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STUDIES ON A LIVE VIRUS VACCINE AGAINST INFECTIOUS BRONCHITIS

(With 10 Table)

By

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دراسات على اللقاح الحى ضد مرض الالتهاب الشعبى

أنصاف خشبه ، سلوى الأصيل ، فكريه البردينى ،
الهام الإبيارى ، نادىه حسن

تمت دراسة خصائص اللقاح الحى لفيروس مرض الالتهاب الشعبى باستخدام عترة ماسوشتس وقد أدت النتائج إلى : كان أحسن تخفيف للقاح هو ١٠ وكانت أعلى قوة عيارية وصلت بعد ٤٠ ساعة من الحقن . وجد من النتائج أن أحسن مكان لتكاثر الفيروس هو السائل الامينوسى للجنين حيث وصلت القوة العيارية إلى ١٠ /مل بينما كانت القوة العيارية لتكاثر الفيروس على الغشاء الالنتوسى أو فى التجويف الالنتوسى هى ١٠ ، ١٠ على التوالى. وجد أن أحسن طريق لحقن الفيروس كان عن طريق الحقن فى التجويف الالنتوسى حيث وصلت القوة العيارية الى ١٠ بينما كانت القوة العيارية عندما تم الحقن على الغشاء الالنتوسى أو فى كيس المخ هى ١٠ ، ١٠ على التوالى. أظهرت نتائج اختبارات مقاومة الحرارة أن تأثر القوة العيارية للفيروس عند وضعه فى درجة حراره ٥٦ م أنخفضت من ٩٠١٦ إلى ٤٠٦ ، ٣٠٣٥ ، ٢٠ ، صفر عندما وضع اللقاح عند مدد ٥ ، ١٠ ، ٣٠ ، ٤٥ دقيقة على التوالى. كما وجد أن أحسن طريق يتم بها حفظ اللقاح سائل هى عند درجة حرارة - ٢٠م حيث أن القوة العيارية لم تتأثر بحفظ اللقاح لمدة سنه بينما أنخفضت القوة العياريه من ٩٠١٥ إلى ٣٠٣٧ فى خلال يومين عند حفظ اللقاح السائل عند درجة حراره ٣٧م. أما اللقاح المجفف عندما تم حفظه عند درجة حرارة ٤م لمدة ١٠ أشهر أنخفضت القوة العياريه الى لوح ١٠٦٥ بينما عند حفظه مجفف عند درجة حرارة ٣٧م لمدة ٢١ يوم أنخفضت القوة العيارية حوالى لوح ٦٠١٦ بينما وصل الأنخفاض إلى لوح ٦٠٣ عند حفظ اللقاح مجفف لمدة ٣ شهور فى درجة حرارة الغرفه.

SUMMARY

The properties of infectious bronchitis virus strain Massachusetts were investigated. It was found that; the best dilution for IBV vaccine was 10^{-3}. The peak titer reached after about 40 hours of inoculation. Concerning the predilection site of IBV multiplication was amino - allantoic fluid (AAF) where LD50 titer reached $10^{9.5}$/ml, while it was $10^{7.7}$ and $10^{4.48}$/ ml in both embryo and chorioallantoic membrane (CAM), respectively. Dealing with the route of inoculation the highest titer was achived in allantoic sac ELD50 $10^{8.5}$ and $10^{8.5}$/ ml, while CAM and yolk sac Y/S reached ELD50 and 10^{6}/ ml, respectively. The results of thermostability of the IBV virus at 56°C revealed that the titer decreased from 9.16 to 4.6, 3.35, 2 and zero when the vials held for 5, 10, 30 and 45 minutes, respectively. The keeping quality of the allantoic fluid at + 4°C revealed that peak titer declined quickly at short time and that kept at 37°C dropped sharply from $10^{9.16}$ to $10^{3.37}$/ ml in 2 days. On the other hand freeze dried live IBV stored at -20°C did not show any significant loss in virus titer kept for more than one year. In freeze dried ampoules kept at + 4°C for 10 months, the reduction in virus titer was 1.56 Log while those kept at 37°C, the drop in virus titer reached 5.16 Log in 24 days. The loss in virus titer in ampoules at room temperature for about 3 months was 6.3 Log.

