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STUDIES ON INFECTIOUS LARYNGOTRACHEITIS IN CHICKENS I. ISOLATION AND IDENTIFICATION OF INFECTIOUS LARYNGOTRACHITIS FROM SOME CHICKEN FLOCKS.

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SUMMARY

Infectious laryngotracheitis virus (ILTV) was detected in chicken laryngeal and tracheal samples from outbreaks in 4 governates in Egypt (Kalubia, Sharkia and Gharbia). The viral antigen was detected by AGPT and FAT in 7 isolates, which subjected to physicochemical, biological characterization and pathological investigations. Isolated ILTV produced characteristic pock lesions on CAM of Embryonated Chicken Eggs(ECEs) and characteristic intranuclear inclusion bodies by direct FAT and histopathological examination in infected CAM and tracheal smears.

The isolate S3 was the most pathogenic strain (by pathogenicity test of the isolates on ECEs and susceptible chickens) and had one more protein band than the other isolates as compared to the standard virus (by SDS-PAGE). Ether sensitivity and thermo-stability of the isolated viruses at 56°C indicated that the local isolate loss its infectivity to ECEs after treatment with ether for 24 hours and 15 min. at 56°C as well as the standard control ILTV. By cross neutralization, the results revealed that the isolated strains appeared to be related to the standard ILTV through neutralization index between each other. Infectivity titre of the virus was increased by passaging it in SPF eggs till reaching the highest titre by the 5th passage.

INTRODUCTION

Laryngotracheitis (LT) is a respiratory tract infection caused by an alphaherpes virus. It is principally a disease of chickens, but it can also affect pheasants, partridges, and peafowl, and may result in severe production losses due to mortality and/or decreased egg production (Ollis 2004). Infectious laryngotracheitis virus (ILTV) was firstly described in USA by May and Tittsler (1925). Since that time, the virus had been identified world-wide and remained a serious disease where

susceptible poultry population occurs, especially in areas of intensive production (Biggs, 1982).

Laryngotracheitis virus (ILTV) recorded for the first time in Egypt by Tantawi et al. (1983), where the virus was isolated from outbreaks of haemorrhagic trachitis occurred in layers 4-12 months in age in several farms in Cairo and Giza Governorates with rapid spread, and 5% and 35% drop in egg production with 0.5% to 19.8% mortality. Repeated virus isolation and survey studies have proved the wide spread existence of the clinical and sub-clinical forms of the disease among commercial layer and broiler flocks (Amer, 1984; Shakel, 1986; Madbouly et al., 1996 ; Sultan and El-Gohary, 1999 and Shehata, 2004). However; problems could rise from the use of the vaccines, including spread of vaccinal virus to non-vaccinated birds with potential reversion of live vaccine viruses to parental-type virulence contributing to sporadic outbreaks and spread of the disease (Guy et al., 1991). Now in Egypt, the increased incidence of ILTV outbreaks in commercial broiler, layer and breeder flocks was observed in several governorates and the main problem in poultry farms lies in the appearance of the disease in vaccinated layer birds, leading to decrease in egg production.

Accordingly, the present work was planned to update the isolation and identification of the circulating field causative agent of ILT in Egypt and determination of its pathogenicity.

The identification of the isolates was done physically using treatments with ether and heat (56oC), and biologically using agar gel precipitation test (AGPT), direct fluorescent antibody test (FAT), and histopathological examination of the affected organs as well as comparison of protein profile of the isolates with different vaccinal strains through sodium dodecyl sulphate poly-acrylamide gel electrophoresis (SDS-PAGE).

MATERIALS AND METHODS

1- SAMPLES FOR ISOLATION:

Tracheal, larynx and lung tissue samples were collected from freshly dead and/or naturally diseased broiler and layered chickens showed severe respiratory disorders. These samples were collected from chicken flocks reared in Kalubia, Sharkeia, and Gharbia governorates.

