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INFECTIOUS LARYNGOTRACHEITIS IN CROSS-BRED AND BROILER BREEDER CHICKENS

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SUMMARY

Outbreaks of infectious laryngotracheitis (ILT) were diagnosed in cross- bred (6-10 weeks of age) and broiler breeder chickens (19-20 weeks of age) with mortality rates ranged from 4.4-12.5% and 1.5-2 % per week, respectively. Four field isolates of laryngotracheitis virus (LTV) were recovered from suspensions of pooled tracheas of affected flock after 1-2 serial passages into 10- day-old specific-pathogen-free (SPF) chicken embryos, and were identified by agar gel precipitation test (AGPT) using reference monospecific LTV antiserum. In addition, the pathogenicity study of LTV which isolated from cross-bred chickens, in 50- day- old cross- bred chickens and in chicken embryos revealed that this isolate was a virulent strain based on intratracheal pathogenicity index (ITPI), tracheal lesion scores and mortality index for chicken embryos (MICE). Assessment of protection against this isolate using either chicken embryo origin (CEO) or tissue culture origin (TCO) vaccines revealed that chickens receiving two vaccinations had lower lesion scores and higher ELISA antibody titers than those vaccinated once. These results suggest that a priming vaccination followed by a booster dose offers better protection against circulated wild virulent LT than a single vaccination alone, moreover, the epizootiology of the disease was discussed.

INTRODUCTION

Infectious laryngotracheitis (ILT) is an acute respiratory disease affecting mainly chickens, but also pheasants, Peafowl and guinea fowl. The classical form of ILT is characterized by dyspnea,

gasping, coughing and expectoration of bloody exudate (Whiteman and Bickford, 1989). Recently, LTV was isolated from an acute outbreak in ostriches (Yuoangen et al., 2002). The disease is caused by Gallid herpes virus 1 (Roizman, 1982), epizootic forms of ILT were often described in earlier years associated with mortalities of 20% or higher, while the benign forms associated with low mortalities (0.1- 2%) (Guy and Bagust, 2003).

Like other herpes viruses, LTV, including the virulent infection and vaccine strains can establish a latent infection in the trigeminal ganglia of chickens (Bagust, 1986; Williams et al., 1992). Although clinical signs were hardly detected from chickens with a latent infection states, the virus can persist in chickens and can be reactivated by stress factors including re-housing with unfamiliar birds or the onset of lay (Hughes et al., 1989).

Immunity to LTV is mediated mainly by cell-mediated immunity (Fahey et al., 1983) and the humoral antibody might play a role in contributing immunity to ILT (Fahey and York, 1990). Several commercial LTV vaccines are available and the immunity including the antibody titer induced by LTV live vaccines was different depending on the type of vaccine, the route and frequency of administration (Fulton et al., 2000).

ILT has been reported for the first time in Egypt in August (1982), when acute outbreaks occurred in layers on several farms in Cairo and Giza areas, with rapid spread of the virus to adjacent localities (Tantawi et al., 1983). Repeated virus isolation and survey studies have proved the wide spread and existence of the clinical and subclinical forms of the disease among laying and broiler flocks (Mohamed, 1986).

Recently, severe outbreaks of ILT have emerged in both broiler and replacement layer pullets in several Governorates. These outbreaks were associated with high mortalities up to 18% and 40% respectively, (Sultan and El-Gohary, 1999; Aaya Amer, 2001). The present study was planned to update the epidemiological situation of ILT in chicken farms, isolation and identification of the causative agent and determination of the pathogenicity of some field isolates compared with live attenuated LTV vaccine types (CEO and TCO), and evaluation of some vaccination programs.

MATERIALS AND METHODS

History of examined chicken farms:

A total of 9 chicken farms naturally infected with LTV were investigated from February 2001 till May 2003. They included 7 cross-bred broiler and 2 broiler breeder chicken farms located in 3 governorates (El-Behera, Giza and kalyoubia). Further details on history of examined farms are given in table (1 and 2).

