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Identification and Purification of a Local Isolate of Infectious Laryngotracheitis Virus

H.M. Madbouly*, M.A.B. El- Sagheer, Nagwa S.S. Ata**, M.A. Kutkat ** and Kawther S.A. Zaher****
**Faculty of Veterinary Medicine, Cairo University (Beni-Seuif Branch), and **National Research Center, Dokki, Cairo, Egypt.*

LOCAL INFECTIOUS laryngotracheitis (ILT) virus strain was isolated on ECE for ten passages. The isolated virus produced characteristics pock lesions on chorio-allantoic membranes of the embryonated eggs, which increased in size and number when the number of passage increases. Moreover, the isolated virus was further propagated on CER and Vero cells where the virus gave high titers only when propagated on Vero cells. The virus was purified by sucrose gradient ultra centrifugation where a white band was collected at the end of the centrifugation cycle. The isolated ILT virus was identified by means of Electron Microscopy, Dot-ELISA and serum neutralization.

The causative agent of ILT is a virus belongs to subfamily alphaherpesvirinae and initially propagated in the chorio-allantoic membrane (CAM) of developing chicken embryos. Laryngeotrachietis has been recorded as early as March 1920 in the United States. In 1926, outbreaks in California chicken flocks were reported in which fowl pox apparently coexisted. Clinical signs were coughing with expulsion of blood and mucus as well as severe dyspnea (Beach, 1926). The term infectious laryngeotrachietis (ILT) was adopted in 1931 by a special Committee on Poultry Diseases of the American Veterinary Medical Association. Egypt remained free from ILTV until August 1982, when serious acute outbreaks occurred in layers 4-12 months old in several farms in Cairo and Giza areas with wide spread to adjacent localities. Therefore, it has been suggested that the disease was introduced to Egypt through importation of pullets from endemic countries (Tantawi, *et al.*, 1983).

With the advent of cell-culture techniques, researchers quickly established that ILTV would replicate in adult chicken kidney cells as well as in a variety of chicken embryo-epithelial cells such as kidney, liver and lung (Hanson and Bagust, 1991).

Although primary cell culture is still used as host for ILTV, it cannot be maintained in vitro. Thus, a continual supply of fresh harvested cells is needed, which results in an inherent degree of variability. Moreover, such cultures are invariably contaminated with fibroblastic cells, which cannot be productively infected by ILTV (Rossi, *et al.*, 1969) In order to circumvent the problems associated with primary cell cultures, the ability of many cell lines to support the replication of ILTV was evaluated like chicken LMH, Vero cells, quail cell line (QT.35) and IQ1A (Kawaguachi, *et al.*, 1987, Schnitzlein, *et al.*, 1994).

In the year 2000 poultry industry has encountered a great economic losses where several outbreaks of high mortalities (25% and may reach 75%) respiratory disease. The clinical picture was severely progressive and in the form of coughing with expulsion of blood and mucus with extension of the head and neck as well as severe dyspnea and conjunctivitis and sinusitis in some affected flocks, this sign was so prevalent that the walls and equipment were spattered with dried blood. The survived cases failed to gain the presumed body weight in case of meat production and drastic decrease in egg production during the disease course in case of flocks for egg production as the disease infect poultry at any age.

Our aim was to isolate and identify the causative agent of this disease for furthermore study to solve this problem by producing a vaccine.

Material and Methods

Tracheal tissue

ILTV was isolated from tracheal tissue of chickens showing hemorrhagic inflammation of larynx and trachea and suffered from bloody coughing and various respiratory disorders. The chickens came from seven farms with different ages; two farms at the age of approximately three weeks (meat production), two farms at the age of approximately six to seven weeks (meat producing) and three at the age of approximately four months (egg production). Four of these farms were given the vaccine in their vaccination program. The tracheal tissue was crushed by sand by the addition of sterilized PBS in 10% suspension with freezing and thawing for at least three times, then centrifuged at 3000 rpm for 10 min at 4°C.

