Effect of Stabilizers and Lyophilization on the Efficacy of Avian Infectious Laryngeo-Tracheitis Vaccine Virus

Ahmed Abd El- Samie H. Ali *; Abou Zead, A. A. ** and Mustafa, A. M. **
*Dept of Virology, Faculty of Veterinary Medicine, Zagazig University, Egypt.
** Poultry Disease Unit, Animal Health Research Institute, Zagazig Branch,

ABSTRACT

Avian infectious laryngeo-tracheitis virus (AILTV) is a dsDNA gallid herpes virus-1. It affects chickens causing variable respiratory manifestations. Live AILTV vaccine prepared from cover strain (**SPAFAS**) on chicken embryos is mixed with different stabilizers included, maize starch peptone, skimmed milk **5%, 10%, 20%**, gelatin **10%, 20%** and lacto-albumin-sucrose. The vaccine virus preliminary titers on fertile eggs were $10^{5.5}$, $10^{5.5}$, $10^{5.3}$, and $10^{5.2}$ EID_{50}/ml with the stabilizers. The vaccine virus was exposed to lyophilization and preserved at temperature ranged from **-60°C, -20°c, 4°c, 25°c, +37°C** followed by corresponding titration. Stabilizers of starch-peptone and skimmed milk **20%** progressively preserved and kept the highest titers of the vaccinal virus preserved at temperatures ranged from **-60°C** to **37°C**. Potency of AILTV vaccine virus was assessed by antibody response in chickens sera post vaccination on weekly intervals using ELISA. The titer means were developed from 1st week **(1174)**, peaked to the 5th week **(4996)** and then declined to **(550)** at 24th week. It assumed that the addition of stabilizers associated with lyophilization preserve the vaccine virus productivity.

INTRODUCTION

Avian Infectious Laryngeo-Tracheitis Virus (AILTV) is classified as ***Gallid herpes virus***-1, within ***Alphaherpesvirinae*** of the ***Family Herpesviridae*** of DNA genome, **80-100** nm in diameter and has icosahedral symmetry. The virus glycoproteins are responsible for upregulation and stimulating humoral and cell mediated immune responses. AILTV strains appear to be antigenically homogenous with minor antigenic variation against antisera. AILTV thymidine kinase gene is directly related to the virulence. AILTV affecting chickens of 3-9 weeks old but the typical form usually occurs in adult birds. Also pheasants, partridges and peafowl are affected *(1& 2)*.

AILTV is not an egg borne infection. Infection mainly occurs via the upper respiratory tract or conjunctiva. Viral replication is limited to the epithelium of trachea, larynx, nares and oropharynx. Rarely viremia may occur.

The clinical disease ranges from subclinical to acute respiratory manifestations includes moist rales, coughing, gasping, bloody expectorations, often associated with morbidity up to **100%**, variable mortality (10-40%) or higher and egg production losses depending on the immune status of the flock, with the fate of persistent infection with frequently intermittent shedding of virus *(1,3,4)*.

Outbreaks of AILTV were reported in different countries including Egypt *(5)*; England *(3)*; Northern Ireland *(6)*; USA *(7)*; Australia *(8)*; and Finland *(9)*.

It has been proposed that viral clearance is mediated primarily by cellular immune mechanisms. However, chickens infected with AILTV produce specific IgG, IgM and IgA antibodies in their sera; and in tracheal washings within 5 to 7 days post exposure with peak around 21 days, and then persist in low level for a year or more *(10)*. Vaccination against ALTV upregulates the mucosal immunity which inhibits the replication of the virulent virus *(11)*. Passive transfer of maternal anti-AILTV antibody IgG to the progeny via the egg does not confer resistance to infection or interfere with vaccination with AILTV among chicks of 2 days to 4 week old or even in ovo *(12)*. The

current freeze-dried live attenuated ILTV vaccines administered via eye-drop and drinking water *(13).* The lyophilization is a method of preservation that greatly preserves virus viability. The freeze-drying process can be divided into three successive stages: freezing, primary drying (sublimation involves lowering pressure and supplying heat for water vapor) and secondary drying during which the residual moisture evaporates from the dried material *(14).* This work was aimed to investigate the effect of lyophilization and stabilizers on the efficacy of AILTV Vaccine