Samples for LTV isolation:

Postmortem examination was performed on a

variable number of freshly dead birds which succumbed to the disease after onset of mortality on the examined farms. Gross lesions were recorded and birds with typical gross LTV lesions which mainly haemorrhagic tracheitis and conjunctivitis were used for virus isolation. For this purpose, trachea from 5-10 birds per farm were collected, pooled, labeled and stored until processed for virus isolation.

Fertile chicken eggs and chicks:

SPF embryonated chicken eggs were supplied by National Agricultural Research Center for SPF production (koam Osheim, El -Fayoum province). These eggs were used for virus isolation, virus titration, virus re-isolation, determination of MICE and antigen preparation for AGPT. Day - old- cross-bred chicks originated from commercial broiler parent stock in El- Behera governorate, the flock had a previous history of routine prophylactic vaccination against ILT using live vaccines during the growing period, were not vaccinated against ILT and used for pathogenicity studies and laboratory vaccination trials.

ILT viruses and antisera:

1- Live chicken-embryo-origin (CEO) commercial LTV-vaccine (Intervet. Inter. B.V Boxmeer, Holland) and live commercial tissue - culture (TCO) LTV vaccine (Fowl laryngotracheitis vaccine, Schering Plough Animal Health) were obtained from local agency. They were used for preparation of AGPT- antigen, pathogenicity tests and laboratory vaccination trials.

2- Field isolate no.1 designated Behera/ 2001was used for pathogenicity tests. It was passaged once on CAMs.

3- ILT-antiserum (Intervet Inter. B. V. Boxmeer, Holland) was used in AGP test.

4- Chicken anti-tenosynovitis serum (Intervet Inter. B.V. Boxmeer, Holland) and chorioallantoic membranes (CAMs) of normal and non infected eggs were used as negative serum and antigen controls in AGPT, respectively.

Agar gel precipitation (AGP) antigen:

LTV antigen for AGPT was prepared from infected CAMs of embryonated chickens eggs 5 days after inoculation with 1/10 diluted LTV vaccinal strain (CEO). The membranes with pock-like lesions were homogenized, then centrifuged at 3000 rpm for 15 minutes and the supernatant fluid was used as antigen (Anon., 1990).

Indirect enzyme linked immunosorbant assay (ELISA):

Serum samples were screened by ELISA for antibodies to LTV, using commercial ELISA Kits supplied by Synbiotic Corporation 11011 VIA San Diego, CA (92127). Application and interpretation of the test were carried out according to the instructions of the Kits manufacturer.

Virus isolation in embryonated chicken eggs:

This was carried out by inoculation of 10% tracheal homogenate suspension in PBS (PH 7.2) containing 1000 IU penicillin and 1 mg streptomycin per ml into chicken embryos at 10-11 days of age by the CAM in the usual way (Anon., 1990). Five embryonated eggs per sample were inoculated each with 0.1 ml of the suspension and then incubated at 37°C . Daily candling was performed and embryonic mortalities were recorded for 7 days PI. Mortalities during the first 24 hours were considered non specific and discarded, while mortalities during 2-7 days PI and embryos that survived till the 7th day PI were examined for pock lesions on CAMs. The amnio - allantoic fluids (AAFs) were checked for the presence of haemagglutinating (HA) agent against 10% chicken RBCs by the rapid HA test (Anon., 1990). When the pock lesions were absent, a second passage was done using 10% CAMs suspension.

Virus titration:

Virus infectivity end point of local field isolate Behera/ 2001 as well as live LTV vaccines (CEO and TCO) were carried out according to Hitchner et al. (1958) by inoculation of ten fold (10^{-1} - 10^{-10}) virus dilutions in sterile PBS on CAMs of 10-11 days old chick embryos using 5 embryos per dilution as well as, the inoculum was 0.1 ml per egg. The eggs were candled daily for five days and deaths within the first 24 hours were considered non specific. The CAMs of dead and survived embryos were examined for pock lesions .Calculation of 50% embryo infective dose (EID_{50}) was carried out according to Reed and Muench (1938).