Virus vaccine

As egg adapted live attenuated virus vaccine (TAD ILT vaccine 1000ds, Lohman animal GMH & Coikg, Heinz-Lohman str. 4.2-7471 Cuxhiven Germany, Batch no 1071121) was used for preparation of anti-ILT virus hyperimmune serum.

Chicken

Ten-4week-old meat type chickens were used for preparation of hyperimmune serum. The serum of these chickens was free from ILT virus antibodies by ELISA which was performed according to (Madbouly, 1989).

Fertile eggs

Commercial and SPF- egg were purchased from faculty of agriculture, Cairo univ., and used for virus isolation and propagation.

Cell culture

Vero cells (African green monkey kidney cells) as well as chicken embryo rough cells (CER) that were purchased from VACSERA institute, Agosa, A.R.E. these cells were used for virus propagation.

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Isolation

The tracheal tissue suspension was concentrated using poly ethylene glycol (PEG). The virus was isolated firstly on commercial embryonated chicken eggs for ten passages then the passage was propagated on SPF- embryonated egg for one time to reduce the cost of this technique.

Anti-ILT hyperimmune virus serum preparation

a- Purification of egg-adapted vaccine strain

The vaccine strain of ILTV, which was propagated on Vero cells of titer 10^3 that incubated for 30 hours in 37°C, freezed and thawed (thawing at 4°C) at least 3 times to release the intracellular virus. The culture fluid was centrifuged at 3000 rpm for 15 minutes at 4°C to remove cell debris; the supernatant fluid was over laid by 30% sucrose and centrifuged at 100.000 rpm (equal to 45.000 Xg) for 2 hours at 4°C to pellet the virus. The virus pellet was resolved in 1ml PBS. The concentration of viral protein in milligrams per milliliter was calculated using a spectrophotometer at A_{260} according to (Burleson, *et al.*, 1989). The virus solution was stored at -70°C until used for immunization and for ILTV identification.

b- Preparation of anti- ILT hyperimmune serum

100 µg of purified ILT antigen mixed with 100 µl of complete Freund's adjuvant was injected subcutaneously (S/C) in the left side of the neck of chicken aged four weeks. The chickens were boosted three times at the second, third and fourth weeks from the first injection by the same mixture but with incomplete freund adjuvant instead of the complete one. After two weeks from the last injection, the chickens were bled and the serum was collected and stored at -20°C.

Purification of the isolated virus

The isolated virus was purified according to (Cruickshank, *et al.*, 1963 and watrach, *et al.*, 1963) using sucrose gradient ultracentrifugation. The sucrose used in concentration of 5,10,15,20,25,30,35,40 and 45%.

Propagation of the isolated virus

The purified virus was propagated on Vero as well as CER cells.

Electron microscopy for the isolated virus

The purified isolated virus was examined by Electron microscope according to (Wu, *et al.*, 1996) where one drop of virus suspension was stained by sodium phosphotungstate.

Identification of the isolated virus

The isolated virus was also identified using Dot-ELISA according to White and Fenner (1986) where one part was added to equal volume of triton x100 and

egg and Vero cells according to Hitchner, *et al.*, 1975 and Hsiung, 1973 respectively.

Results

Results of virus isolation on ECE

Data presented in Table 1 revealed that lesions began small in size and of low number then increased in size and number gradually from the 6th passage till the 10th passage. During virus propagation, the pock lesions were large in size 3- 5 mm with depressed gray center and the embryo was stunted in growth Photo 1.

TABLE 1. Virus isolated on ECE.