2- LABORATORY ANIMALS:

2-1- Specific pathogens free (SPF) fertile chicken eggs:

10-12 days old, were imported from SPA FAS, Inc., USA; and used for virus isolation, re-isolation, virus titration and for serum neutralization test.

2-2- Chickens:

Six week-old Hubbard susceptible chickens were obtained from Egyptian Poultry General Company. They were fed on a balanced commercial ration and used for preparation of anti-ILTV hyper immune serum, and pathogenicity

tests of the isolated virus. The serum of these chickens was proved to be free from antibodies against ILTV by ELISA.

3-ILT VIRUSES:

3-1- Standard ILTV strain:

It was kidney supplied by the NewCastle Department, Vet. Ser. and Vac. Res. Institute, Abbasia, Cairo. It was in the form of a lyophilized powder, with titre 106.5 EID50 / ml.

3-2Egg adapted lyophilized ILTV vaccines: Two different commercial live attenuated egg adapted ILTV vaccines, one imported and the other locally prepared (Vet. Ser. Vac. Res. Ins.), were used to compare with the local virus isolates.

4 Infectious Laryngotracheitis Antisera:

a- Reference hyper immune serum against ILT virus was kindly supplied by NewCastle department, Vet. Ser. Vac. Res. Ins., Abbasia, Cairo. It was used for identification of ILT virus field isolates.

b- Hyper-immune serum against ILT virus was prepared in susceptible chickens (compared its specificity with the standard one) according to Schmidt (1970). This serum was used as positive serum in the serological tests.

c- Reference hyper immune serum against ILT virus was conjugated with fuorescin isothiocyanate (FITC) to be used in fluorescent antibody technique (FA) for virus detection according to Hitchner et al. (1977).

4- STAINS:

1- Haematoxyline & Eosin, was used for histopathological examination.

2- Phloxine Tartrazine, was used for detection of intranuclear inclusion bodies.

5-AGAR GEL PRECIPITATION TEST (AGPT): According to Beard (1982).

6-SODIUM DODECYLE SULPHATE POLYACRYLAMIDE GEL ELECTROPHORESES (SDS-PAGE): According to Sambrook et al. (1989).

7- Enzyme Linked Immuno-Sorbent Assay (ELISA):

According to York et al. (1983) and Leong et al. (1994).

8-Fluorescent Antibody Technique (FAT): According to Hitchner et al. (1977), infected cells of CAMs and tracheal epithelium were smeared on microscopic slides, fixed with cold acetone for 10 minutes. Fixed cells were floated with 1:10 fluorescene conjugated anti ILTV hyper immune serum diluted 1:10 and incubated at 37oC in humid chamber for 30 min. After incubation time, the slides had been washed then examined under fluorescent microscope.

9- Cross Neutralization Test:

According to Tripathy and Hanson (1989), ILTV was identified by the cross neutralization test against reference ILT antiserum. The end point (EID50) for each virus sample was calculated according to Reed and Muench (1938).

10- Virus Pathogenicity:

i. In chickens: Six weeks old chickens tested to be free from antibodies against ILTV by ELISA were inoculated intratracheally with the field isolates (ten chickens for each isolate) and leaved for observation of clinical signs for 10 days. All chickens showing obvious clinical signs or dying after 2 days post infection (PI) were subjected to post-mortem (PM) examination, and tracheal smears were examined for ILTV inclusions by FA and histopathology.

Tracheas showed positive FA, and inclusion bodies were collected and homogenized with sterile sand then prepared as suspension in sterile PBS, the homogenized tracheas were subjected to 3 cycles of freezing and thawing then centrifuged at 3000 rpm for 15 min. by cooling centrifuge. The supernatant were collected and re-inoculated intratracheally in susceptible chickens for 5 successive passages.

ii. In embryonated chicken eggs (ECE): According to Izuchi and Hasegawa (1981), ten days old ECEs were inoculated via the allantoic cavity with 0.1ml of the field isolates virus suspension. The mortality index for chicken embryos (MICE) were calculated. MICE = the cumulative numbers of embryos dying up to 7 days PI / cumulative numbers of live embryos for 7 days PI.