MATERIAL AND METHODS

AILTV virus: Reference AILTV (Cover strain), was obtained from Charles River Lab. **SPAFAS**, Avian Products and Services, USA in the form of allantoic fluid from SPF chicken embryos infected with ILTV. It was positive in AGPT against AILTV immune serum.

Vaccine stabilizers

a- Skimmed milk (SM): A different working dilutions includes 5%, 10% and 20% were prepared in distilled water and sterilized by autoclaving at 121°C for 15 minutes.

b-Gelatin; Difco, USA, was prepared at different dilutions of 10% and 20%, in distilled water and sterilized by steaming at 100°C for 10 minutes on 3 successive times (Salwa et al., 1993).

c-Lacto albumin-sucrose (LAS): The working mixture solution was prepared of 5 parts of 5% Lacto albumin hydrolysate (Difco,USA) to 1 part of 2.5% sucrose (Sigma) in distilled water and sterilized by filtration.

d-Maize-Starch Peptone (MSP): It was prepared by mixing equal parts of maize starch (10%) and peptone (1%) in distilled water (**15**) and sterilized by steaming as above.

Bacteriological culture media for the vaccine sterility:

a-Bacto PPLO broth or agar (Difco), for the detection of Mycoplasma contamination.

b-**Thioglycollate medium (Oxoid)**: for the detection of both aerobic and anaerobic bateria

c-Bacto-Sabaroud's maltose agar (Difco).

Chickens: susceptible broiler chickens of 12 weeks old were used for safety and potency assessment of ILTV vaccines.

Propagation and preparation of AILTV vaccinal strain in SPF eggs

A 0.1 ml of AILTV Cover strain was inoculated into 5 SPF eggs via chorioallantoic membrane (CAM) by 0.2 ml per egg. The propagated virus was titrated after two successive closed passages till yielded $10^{5.9}$ EID_{50}/ml. The virus was inoculated in SPF eggs, incubated at 37°C with daily candling. At the 5th day post inoculation, the eggs were chilled; then the allantoic fluid (AF) and CAMs were aseptically harvested, antibiotic mixture and mycostatin were added, homogenized and exposed to 3 times freezing and thawing, then clarified by centrifugation at 2500 rpm for 20 minutes. The supernatants were collected and were used as virus stocks divided into five portions to be mixed with stabilizers, the 1st part was mixed with equal volumes of 5%, 10% and 20% SM; the 2nd was mixed with 10% and 20% gelatin, the 3rd was mixed with LAS, the 4th part was used as AILTV mixed with 10% maize starch + 1% peptone and the 5th was used as AILTV control.

Sterility tests

The produced ILTV vaccine pre- and post-lyophilization with different stabilizers was screened for aerobic and anaerobic bacteria, Mycoplasma and fungal contamination *(16).*

Titration of AILTV vaccine before Lyophilization: several batches of the attenuated AILTV vaccine with the corresponding stabilizers were titrated before lyophilization. Serial ten-fold dilutions were done. Dilutions **10^{-3}, 10^{-4}, 10^{-5} and 10^{-6}** were inoculated into SPF chicken embryos via CAM, 5 eggs per dilution as well as 5 additional eggs inoculated with the used sterile

diluents as control inoculated embryos were examined at 5th day for AILTV lesions on CAMs and the end point was calculated *(17)*.

Lyophilization and titration of the vaccine at different freezing temperatures

The vaccine batches were lyophilized, AILTV vaccine was kept at variable preserved temperatures of -60°C, -20°C, 4°C, 25°C and 37°C over monthly intervals *(17)*.