Number of passage	Intensity of pock lesions	Size of pock lesion
1	+	Small
2	+	Small
3	+	Small
4	+ +	Small
5	+ +	Small
6	+ + +	Medium
7	+ + +	Medium
8	+ + +	Medium
9	+ + + +	Large and scattered
10	+ + + +	Large and scattered

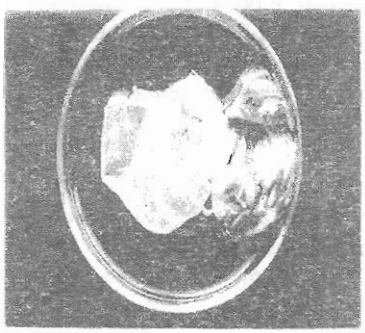

Photo 1. ECE infected with the isolated virus showing stunted embryo and the CAM showing numerous pock lesions with large size (10^{th} passage).

Results of virus isolation on CER and Vero cells

CER cells were poorly productive (refractory), giving low virus titer, with limited CPE, when infected with the isolated virus. The infectivity titer remains very low even after 10 passages (3.6 $\log_{10}$). While the virus infectivity titers propagated on Vero cells increased gradually from the first passage (3.83 $\log_{10}$) until the 9^{th} passage (7.56 $\log_{10}$) then decreased gradually from the tenth passage (5.3 $\log_{10}$). Finally, the infectivity titer reaches (3 $\log_{10}$) in passage 14. Infected

cells with the isolated virus become rounded and aggregated showing syncytial formation. Cytoplasmic fusion results in formation of multinucleated cells then dispatched areas were appeared on later stage of infection and most of the cells degenerate with few scattered cells after 72 hours P.I. This was clear in Photos 2-3 and Table 2.

TABLE 2. Virus propagation on CER and Vero cells cells.

Number of passages	CER cells		Vero cells	
	TCID50/0.1 ml Log10	Time of appeared CPE/ hours	TCID50/0.1 ml Log10	Time of appeared CPE/ hours
1	207	12	3.83	15
3	3	12	5	12
4	3	12	5.33	10
6	3	12	6 33	10
8	3 5	12	6 84	10
9	3 3	12	7.56	8
10	3.6	12	5 3	12
12	3 6	12	3 3	15
14	3.6	12	3	15

Photo 2. Vero cells infected with the Isolated virus after 27 hours. Very clear syncytial and protoplasmic threads (Crystal violet stain, X 200).

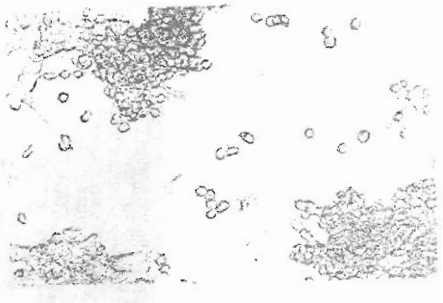

Photo 3. Vero cells infected with ILT virus after 48 hours. Cell detachment and destruction of cells and the cells appears in island of small number. (Crystal Violet stain, X 200)

Viral examination by Electron microscope (EM)

Sample of the isolated virus was examined by EM according to (Wu, *et al.*, 1996) and the observed virus particles had an irregular envelope of a diameter of 195-250 nm. The surface of the envelope contains projections. The shape of the virus was clear in Photo 4.

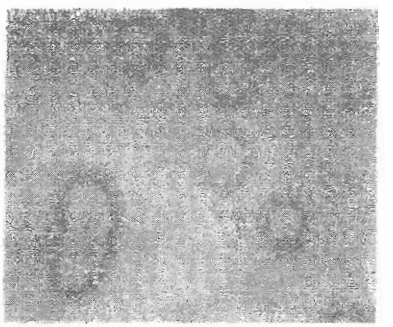

Photo 4. Electron micrograph of ILT virus showing the size approximately 200 nm and the fine projection on the surface. (Sodium phosphotungstate stain, X 75000).