11-ILT Virus Stability:

The following tests were carried out to study the physicochemical properties of the ILT local isolates:

i. Ether Sensitivity: According to Sharma et al. (1974).

ii. Stability at 56oc: According to Schalm and Beach (1935).

12-Comparison between protein bands of local isolates with standard ILT viruses using Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE): The protein antigen was identified by its electropheretic mobility in 10% polyacrylamide gel according to Sambrook et al. (1989).

13- Statical analysis: According to Steel and Torrie, (1960).

RESULTS AND DISCUSSION

The epidemiological investigation of viral laryngotracheitis infection in chickens was carried out on 20 specimens which were collected from

chicken flocks showed nasal discharge, gasping, coughing and/or expectoration of bloody exudates) from different regions (9 samples from kalubia governorate, 5 samples from Sharkia and 6 samples from Gharbia). The outbreaks were occurred at 4-6 weeks of age in broilers, 7-8 weeks or 20 weeks of age in commercial layers and average 10 weeks of age in breeder chickens (table 1). The range of susceptible age agreed with the previous reports (Seddon, 1952; El-Zanaty and Ahmed, 1995; Madbouly et al., 1996; Sultan and El-Gohary, 1999; Amer, 2001 and Shehata, 2004). The affected birds showed respiratory signs, conjunctivitis, and haemorrhagic Tracheitis (epizootic form) from flocks in kalubia and Gharbia, and yellow caseous materials in the trachea (enzootic form) from flocks in Sharkia governorate. These findings agreed with results described by several investigators for ILT infection (James and Trevor, 2003). The morbidity rate was 38 – 63 % and mortality rate was 11 – 21 % in flocks at kalubia. The morbidity rate was 22 – 44 % and mortality rate was 8 – 16 % in flocks at Sharkia (table 1). The morbidity rate was 30 – 56 % and mortality rate was 10 – 15 % in flocks at Gharbia. The mortality rates ranged 12 – 16 % in broilers, 8 – 21 % in commercial layers and average 11 % in breeder chickens. Variable mortalities may be due to variation in pathogenicity of LTV strain involved, however flocks vaccinated against ILT showed the lowest mortality rates.

For the detection of ILT virus in collected tracheal samples, AGPT and FAT were applied. Data presented in table (2) showed that ILTV was detected by AGPT in 2 samples which collected from broiler flock (S3) and breeders flock (S9) in kalubia. On other hand, FAT revealed that ILTV was detected in 7 samples which collected from broiler flocks (S2, S3), commercial layer flock (S5), and breeders flock (S9) in kalubia, commercial layer flock vaccinated with live ILT vaccine (S14) from sharkia, and commercial layer flock (S16) and broiler flock (S19) from Gharbia. These results indicated the highest sensitivity of FAT for the detection of ILT virus than AGPT. The findings are in agreement with those York et al. (1983) and Jones (2001) who conducted the lowest sensitivity of AGPT. While, the highest sensitivity of FAT was conducted by Hitchner (1975) and Goodwin et al. (1991).

Beside the simplicity of application and the accurate results taken by FAT, it triggered us to say that this technique was preferred to use for detection of the virus in infected tissues as rapid and accurate diagnostic tool. In the suspected outbreaks of the disease, from which no virus was isolated, it is possible that the samples had deteriorated in transportation or had been collected too late in the course of the disease. Alternatively, the ILT virus may not have been involved and or there was other possible cause of the outbreaks (Hughes et al., 1991).