Measurement of Vaccine induced antibody; ELISA; Proflok AILTV ELISA kit

Measurement of humoral immune response of serum samples collected from chickens12 weeks old vaccinated with attenuated ILTV virus vaccines with different stabilizers using ELISA *(***Proflok AILTV ELISA kit** *& 18)* .

Interpretation of the results: The means of **OD** for both negative and positive controls were calculated and the corrected **OD (cOD)** was calculated. The sample positivity or negativity for AILTV antibody was calculated from Sample to Positive **(S/P)** ratio using the following equation format: **S/P ratio =**

$$\frac{\text{OD Sample Absorbance - OD Negative Absorbance Mean}}{\text{Corrected Positive Control Absorbance (COD)}}$$

A serum considered positive when the calculated **S/P ratio** was higher than **> 0.150** as:

Negative sera for ILTV antibody	Positive sera for ILTV antibody
S/P ≤ 0.150	S/P > 0.150

AILTV ELISA end points and titers were calculated using the following equations:

Log_{10} Titer = (1.450 X Log_{10} Sp) + 3.726, Titer = Antilog of Log_{10} Titer.

RESULTS

Propagation, preparation and titration of AILTV vaccinal strain

The propagated AILTV was expressed on $ED_{50}= 10^{6.4}$ (Table 1).

Table 1. Titration of lyophilized AILT vaccine virus in SPF –ECE.

Stabilizer	Stabilizer %	Vaccine Dilution	Clinical Score of infectivity		Vaccinal Virus titer (EID_{50}/ml)
SM	5	10^{-3}	0/5	No Lesion	0.00
		10^{-4}	0/5		
		10^{-5}	0/5		
	10	10^{-3}	5/5	Thickening of CAMs	4.90
		10^{-4}	1/5		
		10^{-5}	1/5		
	20	10^{-3}	5/5	Thickening of CAMs pock lesions	5.50
		10^{-4}	2/5		
		10^{-5}	2/5		
G	10	10^{-3}	5/5	Thickening of CAMs	4.90
		10^{-4}	1/5		
		10^{-5}	1/5		
	20	10^{-3}	5/5	Thickening of CAMs	5.30
		10^{-4}	2/5		
		10^{-5}	2/5		
LAS	5 + 2.5	10^{-3}	5/5	Thickening of CAMs	5.20
		10^{-4}	1/5		
		10^{-5}	0/5		
MSP	10 + 1	10^{-3}	5/5	Thickening and pock lesion of CAMs	5.50
		10^{-4}	3/5		
		10^{-5}	2/5		

SM: Skimmed Milk; G: Gelatin; LAS: Lactoalbumin Sucrose; MSP: Maize Starch-Pepton

The AILTV vaccine was prepared and proven to be free from other microbial contaminations. The vaccine virus titer was significantly recorded with most stabilizers and includes as $10^{5.5}$; $10^{5.5}$; $10^{5.3}$. $10^{5.2}$ with MSP, SM20%, gelatin 20%, LAS (Table-2)

Table 2-A. Titration of lyophilized vaccinal ILTV stored at variable preservation temperature