Results of identification of the isolated virus by Dot- ELISA and SNT

The tested purified virus sample, from CAMs of infected ECE, tissue culture, reference ILT virus and negative control antigen was added on the circles of the NCM. Blue dots appear on the circles of tested purified ILT virus and virus obtained from CAMs, Vero cells and reference ILT virus. Purified ILT virus did not give positive results except after addition of NP-40 and Triton X 100 Photo 5.

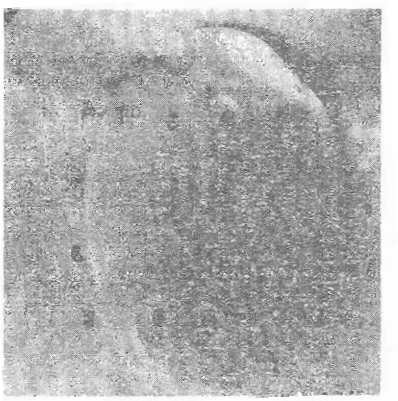

Photo 5. Dot ELISA test for the purified tested ILT virus showing blue dots: a-Tested purified ILT virus. b-Tested ILT virus suspension obtained from CAM of infected ECEs. c-Tested ILT virus suspension obtained from infected Vero cells. d-Reference ILT virus as positive control. e-Negative antigen as negative control.

No pock lesion or CPE were observed in both used system after neutralization of the isolated virus by the anti-ILT virus hyperimmune serum.

Discussion

ILT is an acute viral disease affects chickens with high flock morbidity and mortality. The affected chicken showed nasal discharge, lacrimation and moist rales, followed by gasping, in the later stages dyspnea occurs with extension of the head and neck and expectoration of bloody mucus (Bagust, 1986 and Bagust, *et al.*, 2000). The observed clinical findings come in agreement with the picture seen in a farm located in El-Qaluobia governorate.

During the follow up of this farm and on the post mortem examination, acutely affected birds showed mucoid inflammation and necrosis and desquamation of the inflamed tracheal mucosa, which in sever cases, clotted blood occluded the tracheal lumen resulting in asphyxiation and this picture agrees with (Cover and Benton, 1958). The trachea of infected chicken in this farm were taken and subjected for virus isolation, identification and for further vaccine preparation.

The virus was isolated on CAM of ECEs of 10-day-old, which produces foci of pock lesions having an opaque raised edge and a grey central area of necrosis, which increases in size and number with several passages (Table 1 and Photo 1). This result was also described by (Hanson, 1984; Hanson and Bagust, 1991; and Madbouly *et al.*, 1996).

The isolated virus was propagated on CER and Vero cell lines because primary cell culture were expensive, cannot be maintained in a continual supply of fresh harvested cells and may results in an inherent degree of variability (Rossi *et al.*, 1969).

Data presented in Table 2 showing that the infectivity titer of the isolated virus on CER cells did not increase than 3.6 $\log_{10}$ even after the 10^{th} passage and the showed CPE was rounded cells begin after 12h and end after 72 hours without progressive CPE. In contrast, the infectivity titers on Vero cells were 7.65 $\log_{10}$ after the 9^{th} passage and then decreased gradually from the 10^{th} passage till 14^{th} passage. The observed CPE began with rounded cells that aggregated showing syncytia followed by Cytoplasmic fusion, which resulted in multinucleated cells and ended with dispatched areas (Photos 2- 3). From these obtained results, it is very clear that, Vero cells appeared to be both permissive and productive for ILT virus multiplication while CER was poorly productive as they were giving low virus titer with very limited CPE. These data come in agreement with (Hanson and Bagust, 1991; and Hughes and Jones, 1968). The virus titer increased gradually till reaching 7.65 $\log_{10}$ (Table 3), then decreased again till reaching (3 $\log_{10}$) probably due to virus mutation. This mutation may be due to point mutation (deletion of some nucleotide in the viral genome). Some factors play an important role in virus mutation including prolonged propagation on cell culture, changing the host system used for virus propagation (Madbouly, *et al.*, 2001), here propagation on ECE for 10 passages followed by propagation on Vero cells for 14 passages may be the main reason for mutation, but Vero cells has the disadvantage that propagated virus decrease in its infectivity titers after several passages on this type of cells.