The prepared tracheal tissue suspension samples

S2, S3, S5, S9, S14, S16, and S19 gave positive results by FAT were separately separately inoculated on CAM of 12 day-old SPF embryonated eggs. Only 2 inoculated samples (S3, S19) gave the characteristic pock lesions for ILTV as rounded foci 3-5 mm in diameter with opaque raised edge and depressed gray central area of necrosis with generalized edema on the CAM and area of opacity at the site of inoculation (Fig. 1). The character of the observed pock lesions are similar to those ILTV described by Alls et al. (1969) and Hanson (1984). While, the other 5 inoculated samples (S2, S5, S9, S14 and S16) gave large number of small foci ranged from 1-2mm. in diameter scattered all over the CAM and agrees with those described by Herceg et al. (1971) and El-Mahdi (1986). Furthermore, these pock lesion may be due to attenuated ILTV as stated by Yamanaka et al. (1982) who pointed out that the attenuated virus formed atypical small pocks (about 2mm. in diameter) on the CAM of ECE.

Histopathological examination still considered as one of the most important diagnostic tool for ITLV and detection of intranuclear inclusion bodies cyncetia formation and inflammatory cells infiltration in infected tracheal epithelial cells and CAM as described by keller and Hebel (1962) and Humberd et al. (2002). So, tracheal epithelium smear from birds inoculated by prepared tissue samples for virus re-isolation gave positive results by FAT and confirmed histopathologically by the presence of characteristic intranuclear inclusion bodies which agreed with the findings of Idc (1978) and Goodwin et al. (1991) who reported that FAT is as effective as histopathology for the diagnosis of ILT virus infection.

The results of table (2) showed clearly that the AGPT was less sensitive test for virus identification as gave negative results with two isolates (S14 & S16) which were given pock lesion on CAM and were positive by FAT and histopathology. These results agreed with Van-Kammen and Spradbrow (1976) and Badr El-Din (1985) who declared the lower sensitivity of AGPT in detection of ILTV infection.Therefore, the identification of ILT virus by fluorescent antibody technique (FAT) was used for the identification of ILT virus in infected CAM section, where very clear intranuclear green shiny fluorescence granules were appeared with different in density, as well as by histopathological examination for the detection of intranuclear inclusions bodies was applied (Fig. 2).

The pathogenicity of each identified virus isolate was detected by inoculating 11 day-old chicken embryos to distinguish the virulent strains of ILT virus. The mortality index for chicken embryos (MICE) less than 0.16 indicate low pathogenicity or no pathogenicity for chickens and those with MICE more than 0.27 can be highly pathogenic (Izuchi and Hasegawa, 1981). So, results in table

(3) showed that ILT isolates S3 & S19 considered highly pathogenic as the MICA are 0.92 and 0.64; respectively and S2, S5 & S9 were moderate pathogenic as MICA was 0.21, 0.31 and 0.23; respectively, while S14 and S16 were considered apathogenic (MICA 0.15 and 0.12; respectively). Our findings agreed with Yamanaka at el., (1982) who found that chicken embryos inoculated with wild type of ILTV had higher mortality rates than those inoculated with modified virus strain which gave no adverse reaction after administration to chickens.

When studying the pathogenicity of ILTV isolates, each virus isolate gave positive result by FAT, when it was inoculated intratracheally on susceptible 6 weeks old chickens. On the 4th day post inoculation (PI) the inoculated chickens were killed and tracheas were collected for examination to detect the virus pathogenicity. As shown in table (4) only S3 virus isolate gave severe respiratory symptoms and one bird died at the 7th day PI with haemorrhages in larynx and trachea in PM examination (Fig. 3). The clinical symptoms and PM lesion of chickens inoculated with isolate S19 gave moderate reaction, and isolates S2, S5, S9 gave only mild symptoms and PM lesion. While no signs could be detected in S14 and S16 . The isolates S9 and S14 were isolated from vaccinated flock, so, these isolates could be vaccinal strains. It remains uncertain whether the other isolates (S2, S5 and S16) have their origin as wild field viruses or as virulence reversed modified vaccinal viruses. From these results it was very clear that S3 isolate was the highly pathogenic isolate followed by S19 isolate which is less pathogenic as it failed to induce death in inoculated birds.