Time interval	Preservation temperature														
	+4°C					-20°C					-60°C				
	SM	G	LAS	MSP	No stabilizer	SM	Gelatin	LAS	MSP	No stabilizer	SM	Gelatin	LAS	MSP	No stabilizer
0 D	5.5	5.5	5.5	5.5	5.5	5.5	5.5	5.5	5.5	5.5	5.5	5.5	5.5	5.5	5.5
1 M	5.5	5.5	5.5	5.5	5.5	5.5	5.5	5.5	5.5	5.5	5.5	5.5	5.5	5.5	5.3
3 M	5.5	5.4	5.4	5.5	5.3	5.5	5.5	5.5	5.5	5.4	5.5	5.5	5.4	5.5	5.1
5 M	5.5	5.3	5.3	5.5	5.0	5.5	5.5	5.4	5.5	5.3	5.5	5.5	5.4	5.5	5.0
7 M	5.5	5.1	5.0	5.5	4.8	5.5	5.2	5.3	5.5	5.2	5.5	5.5	5.5	5.5	4.8
9 M	5.5	5.0	4.9	5.5	4.4	5.5	5.1	5.2	5.5	4.5	5.5	5.4	5.3	5.5	4.4
12 M	5.5	5.0	4.8	5.5	3.9	5.5	5.1	5.0	5.5	4.0	5.5	5.4	5.3	5.5	4.0
Mean	5.5	5.3	5.2	5.5	4.9	5.5	5.3	5.3	5.5	5	5.5	5.4	5.3	5.5	5

D: Day; M: Month

The means of virus titers post lyophilization and preservation at temperature of 4°C and with the stabilizers of MSP, SM,G 20% and LAS were at 4°C were $10^{5.5}$, $10^{5.5}$, $10^{5.3}$, and $10^{5.2}$ EID_{50}/ml ; **at -20 were** $10^{5.5}$, $10^{5.5}$, $10^{5.3}$, and $10^{5.3}$ EID_{50}/ml **and at -60** $10^{5.5}$, $10^{5.5}$, $10^{5.4}$, and $10^{5.3}$ EID_{50}/ml respectively. The lyophilization with the used stabilizers was variably preserve and improved the efficacy of the AILTV vaccinal virus titers as SM 20% > MSP > Gelatin> LAS which yielded titers of **5.5, 5.4, 5.3,5.2** respectively at preservation temperature of **–60**°C to **–20**°C (Table 2-A).

Table 2-B. Titration of lyophilized vaccine AILTV stored at variable preservation temperature.

Temp.	Days Titer								
37°C	0	3	5	7	10	15	20	25	30
	5.5	5	4.4	3.9	3.3	2	1.0	0.0	0.0
25°C	5.5	5.2	5.0	4.5	4.2	3.5	3.0	3.0	2.1

Preservation of AILTV vaccine even lyophilized with stabilizers was deteriorated rapidly at 37°C than 25°C, (Table 2-B).

Measurement of antibody response to lyophilized AILTV vaccine using ELISA

The chickens vaccinated with lyophilized live **AILTV vaccine with** MSP stabilizer showed antibody titers increased from the 1st week to reach the highest titer at the 5th week, then declined until at 24th week of the end of the experiment by titers reported as **1174, 4996** and **550** respectively, (Table 3).

Table 3. Measurement of AILTV vaccine induced titers in vaccinated chicken sera.

Time Interval/ Week	Mean of ILTV Ab titer
1st	1174
3rd	1842
5th	4996
10th	3650
16th	2064
24th	550

DISCUSSION

A lyophilization is process involved the freezing of the vaccine followed by withdrawal of water content. In detail, it can be divided into three steps: freezing, primary

drying (sublimation of ice) and secondary drying (absorption of water) with a residual moisture content of 5-10% and 1.3% during primary and secondary drying into the solid matrix of vaccine and excipient.

The protecting stabilizer media includes MSP, SM, G and LAS were recommended in the preparation of AILTV vaccine. Several reports *(15, 19 & 20)* also indicated that MSP as a stabilizer base in Newcastle disease virus strains and fowl pox virus vaccines.

AILTV vaccine either before or after lyophilization was freedom from bacterial (aerobic and anaerobic), fungal and Mycoplasma contaminations and in similarity with previously recorded *(16).*

Keeping quality of the AILTV allantoic fluid in **4°C**, **-20°C** and **-60°C** affect its infectivity titre. It was reduced about 1.2 log at 4°C and 0.5 log at -20°C and at - 60°C did not reduced; which revealed the loss in titre lyophilized vials of ILTV kept at **37°C**. There was loss in titer of **1.3** log in **5** days, demonstrated that keeping quality of ILTV at **25°C**, referred to reduction of infectivity titer 0.5 log at the same time.