Following propagation, the isolated virus was purified by sucrose gradient ultra centrifugation, where purified virus was collected in the form of a white band at the end of centrifugation. This comes in agreement with (Cruickshank, *et al.*, 1963 and Wartch, *et al.*, 1963).

The purified virus was examined by electron microscopy. The mature virus particles appeared as hexagonal virions with irregular envelope, which contain fine projection on its surface Photo 5. This observation conform those obtained by (Cruickshank, *et al.*, 1963, and Wu, *et al.*, 1996).

The isolated virus was also identified by Dot-ELISA using the prepared ILT virus from the vaccine strain. Triton X100 and/or NP-46 were essential for appearance of blue dots. This comes in agreement with (Lin, *et al.*, 1996). The intensity of the blue dots were very clear and dark with peroxidase conjugate concentration 1: 100 instead of 1: 1000, and the solubility of the viral antigen liberated by using Triton X100 or NP-40.

Moreover, the isolated virus was identified by serum neutralization test using ECE and Vero cells where no pock lesions nor CPE were observed, and this agree with Hitchner, *et al.*, 1975 and Hsiung, 1973 respectively.

References

Bagust, T.S. (1986) *Laryngotracheitis (gallid-1) herpes virus infection in chicken.* (4) latency establishment by wild and vaccine strain of ILTV. *Arian Pathol.*, **15**, 581.

Bagust, T.S., Jones, R.C. and Guy, S.S. (2000) Avian infectious laryngotracheitis. *Rev. Sci. tech., Aug.;* **19** (2), 483.

Beach, S.R. (1926) The virus of infectious laryngotracheitis. *J. Am. Vet. Med. Assoc.;* **68,** 570.

Burleson, F.G., Chambers, T.M. and Wiedbrauk, D.L. (1989) Antisera production in virology: A laboratory Manual. *Acad. Press, INC, Harcourt Brace and Jovanovich; Publishers, New York,* 229.

Cover, M.S. and Benton, W.S. (1958) The Biological variation of ILT virus. *Avian Dis.;* **2** (2), 375.

Cruickshank, J.G., Berry, D.M. and Hay, B. (1963) The fine structure of infectious laryngotracheitis virus. *Virol.*, **20**, 376.

Hanson, L.E. (1984) Laryngotracheitis "*Disease of poultry*" Hoftsted, M.S. and others (Ed.), 8th ed. p. 440, Iowa State University Press, Ames, Iowa, USA.

Hanson, L.E. and Bagust, T.J. (1991) Laryngotracheitis in "*Disease of poultry*" Bu Calnek, H.S. Barnes, C.W. beard, W. M. Reid and H.W. Yoder (Ed.), 9th ed. p.485, Iowa State University Press, Ames, Iowa, USA.

Hitchner, S.B., Catherine A. Shea and White, P.G. (1975) Studies on the serum neutralization on test for the diagnosis of laryngotracheitis in chickens. *Acian Dis.;* **2**,258.

Hsiung, G.D. (1973) *Diagnostic Virology–An illustrated Handbook.* Yale University Press, New Haven and London., p. 22.

Hughes, C.S. and Jones, R.C. (1968) Comparison of cultural methods for primary isolating of infectious laryngotracheitis virus from field materials. *Avian Pathol.;* **17**,295.

Kawaguachi, T., Nomura, K., Hirayama, Y. and Kitagawa, T. (1987) Establishment and characterization of a chicken lepato cellular carcinoma cell line, LMH. *Cancer Res.;* **47**, 4460.

Lin, M.Y., Wu, H.H., Huang, K.S. and Sung, H.T. (1996) Simple dot immuno assay for serum antibody against infectious bursal disease, infectious laryngotracheitis, infectious bronchitis and avian reovirus. *Taiwan Journal of Veterinary Medicine and Animal Husbandry;* **66** (4), 343.