Determination of mortality index for chicken embryos (MICE)

The pathogenicity of local field isolate no. 1 and LTV vaccines (CEO and TCO) were evaluated by inoculating them into 10-day-old embryonated SPF chicken eggs. Each egg was inoculated via allantoic cavity with 0.1 ml of appropriate dilutions of virus suspension and re-incubated at 37°C. They were observed once daily for death for 7 days PI. All deaths occurring within 2 days were considered non-specific. MICE is calculated according to Isuchi and Hasegawa (1982) by dividing the cumulative no. of dead embryos for 7 days PI by the cumulative no. of live embryos for 7 days PI.

Agar gel precipitation test:

The test was used to demonstrate the presence of antibodies to LTV in chicken sera and for detection of LTV antigen (s) in CAMs of inoculated eggs as described by Beard (1982). Control positive and negative antigens or antisera were included in each plate, depending on the objective of the test (antibody or antigen detection). The plates were examined for specific precipitin line (s) after 24 hours incubation at 37°C and rechecked for final reading after 48 hours.

Histopathological examination:

Tracheas from experimentally infected birds that died 5-7 days PI controls and vaccinated birds were fixed in 10% neutral formalin buffer solution and processed for paraffin sections in the usual way. Sections were stained with Hematoxlin and Eosin stains (Culling, 1974) and evaluated according to the method described by Guy et al. (1990) for calculation of lesion scores as follow: 0 = normal epithelium; 1= normal epithelium with very mild lymphocytic infiltration in lamina propria; 2= mild changes of epithelium and mild inflammatory cell infiltration by lymphocytes; 3= moderate changes of epithelium and moderate lymphocytic infiltration and numerous syncytia with intranuclear inclusion bodies, and 4= severe desquamation of epithelium with numerous intranuclear inclusion with severe lymphocytic infiltration.

Pathogenicity test and intratracheal pathogenicity index:

Behera/2001 field isolate and two LTV vaccines (CEO and TCO) were used for these tests. The field isolate was passaged once on CAMs. Infected CAMs were harvested 120 hours PI, frozen at -20°C and thawed once then homogenized and suspended in amnio-allntoic fluid (AAF) of infected eggs (vol. /vol.), and finally centrifuged at 1000 rpm for 10 minutes. The supernatant fluid was decanted and kept in small aliqoutes at -20°C until they were used. Virus titration test in chicken embryos proved titers of $10^{3.2}$, 10^{5}, and $10^{5.4}$ EID_{50}/ 0.1 ml for Behera/2001 isolate, CEO and TCO vaccines, respectively. Each challenged bird was given, via micropipette instillation directly into the trachea, a dose of $10^{3.2}$, 10^{3} and $10^{3.4}$/ 0.1 ml of Behera/2001 isolate, CEO and TCO, respectively (Anon., 1990). Details of the pathogenicity test are summarized in the following table.

Laboratory vaccination trials

Sufficient one-day-old, cross- bred chickens were used for determination of the serological response and degree of protection following vaccination of cross-bred chickens with ILTV vaccines (TCO and/ or CEO). At 35 days of age, they were divided into groups. Vaccination and/ or challenge were done according to experimental design summarized in the following table.

Statistical analysis:

Data were analyzed by the Students - test after Steel and Torrie (1960) to determine the significance of differences between individual treatment and corresponding controls.

Experimental design for pathogenicity studies of local isolate Behera/2001 compared with chicken embryo and tissue culture origin vaccines in 50- days- old cross bred chickens.