Further investigations for more identification were carried out on the isolated ILT viruses. The inability to demonstrate pock lesion on inoculated CAM with ILTV after ether treatment indicates that these isolates are ether sensitive. Similar finding have been reported for some ILTV strains of ILT virus by Fitzgerald and Hanson (1963) and Sharma et al. (1974).

Concerning to the results of thermal stability at 56oC, enveloped viruses rendered non-infections after incubation at 56oC for different times due to protein denaturation and tend to be more heat labile. The ILT virus isolates were rendering non-infectious after incubation for 10-15 min. at 56oC as showed in table (5). Similar results were achieved by Schalm and Beach (1935), when the virus kept at 55oC, it became avirulent within 15 min. Also, Sharma et al. (1974) and Meulemans and Halen (1978) found that most ILT virus isolates were sensitive at 56°C for 30 min.

Protein content of the pathogenic ILTV isolates and of standard ILTV strain was purified and subjected to SDS-PAGE to compare between their protein profiles. The electrophoretic migration

pattern of virus isolates protein revealed that the more bands were used for the vaccine preparation. Polypeptide profiles of ILTV isolates was sharing the standard one in all major bands with demonstration of minor variation between the local isolates and the standard one as they have less dense in protein band (55 KDa), while the virus isolate S3 was having protein band with 22 KDa more than the standard one and more dense in protein band 112 & 60 KDa; (Fig. 4). Simillar result was reported by York et al. (1987) and Yurov et al. (1993) who reported that ILT virus have five major envelope glycoproteins with molecular weights of 205, 160, 115, 90 and 60 KD, which considered the major immunogens of the LTV, also, detected dimmer of molecular weight of 112 KD with a disulfide bond.

Moreover, virus cross neutralization assay was used for detection the relation between ILTV isolate strain (S3) and the standard one. It indicated minor antigenic variations between the two viruses, as there was no significant difference between the neutralization indexes between each other at $P \leq 0.05$. As shown in table (6), the cross neutralization test between standard and local ILTV isolate (S3) gave neutralizing index 2.9, 3.7, 2.7, and 3.5 by the isolate / the prepared antiserum, isolate / the standard antiserum, standard ILTV /the prepared antiserum, and standard ILTV / standard antiserum; respectively. So, the isolated strain (S3) was closely related to the standard ILTV through the neutralizating activity.

As resulted by Fitzgerald and Hanson (1963) and Russell and Turner (1983) in comparing five isolates of ILTV and found that all viruses were antigenically close related by kinetics of neutralization but 2 viruses were not homogeneous with the other three viruses when neutralized by standard antiserum.

Table(1): History of the examined suspected ILTV infected chicken flocks.

Location	No. of flocks	Sample No.	Type of birds	Flock capacity	Age of birds	Disease* Form	Morbidity rate	Mortality rate	vacci ag IL
kalubia	Flock 1	S1	Commercial layer	10000	5 w	Epizootic * Form.	63%	21%	
		S2							
		S3							
	Flock 2	S4	Broiler	8.000	7 w		49%	12%	
		S5							
		S6							
	Flock 3	S7	Breeders	6.000	10 w		38%	11%	At 5
		S8							
		S9							
Sharkia	Flock 1	S10	Broiler	7.000	4 w	Enzotic * Form.	44%	16%	
		S11							
	Flock 2	S12	Commercial layer	10.000	20 w		22%	8%	At 6
		S13							
		S14							
		S15							
Gharbia	Flock 1	S16	Commercial layer	8.000	8 w	Epizootic* Form.	30%	10%	
		S17							
		S18							
	Flock 2	S19	Broiler	6.000	6 w		56%	15%	
		S20							

S= sample W= week * disease form according to James and Trevor (2003).

Table (2): Detection of ILTV in suspected samples.