Madbouly, H.M. (1989) A simple method for coating ELISA plates with Newcastle disease virus. *J. Egypt. Vet. Med. Ass.;* **49** (4), 1129.

Madbouly, H.M., Ensaf, M. Khashaba and Nadia, M. Ebraheem (2001) Virus vaccine from recent Egyptian isolated and adjuvanted with Nigella sativa oil. *J.Vet. Med. Giza;* **49** (4), 553.

Madbouly, H.M., Ismail, M.R. and Tamam, S.M. (1996) Role of infectious laryngotracheitis virus vaccine in spreading of ILT disease among and in between layer and broiler birds 1-Isolation and identification of the virus. *Beni-Suef Vet. Med. Res.;* **6**, 27.

Rossi, C.R., Reynolds, H.A. and Watrach, A.M. (1969) Studies of laryngotracheitis virus in avian tissue cultures plaque assay in chicken embryo kidney tissue cultures. *Arch. Gesamta Virusforsch;* **28**, 219.

Schnitzlein, W.M., Jane Radzevicius and Deoki, N. Tripathy (1994) Propagation of infectious laryngotracheitis virus in an avian liver cell line. *Avian Dis.;* **38**, 211.

Tantawi, H.H., Batrawi, A.A., El Bastami, M.A. Youseef, Y.I. and Fawzia, M.M. (1983) Avian infectious laryngotracheitis in Egypt (Epidemiology, virus isolation and identification). *Vet. Res. Communication,* **6** , 281.

Wartch, A.M., Hanson, L.E. and Watrach, M.A. (1963) The structure of infectious laryngotracheitis virus. *Virol.;* **21**, 601.

White, D.O. and Fenner, F.S. (1986) *Medical Virology,* Academic Press, Inc., Orlando, 325.

Wu, Hong Zhuang, Lu, Fu An, Wu, H.Z. and Liu, F.A. (1996) Purification and electron microscopic observation of infectious laryngotracheitis virus. *Chinese J. of Vet. Med. Sci. and Tech.,* **26** (3), 21.

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تنقية وتوصيف عتره فيروس مرض التهاب الحنجرة والقصبة الهوائية المعدى المعزولة محليا

حنفي محمود مدبولي*، محمد الصغير أحمد بكر، نجوى سيد سيد عطا**، محمد عبد العزيز قطفاط** و نوش سيد علي زاهر**.**

*كلية الطب البيطري ، جامعة القاهرة (فرع بني سويف) و** المركز القومي للبحوث، الدقي -- مصر.

تم عزل عترة محلية من الفيروس المسبب لمرض التهاب الحنجرة والقصبة الهوائية المعدي في الدواجن على الأغشية اللقانيفية للبيض المخصب الخالي من المسببات المرضية وذلك بالتمرير لمدة ١٠ تمريرات عند عمر ١٠ أيام وقد أظهرت الحرشوفيات الخاصة بالمرض على الغشاء اللقانيقي صغيرة الحجم عند التمريرة الأولى ثم ازدادت في العدد والحجم بعد ذلك ثم تم تمرير وإكثار الفيروس على خلايا الفيرو والـ سي إي أر (CER) وأزداد عيارية الفيروس فقط عند تمريره على خلايا الفيرو حتى وصل إلى العيارية لو.، $^{٧.٥٦}$ التمريرة التاسعة ثم تناقص عيارية الفيروسات بعد ذلك.تم تنقية الفيروس بواسطة الطرد المركز السريع وبعد ذلك تم التعرف عليه بواسطة الميكروسكوب الإلكتروني والاختبار الأنزيمي المناعي الأرتباطى النقطي Dot-ELISA وتعادل المصل العالي في الأجسام المناعية الخاصة للفيروس.