INTRODUCTION

Infectious bronchitis virus (IBV) is the cause of a common, highly contagious disease of the respiratory and urogenital tract of chickens. Young chicks develop respiratory disease whereas adult hens experience reduced egg production and shell quality with or without coughing, sneezing and rales (*HOFSTAD, 1984*).

WINTERFIELD and HITCHNER (1962) reported a nephrosis condition associated with some outbreaks of IB. This syndrrom was also reported from Australia and appeared more severe than USA (*COMMING, 1963*).

Numerous IB serotypes have been identified on the basis of virus neutralization test. *JUNGHERR* et al. *(1956)* described the immunological differences between 2 strains of IBV and proposed

that they may be designated Massachusetts and Connecticut types for sometime therefore they were accepted as the only immunological types of IBV. Recently other virus types have been described *HOFSTAD (1958) and WINTERFIELD* et al. *(1964).*

DUBOSE and GRUMBLESS (1955) have suggested thermostability tests at 56°C for 30-45 minutes to differentiate between IBV and quail bronchitis virus, the latter being stable at 56°C for periods upto 60 and 90 minutes.

SINGH and GUNNINGHAM (1978) exposed IBV to 56°C for 75 minutes and determined thermal sensitive S virions and thermal resistant R virions, 98 % were sensitive.

MATERIALS AND METHODS

1- a- Embryonated chicken eggs:

7-10 days old embryonated chicken eggs were used for inoculation by three different routes and also for virus propagation and titration.

b- Speccific pathogen free eggs:

F r ee (SPF) eggs 9-10 days old were imported for preparation of the seed virus.

2- Vaccinal strain of IBV:

Infectious bronchits virus vaaccine Massochust was recevied as allantoic fluids from Department of Animal Science and Agricultural Biochemistry, University of Delaware, New Wark, USA.

3- Propagation of IBV egg propagated vaccine:

Stock virus first employed, was prepared by (0.1-0.2 ml / embryo) 10 days old SPF embryos via the chorioallantoic cavity with 10^{-3} dilution. Allantoic fluid was collected from embryo (after chilling) 40 hours post inoculation *(CUNNINGHAM, 1973).* Ten percent of skimmed milk was added as stabilizer on the allantoic fluid and dispensed into ampoules, each contain 1 ml and lyophilized and kept at -20°C its ELD_{50} titer was $10^{8.16}$/ ml.

4- Vaccine titration:

It was carried out according to *CUNNINGHAM (1973).* Titers were expressed as the embryo lethal dose (ELD_{50}) per ml as determined by method of *REED and MUENCH (1938).*

5- Thermal - sensitivity tests of virus at 56°C:

At the time of use several vials containing allantoic fluid were placed in water bath at 56°C, at zero time the first vial was removed. At certain time intervals thereafter, one vial was removed from the water bath and

placed immediately in an iced - bath to chill the fluid throughly before it was tested for infectivity, *HOFSTAD (1956).*

EXPERIMENTS

I- Determination of the best dilution for IBV inoculation:

Serial ten fold dilution (10^{-1} to 10^{-5}) of IBV vaccine were used in inoculation of fifty embryonated eggs, ten eggs per each dilution. The allantoic fluid of each dilution was harvested, then titrated and the EID_{50} was determined (*HITCHNER, et al. 1975*).

II- Determination of the best time of harvest for IBV:

Thirty five eggs were inoculated with 0.1 ml / egg of dilution 10^{-3} of IBV and incubated at 37°C for 7 days. Five eggs were chilled daily and harvested then titrated to determine the EID_{50} of virus at each day. The previous steps were repeated five times (*HITCHNER and WHITE, 1955*).

III- Best site for IBV propagation:

Thirty embryonated eggs were used in this experiment, inoculated via allantoic cavity A/V with 10^{-3} of IBV vaccine. The inoculated eggs were candled daily, after 48 hours (IP). The amino - allantoic fluid (AAF), chorio - allantoic membrane (CAM) and embryos were harvested separately. The CAM and embryos were suspended weight / volume in saline with antibiotic and ground in blender, exposed to 3 successive cycle of freezing and thawing. Those suspensions were centrifuged for 15 minutes at 3000 r.p.m. and the supernatant fluid and AFF were titrated for EID_{50} of IBV (*CUNNINGHAM, 1973*).

