Table (1).

Choice of the most suitable virus dilution for egg inoculation with reo virus.

Virus dilution	mortalities. Hours post inoculation						EID_{50}/1ml	MDT
	24	48	72	96	120	144		
Concentrated Virus	—	10	3	—	2.	—	$10^{9.3}$	2.6
10^{-1}	—	3	8	2	1	1	$10^{9.1}$	3.3
10^{-2}	—	2	5	3	3	—	$10^{7.3}$	3.5
10^{-3}	—		5	3	3	2	$10^{7.7}$	4.2
10^{-4}	—		1	2	2	1	$10^{7.6}$	4.5

MDT = meane death time.

Table (2): Effect of Route of inoculation on EID_{50}/ml of reo virus

Route	I	II	III	Mean titer
Chorio allantoic membrane CAM	$10^{7.5}$	$10^{7.55}$	$10^{7.6}$	$10^{7.5}$
Yolk sac YS	$10^{7.2}$	$10^{7.6}$	N.D	$10^{7.4}$
Allantoic Sac	$10^{7.6}$	$10^{7.5}$	N.D	$10^{7.5}$

N.D = Not done

Mean... = average of 3 experiments.

Table(3): Determination of the effect of harvest time on EID_{50} of reo virus harvest.

day of harvest	EID_{50}/ ml			Mean EID_{50} 1/ ml
	I	II	III	
1st	$10^{6.75}$	$10^{4.66}$	$10^{4.8}$	$10^{5.4}$
2nd	10^{6}	$10^{6.6}$	$10^{6.8}$	$10^{6.5}$
3rd	$10^{7.2}$	$10^{5.5}$	$10^{6.7}$	$10^{6.5}$
4th	$10^{7.8}$	10^{6}	$10^{6.6}$	$10^{6.8}$
5th	10^{7}	$10^{5.8}$	$10^{6.5}$	$10^{6.4}$
6th	10^{6}	ND	ND	10^{6}
7th	10^{6}	ND	ND	10^{6}

ND = Not done

Mean=average of 3 experiment.

Table(4):Choice of best source for virus multiplication.

Dilution	Route of Inoculation									
	Chorioallantion membrane CAM					Yolk sac				y.s
	embry	yolk	Memb	fluid	Mixt	embry	yolk	Memb	fluid	Mirture
10^{-2}	6.45	7.45	6.45	6.45	6.48	7.05	5.82	7.3	5.58	7
10^{-3}	7.82	7.7	7.2	5.6	8	7.6	5.5	5.85	5.48	7

Table(5): Effect of using different stabilizer on the stability of lyophilized reovirus vaccine

Type of stab.	I	II	III	Mean titer EID_{50}/ ml
Skimmed milk	10^{7}	$10^{7.66}$	$10^{7.5}$	$10^{7.4}$
Lactw albumine	$10^{6.3}$	10^{8}	$10^{6.5}$	$10^{6.9}$
gelatine	$10^{6.6}$	$10^{6.6}$	$10^{6.8}$	10^{7}
vaccine control without stabilizer	$10^{5.8}$	$10^{6.4}$	$10^{6.5}$	$10^{6.2}$

Table (6)

Effect of heat (56 d) on the EID_{50} of reovirus.

Time	EID_{50}	Log reduction
0 (orginal)	$10^{6.8}$	0
15 minutes	$10^{6.37}$	0.4
30 minutes	$10^{6.5}$	0.3
1 hour	$10^{6.4}$	0.4
1½ hours	$10^{6.4}$	0.4
2 hours	$10^{5.8}$	1.0
3 hours	$10^{5.5}$	1.3
4 hours	$10^{5.5}$	1.3
7 hours	10^{5}	1.8
8 hours	$10^{4.5}$	2.3
9 hours	10^{3}	3.8
17 hours	10^{3}	3.8