The mean of AILTV specific antibody titer increased gradually from 1st week (**1174**) until reached the peak at 5th week (**4996**), then declined into (**550**) at the end of sampling time. Similar result was previously recorded *(18).*

REFERENCES

1. ***Bagust, T.J.; Calnek, B.W. and Fahey, K.T. (1986):*** Gallid-1 herpes virus infection in the chicken 3. Re-investigation of the pathogenesis of infectious Laryngeo-tracheitis in acute and early post-acute respiratory disease. Avian Dis., 30: 179-190.

2. ***Kaleta E.F., (1990):*** Herpesvirus of birds-A review. Avian pathol., 19: 193-211.

3. ***Hughest, C. S., Gaskell, R. M., Bradbury, J. M., Jordan, F. W., and Jones R. C., (1991):*** Survey of field outbreaks of AILTV in England and Wales. Vet. Rec. 129:258-260.

4. ***Bagust, T. J., and Guy, J. S., (1997):*** Laryngotracheitis. In: Diseases of poultry, 10th ed. Iowa State of university press, Ames, IA. pp. 527-539.

5. ***Tantawi, H. H., El-Batrawi, A. M., Bastami, M. A., Youssef, Y. I, Fawzia, M. M., (1983):*** AILT virus in Egypt. I. Epidemiology, virus isolation and identification. Vet Res Commun 6: 281-287.

6. ***McNulty, M. S., Allan, G. M., and McCracken, R. M., (1985):*** Infectious laryngotracheitis in Ireland. Irish Vet. J., 39, 124-125

7. ***Linares, J. A., Bickford, A. A., Cooper, G. L., Charton, B. R., and Woolcock, P. R., (1994):*** An outbreak of infectious laryngotracheitis in California broilers. Avian dis 38:188-192.

8. ***Pulsford M.F.; and Stocks, J. (1953):*** ILT in South Australia. Aust. Vet. J. 29:8-12.

9. ***Rislakki, V. (1965):*** Case report: infectious Laryngeo-tracheitis of poultry in Finland.

Avian Dis., 9: 339-342.

10. ***Da Silva Martins, N. R., Mockett, A. P., Barett, A. D., and Cook J. K., (1992):*** Local and systemic antibody class response to an ILTV vaccine strain Avian Pathology: 21: 97-106.

11. ***Fahey, K. J., and York, J. J., (1990):*** The role of mucosal antibody in immunity to infectious laryngotracheitis virus in chicken. J of G Virol 71: 2401-2405.

12. ***Fahy, K. J., Baghust, T. J., York, J.J. (1983):*** LT herpesvirus infection in the chickens: The role of humoral antibody in immunity to a graded challenge infection. Avian Pathol., 12:505-514.

13. ***Sinkovic, B. (1985):*** Australian experiences with mass methods of vaccination against ILT VIIIth. Inter. Congress World Vet. Poult. Assoc., Jerusalem, Israel, Abstract, P. 24.

14.Pikal, M.J.; Shah, S.; Senior, D. and Lang, J.E. (1983): Physical chemistry of freeze-drying: measurement of sublimation rates for frozen aqueous solutions by a microbalance technique. J. Pharmacol. Sci., 72: 635-650.

15.Chauhan, D.S.; Tanwani, S.K.; Pathak, P.N. and Shivdenkar, D.S. (1985): Studies on an alternative method of drying Newcastle disease and fowl pox vaccines using maize starch powder as stabilizer cum base. Ind. Vet. J., 62 (9): 735-792.

16.Anon (1971): Methods of examining poultry biologics and for identifying and quantifying avian pathogens.Nut. Acad. Sci., Washington, D.C., Cited by Amer, M.M. (1984).