IV- Effect of different inoculation routes on virus infectivity titer (EID_{50}):

Sixty enbryonated eggs were used, divided into 3 groups, 20 eggs in each. First group was inoculated via A/C using 9 days old ECE. The second group was inoculated on CAM of 9 days old ECE. The third one was 9 days old ECE and inoculated via Y/S. The AAF of the three groups were harvested after 48 hours from inoculation, the AAF was titrated and EID_{50} was calculated.

V- Thermmostability of the IBV fluid samples to 56°C :

Several vials containing IBV allantoic fluid was placed in a water bath at 56°C at certain time intervals thereafter, one vial was removed from the water bath and placed immediaately in iced bath chill fluid throughly before it was tested for infectivity.

VI- The effect of temperature +4°C and +37°C on the infectivity of allantoic fluid of the IBV :

Allantoic fluid of IBV was distributed into vials and some kept at refrigerator +4°C and others placed in incubator 37°C. At certain time intervals, one vial was removed and kept at -20°C to determine its infectivity.

VII- Determination of the effect of different temperature on infectious Bronchitis freeze - dried vaccine :

IBV antigen mixed with 10 % skimmed milk as preservative was freeze dried and examined for its keeping quality at different temperature degrees. The lyophilized ampoules were kept at deep freeze (-20°C), refrigerator +4°C, +37°C and at room temperature (+6°C - +25°C), titrated at different intervals. Samples were titrated monthly for 13 months for ampouls kept at -20°C and for 10 months for those kept at +4°C, on the other hand ampouls kept at +37°C examined for twenty one days and that kept at room temperature for about 3 months.

RESULTS

Table (1): showed that the best dilution for IBV vaccine inoculation was dilution 10^{-3} in which the titer reached EID50 $10^{9.5}$/ml.

Table (2): illustrates that the meaan peak EID50 titer was $10^{3.2}$/ml reached after 40 hours post inoculation (PI), meanwhile it reached $10^{5.45}$/ml after 168 hours (PI).

Table (3): concerning the best site of IBV multiplication it was clear that the highest titer reached in AAF ($10^{9.5}$/ml) while it was $10^{7.7}$/ml and $10^{4.48}$/ml in both embryo and CAM, respectively.

Table (4): revealed that the best routes of inoculation that gave titer of IBV was A/S EID50 $10^{9.5}$/ml while CAM and Y/S reached EID50 $10^{8.56}$/ml, respectively.

Table (5): showed that there was loss of titer of IBV that exposed to water bath at temperature 56°C. At zero time titer was $10^{9.16}$, then decreased to $10^{4.5}$/ml, $10^{3.35}$/ml, 10^{2}/ml and zero when the vial held in 56°C for 5, 10, 30 and 45 minutes, respectively.

Table (6): cleared the results of keeping the harvested allantoic fluid at refrigerator +4°C EID50 titer decreased from $10^{9.16}$ at zero time to $10^{5.2}$/ml EID50 after 4 days then $10^{4.2}$/ml EID50 in 20 days. On the other hand, the allantoic fluid that kept at +37°C the virus infectivity titer dropped sharply from $10^{9.16}$/ml to $10^{3.37}$/ml during 2 days and reched zero after 4 days on incubation.

Table (7): cleared that after 13 months of storage at -20°C the titer ranged from $10^{8.4}$ to $10^{8.0}$/ml and there is no

significan loss in the virus titer than the original one.

Table (8): Ampoules kept in refrigerator for 10 months there is reduction in the virus titer from $10^{8.3}$/ml to $10^{6.65}$/ml.

Table (9): Ampoules kept at 37°C the virus titer dropped from $10^{8.16}$/ml to $10^{3.0}$/ml in 21 days, the drop of titer reached 5.16. it was found that there was loss in virus titer from $10^{8.3}$/ml to $10^{2.0}$/ml.

Table (10): Ampoules kept at room temperature for about 3 months from January to March and the temperature ranged between +6°C - +25°C and the drop of titer was $10^{6.3}$ after 100 days at room temperature.