Isolates No.	FAT	AGPT	Histpath . Exam.	Isolates No.	FAT	AGPT	Histpath . Exam.	Isolates No.	FAT	AGPT	Histpath . Exam.
S1	-	-	-	S8	-	-	-	S15	-	-	-
S2	+	-	+*	S9	+	+	+	S16	+	-	+
S3	+	+	+	S10	-	-	-	S17	-	-	-
S4	-	-	-	S11	-	-	-	S18	-	-	-
S5	+	-	+	S12	-	-	-	S19	+	-	+
S6	-	-	-	S13	-	-	-	S20	-	-	-
S7	-	-	-	S14	+	-	+				

+FA: Intranuclear fluorescence (yellowish-green) granules. +AGPT: Presence of line of precipitation.

Table (3): Mortality index of ILTV isolates for chicken embryos.

Virus isolates	S2	S3	S5	S9	S14	S16	S19
MICE	0.21	0.92	0.31	0.23	0.15	0.12	0.64

Table (4): Result of pathogenicity testing and virus re-isolation in susceptible chickens.

Virus isolates	Number of birds	Symptoms and PM	Deaths	Re-isolation	FAT	Detectin of inclusion bodies
S2	10	mild#	-	+	+	+
S3	10	sever	1	+	++*	+
S5	10	mild	-	+	+	+
S9	10	mild	-	+	+	+
S14	10	-	-	+	+	+
S16	10	-	-	+	+	+
S19	10	moderate	-	+	++*	+
control	5	-	-	-	-	-

*++: high density of virus FA reaction. # Mild: Depression and conjunctivitis. Increase mucous in the trachea. Moderate: Conjunctivitis with watering and mucoid nasal discharge. Congestion of larynx and trachea with mucoid exudates in tracheal mucosa. Sever: Conjunctivitis, nasal discharge, swelling of sinus, and coughing, Fibrinous laryngotracheitis, following by peticheal hemorrhage in larynx and trachea.

Table (5): Result of virus stability at 56°C.

Time/Min./ isolate No.	5	10	15	20
S2	+	+	+	-
S3	+	+	-	-
S5	+	+	-	-
S9	+	+	+	-
S14	+	+	-	-
S16	+	+	+	-
S19	+	+	-	-
Control	+	+	+	+

+ = virus still have infectivity to CAM (presence of pock lesions)
- = virus loss infectivity to CAM (no pock lesions

Table (6): Cross neutralizing index of standard and isolated ILTV.

Group of neutralization	Virus titer alone	Virus Serum mixture titer	Neutralizing Index
Group I	6.1	3.2	2.9
Group II	6.1	2.4	3.7
Group III	7.5	4.8	2.7
Group IV	7.5	4.0	3.5

Group I: Isolated ILTV + prepared antiserum.
Group II: Isolated ILTV + standard antiserum.
Group III: Standard ILTV + prepared antiserum.
Group IV: Standard ILTV + standard antiserum.