17.Reed, L. J. and Muench (1938): A simple method of estimating fifty percent end point. Amer. J. Hyg., 27: 493-497.

18.York, J. J., Fahey, H. J., and Bagust, T. J., (1983): Development and evaluation of an ELISA for the detection of antibody to ILTV in chickens. Avian Dis. 27:409-421.

19.Salwa A. El-Assily; Ensaf M. Khashabah; Afaf Hamdi and Fekria A. El-Bordeny (1993): Comparative study on the effect of stabilizer dilutions on the Newcastle disease virus vaccines. Zag. Vet. J., 21 (5): 975-981.

20.Mariner, J.C.; House, J.A.; Sollod, A.E.; Stem, E. Van den Ende, M.C. and Mebus, C.A. (1990): Comparison of the effect of various chemical stabilizers and lyophilization cycles on the thermostability of a Vero cell adapted rinderpest vaccine. J. Vet. Microbiol., 21: 195-209.

الملخص العربى

تأثير نوع المثبت والتجفيف على كفاءة لقاح فيروس التهاب الحنجرة والقصبة الهوائية المعدي للدجاج

د /أحمد عبد السميع حسن علي * د.عاطف على أبوزيد ** عبد الحكيم محمد مصطفى

*قسم الفيروسات - كلية الطب البيطرى- جامعة الزقازيق - مصر

**وحدة أمراض الدواجن- معهد بحوث صحة الحيوان – فرع الزقازيق

ينتمي فيروس التهاب الحنجرة والقصبة الهوائية الى عائلة فيروسات الهربس، ويتميز بالانتشار واسع النطاق وإحداثية الكثير من الحالات المرضية التنفسية المتفرقة بين قطعان الدواجن.

تم تحضير لقاح حي للفيروس باستخدام عترة (SPAFAS) بعيارية 10° على بيض الدجاج الخالي من العدوى مع إضافة مثبتات بنسب متفاوتة شملت اللبن منزوع الدسم ٥%، ١٠%، ٢٠%، والجيلاتين ١٠، ٢٠%، وخليط نشا الذرة، وخليط اللاكتوز والسكروز.

تم معيارية اللقاح مع كافة المثبتات قبل التجفيد ووجد أن معدلات العيارية كانت $10^{0.0}$ و $10^{0.0}$ مع $10^{5.3}$ ، مع المثبتات بين الأنواع خليط نشا الذرة والببتون، واللبن المنزوع الدسم ٢٠% وكذلك الجيلاتين ٢٠% ثم اللاكتوز والسكروز $10^{0.2}$ على التوالي.

تم تجفيف اللقاح ثم حفظه عند درجات حرارة متفاوتة ٦٠، -٢٠ ، ٤، ٢٥، ٣٧ $^{\circ}$م. الحرارة السالف ذكرها. أظهرت نتائج العيارية انه لم يحدث أى تأثير سلبى فى قوة اللقاح حيث كانت متوسطات العيارية $10^{5.5}$ ، $10^{5.5}$ ، $10^{5.3}$ ، $10^{5.3}$ وليس هناك فارق جوهري في العيارية عند درجات الحرارة من -٦٠ وحتى ٤$^{\circ}$م، مما يفيد بأن عملية التجفيف تحافظ على قوة وعيارية اللقاح مما يسهل تداول اللقاح عند درجات الحرارة المختلفة.

تم قياس قوة فيروس اللقاح المجفف بالحقن فى الدجاج وقياس الأضداد في المصل باستخدام اختبار ELISA بعد فترات أسبوعين ولمدة ٢٤ أسبوع، وأظهرت النتائج أن الأضداد بدأت بمتوسط عيارية ١١٧٤ فى الأسبوع الأول ووصلت إلي الأعلى ٤٩٩٦ فى الأسبوع الخامس وانحدرت تدريجيا حتى الأسبوع ٢٤ (نهاية التجربة) بمعدل ٥٥٠.