DISCUSSION

Infectious bronchitis is of economic importance to the poultry industry. In laying flocks the major loss is decreased production and poor quality eggs. In young chicks there may by mortality and loss in weight gain and efficiency. So vaccination is very important for countrolling that disease and this study was the first step for production of IBV vaccine.

The results as shown in table (1) recorded that the suitable virus dilution for egg inoculation was 10^{-3} EIDso which gave a higher virus titer reached 1.5 - 1 Log more than dilution 10^{-2},10^{-4}, respectively and that agreed with *HITCHNER* et al.*(1975)*.

Concerning the optimal time for harvesting the virus, table (2) showed that the infectivity titers continued to increase from the first 24 hours (PI) reached its peak titer $10^{8.2}$ EIDso/ml after 40 hours and dropped about 2 Log then remained constant for about 4 days ranging from $10^{6.6}$ to $10^{6.3}$ after that it dropped 1 Log at the end of incubation period. *HITCHNER and WHITE (1955)* studied the growth curve of IBV (20-30 embryo passages) and reported that maximum titers were attained after 24 - 30 hours at 37°C and also reported that inocula of about 10^{3} TC infectious doses or 10^{4} EIDso should give near maximum titers by 36 - 40 hours at 37°C. *CUNNINGHAM (1973)* collected allantoic fluid from embryos killed by IBV within 24 to 36 hours (PI) and from living embryos between 36 and 48 hours. *CUNNINGHAM (1970)* reported that the highest concentration of IBV 10^{7} embryonated doses detected 36 hours after incubation.

Chocce of the best target organ for virus multiplication as indicated in table (3), it was found that allantoic fluid gave the highest titer $10^{9.5}$ EIDso/ml, these results agreed with *CUNNINGHAM (1970)*, who reviewed that the highest

concentration of virus is recovered from the CAM, followed in order by the allantoic fluid, amniotic fluid and liver.

Table (4) recorded the best routes of IBV inoculation, it was found that the A/C, CAM and Y/S gave $10^{9.5}$ EID_{50} /ml, $10^{8.56}$ /ml and 10^{6}/ml, respectively. *CUNNINGHAM and JONES (1953)* found that it varied with the route of inoculation in the following decreasing order amniotic sac, allantoic and chorio - allantoic membranes.

Table (5) illustrates the thermostability of IBV fluid exposed to 56°C, It was found to be sensitive, the infecivity titer dropped about 4.6 Log in 5 minutes and reached to complete inactivation after 45 minutes. *STUART and HOPKINS (1967)* detected that Beaudette 42 strain more stable in IM Mg SO_4 than IM Na Cl for period up to 80 minutes. *HOFSTAD (1956)* stated that most strains of IBV are inactive after 15 minutes at 56°C, few strains survive beyond 45 minutes. *OTSUKI* et al. *(1979)* determined that IBV strain are inactivated afetr 15 minutes at 56°C and after 90 minutes at 45°C. *SINGH and CUNNINGHAM (1978)* studied the effect of 56°C on thermal sensitivity of IBV isolates and found that it was 5 minutes for A3 -25, 17 - 35 and 15 minutes for (40 - 16).

The results in table (6) cleared that keeping of the IBV allantoic fluid in +4°C affect its infectivity titer, it was reduced about 2 Log after 2 days. On the other hand, there is marked reduction of IBV EID_{50} titer of allantoic fluid kept at 37°C which reached zero after 4 days of exposure. It was shown that IBV allantoic fluid was very sensitive to keeping temperature. *HOFSTAD (1984)* reported that avian IBV stores well at -30°C in the form of allantoic fluid.

Freezing dried ampoules stored in -20°C revealed that there is no loss in virus titer during the experimental period 13 months (Table, 7). *HOFSTAD (1984)* found that lyophilized IBV stored in refrigerator remained viable for at least 30 years. Our results of monthly titration of dried vaccine kept at +4°C revealed reduction in virus titer from $10^{8.3}$ EID_{50} to $10^{6.65}$ EID_{50} in a period of time 10 months (Table, 8).

The results in table (9) revealed the loss in titer in freeze dried ampoules of IBV kept at 37°C. There is loss in titer about 3 Log in 8 days. *HITCHNER* et al. *(1975)* denoted that the loss of titer in vials held at 37°C will be between 1 and 2 Log in 7 days. *HOFSTAD (1984)* reported that lyophilized IBV is completely inactivated within 6 months when stored at 37°C.