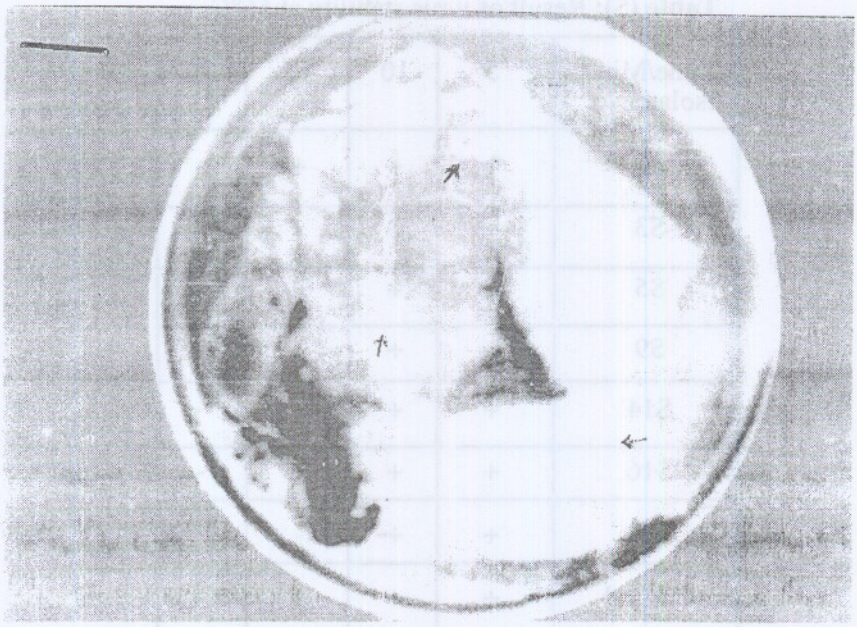


Fig.(1): Inoculated CAM show characteristic pock lesions for IL TV.

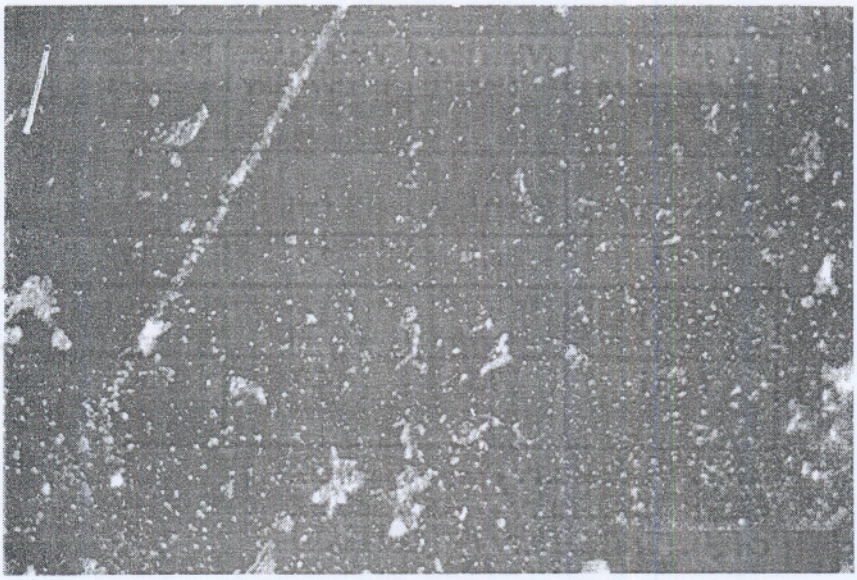


Fig.(2): Infected CAM show intranuclear fluorescence granules.

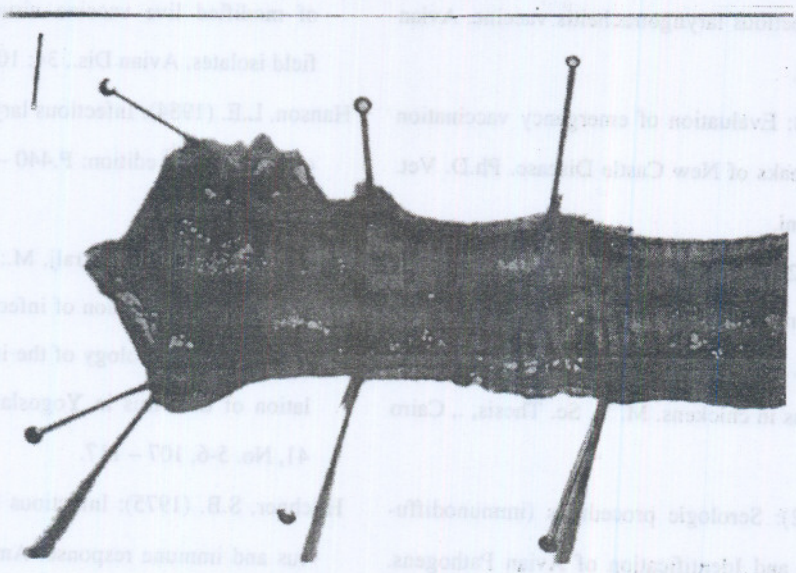
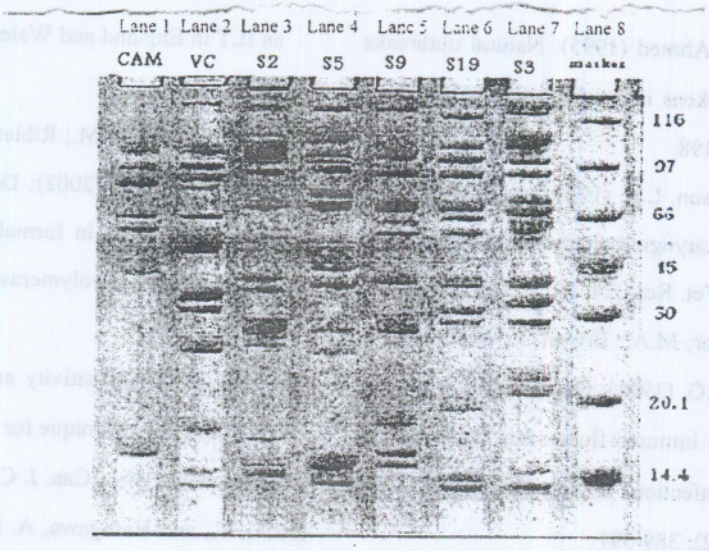


Fig. (3): and trachea of bird experimentally inoculated with IL TV isolate S3, showing peticheal and echymotic haemorrhages.

Fig. (4): Protein profile of ILTV isolates by SDS-PAGE.



CAM: non inoculated chorio-allantoic membrane (-ve control) VC: virus control (standared ILTV strain)

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الملخص العربى

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تم الكشف عن فيروس التهاب الحنجرة والقصبة الهوائيه المعدى فى عينات تم تجميعها من قطعان في محافظات القليوبية والشرقية والغربية، باستخدام اختبار الترسيب في الأجار الهلامي والإليزا.

تم عزل سبع عترات من الفيروس وتمت دراسة الصفات الفيزيوكميانية والصفات البيولوجية للفيروسات المعزولة محلياإالي جانب الفيروس القياسي كضابط.

تسببت العترات المعزولة لفيروس التهاب الحنجرة والقصبة الهوائية المعدي في ظهور بثرات مميزة علي الغشاء اللفافي لأجنة البيض كما أظهرت أجسام محتواه بالنواه مميزة للفيروس عند الصبغ بالصبغات العادية وصبغة الفلوريسين لخلايا الغشاء اللفافي والحنجرة.

أظهرت العترة S3 في اختبار الضراوة بالنسبة لأجنة البيض والفراخ القابلة للعدوى، أنها الأكثر ضراوة في احداث موت للأجنة واظهار الأعراض الأكلينيكية علي الطيور.

عند دراسة الشكل البروتيني للفيروسات المعزولة مع الفيروس القياسي باستخدام طريقة الفصل الكهربائي في الأكريلاميد جيل، ظهر تشابه بين جميع العترات المعزولة والفيروس القياسي مع اختلاف بسيط في الفيروس الضاري المعزول S3.

عند اختبار تأثير الأثير ودرجة حرارة 56^{5}م علي الفيروسات المعزولة، وجد أنه يفقد قوته العيارية بعد 24 ساعة من التحصين مع الأثير و15 دقيقة في درجة حرارة 56^{5}م .

عند اجراء اختبار التعادل المصلي التبادلي بين العترة الضارية المعزولة محليا والعترة القياسية، وجد أنه لا يوجد فرق واضح بين مقياس التعادل لكل منهما.