Table (10) demonstrates the survival of IBV lyophilized ampoules kept at room temperature in winter and spring time in which temperature ranging from +6°C to +25°C, thhe drop in titer reached 3 Log in 30 days in winter. After elabs of 100

days the loss of titer reached 6.3 Logs. *SATYLGANOV (1971)* studied exposure of IBV to different temperatures and humidities during spring, summer and winter, survival was up to 12 days in spring and 56 days in winter.

From the previous results to produce IBV vaccine of high titer, it is recommended to inoculate the IBV seed material via A/C and harvest the allantoic fluid only at 40 hours after inoculation with dilution 10^{-3}. The harvested allantoic fluid must be freeze dried quickly after harvesion and should be stored in deep freezer as there is no loss in the virus titer after more than 13 months.

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Table(1): Determination of the best dilution for IBV inoculation.

Virus dilution	Virus titer EID_{50}/ml
10^{-1}	9.16
10^{-2}	8.00
10^{-3}	9.50
10^{-4}	8.40
10^{-5}	7.33

Table(2): Dtermination of the suitable time for harvesting of IBV.

Inoculation No.	EID_{50}/ml in hours postinoculation (PI)						
	24	40	72	96	120	144	168
1	7.00	7.25	6.16	7.37	6.33	7.33	7.00
2	8.00	8.63	7.30	7.00	6.60	6.60	7.00
3	6.50	8.26	6.29	4.08	5.49	5.80	6.80
4	4.66	8.90	6.50	6.90	6.90	6.90	6.78
5	7.46	8.00	6.76	7.18	7.60	5.35	5.20
Mean	6.72	8.20	6.60	6.50	6.58	6.39	5.46

Table(3): Target organ of the IBV propagation.

Organ	EID_{50}/ml
Embryo	7.70
Membrane	4.48
Fluid	9.50

Table(4): Effect of different routes of inoculation on IBV titer.

Route of inoculation	Virus titer in ECE EID_{50}/ml
CAM	8.56
A/C	9.50
Y/S	6.00

Table(5): Thermostability of infectious bronchitis virus at 56°C.

Time of exposure to 56°C per minute	EID_{50} titer/ml	Loss in titer
Zero	9.16	--
5	4.60	4.56
10	3.35	5.81
30	2.00	7.16
45	Zero	9.16

Table(6): Effect of temperatures + 4°C and + 37°C on the fluid of infectious bronchitis virus.

Temperature	Log EID_{50} titer/ml						
	Days of exposure						
	0	2	4	8	12	16	20
+ 4°C	9.16	7.16	5.20	4.20	3.35	2.70	2.00
+ 37°C	9.16	3.37	Zero	Zero	ND	ND	ND

Table(7): Effect of temperature (- 20°C) on the keeping quality of IBV lyophilized ampouls.

Temperature	EID_{50}/ml monthly titer.												
	1	2	3	4	5	6	7	8	9	10	11	12	13
- 20°C	8.4	8.2	8.3	8.0	8.3	8.0	8.37	8.3	8.16	8.0	8.3	8.2	8.4

Table(8): Keeping quality of IBV lyophilized ampouls kept at + 4°C.

Temperature	EID_{50}/ml titer in days.										
	0	1	2	3	4	5	6	7	8	9	10
- 4°C	8.3	8.0	7.5	7.22	7.25	7.2	7.0	7.0	6.8	6.0	6.5

Table(9): Keeping quality of freeze - dried IBV stored at 37°C.

Temperature	EID_{50}/ml monthly titer.											
	0	1	2	4	6	8	10	12	15	17	21	30
+ 37°C	8.3	8.16	6.5	6.2	5.7	5.5	4.0	3.8	3.5	3.33	3.0	0.0

Table(10): Results of keeping quality of freeze - dried IBV stored at, room temperature (+ 6°C - 25°C).

Time	No. of days during January, February and March												
	0	2	7	20	29	36	43	53	64	72	84	90	100
EID_{50}/ML	8.3	7.5	6.8	5.8	5.0	5.0	5.00	5.0	4.75	4.0	3.5	2.8	2.0