Group No.*	Birds No.	Experimental infection			Criteria adopted for evaluation of pathogenicity			
		Group	virus	Dose	Serology	ITPI***	Virus reisolation	Histopathology
I	30	Infected	Infected CAMs suspension of isolate No.1	0.1ml ($10^{3.2}$) EID_{50}	1- Immune status pre-infection. 2- Sero-conversion at 15 days PI	1- Clinical symptoms 2- Mortality for 8 days observation period PI.	Pool of tracheal swabs in 50% Glycerin PBS at 4 days PI.	Lesion scores **** 0 = normal epithelium. 1= Epithelium normal, mild lymphocytic infiltration in lamina propria. 2=Mucosa thickened, mild edema, mild congestion and mild inflammatory cell infiltration by lymphocytes, heterophils and monocytes. 3= Moderate sloughing of epithelium, reduced mucous glands, marked hyperemia, moderate lymphocytic infiltration and numerous syncytia with intranuclear inclusion bodies. 4= Severe changes. Severe edema, severe desquamation of epithelium, haemorrhages, severe lymphocytic infiltration and INIB.
II	30	Infected	Suspension of CEO vaccine	0.1ml (10^{3}) EID_{50}				
III	30	Infected	Suspension of TCO vaccine	0.1ml ($10^{3.4}$) EID_{50}				
IV	30	Non-treated	**AAF of normal eggs	0.1ml				

* No. = number. **AAF=Amnioallantoic fluid., **** Lesions scoring after Guy et al. (1990).
*** ITPI = intratracheal pathogenicity index, determined by scoring each bird daily for 8 days PI after Hanson (1980) as follows:
0 = Normal , 1 = respiratory signs (congestion, gasping, conjunctivitis and expectoration of blood) , 2 = dead; Indices were determined by dividing the sum of scores by total number of observations.

RESULTS

Characteristics of ILT outbreaks:

During the period of February 2001- May 2003, 9 ILT outbreaks were occurred in three governorates and involved different types of chickens. The majority of these outbreaks occurred 6- 10 weeks of age (average 8 weeks) in cross- bred and 19- 20 weeks of age (average 19.5weeks) in broiler breeder chickens. ILT outbreaks were characterized by mild to severe clinical signs and gross lesions. Mortality rates ranging 4.4- 12.5% (average 8.6 %) in cross- bred and 1.5- 2 % (average 1.75%) in broiler breeder chickens (Table 1 and 2).

Experimental design of determination of the serological response and degree of protection following LTV challenge of vaccinated susceptible cross- bred chickens with tissue culture origin and/or chicken embryo origin LTV vaccines.

Treatment	Birds No.	Vaccination regime[3]			ILTV** Challenge Age/day	Assessment of protection		
		Freq.	Age/ days	Vaccine type		Observation for 15 days Pch.***	Serology	Histopathology
Chall-Vacc.	20	1X*	35	TCO	50	1- Mortality %		Lesion scores
Chall. non vacc	20	--	--	--	50	2- Gross lesions		0 = normal epithelium.
Vacc. non chall.	20	IX	35	TCO	--	for 15 days	Seroconversion	1= Epithelium normal, mild lymphocytic
Non treated	20	--	--	--	--	observation	15 days PI.	infiltration in lamina propria.
						Pch		2= Mucosa thicken, edema, hyperemia
Chall-Vacc.	20	2X	35	TCO				moderate inflammarory cell infiltra-
			50	TCO	65			tion cell infiltration as lymphocytes
Chall. non vacc.	20	--	--	--	65			and heterophils.
Vacc. non chall.	20	2X	35	TCO	--			3= Moderate sloughing of epithelium, re-
			50	TCO	--			duced mucous glands, marked hyperc-
Non treated	20	--	--	--	--			mia, moderate lymphocytic infiltration
								and numerous syncytia with intranu-
Chall-Vacci	20	2X	35	TCO				clear inclusion bodies.
			50	CEO	65			4= Severe changes. Severe edema, severe
Chall. non vacc.	20	--	--	--	65			desquamation of epithelium, haemor-
Vacc. non chall.	20	2X	35	TCO	--			rhages, severe lymphocytic infiltration
			50	CEO	--			and numerous INIB.
Non- treated	20	--	--	--	--			

No= Number , X*= Number of vaccinations , TCO=Tissue culture origin vaccine, CEO=Chicken embryo origin vaccine, ** LTV challenge with local field isolate no. 1, Pch***= Post-challenge. Birds vaccinated via eye drop route with a vaccinal dose/ bird. Serological tests used were AGPTand ELISA.

The affected birds showed nasal discharge, lacrimation, coughing and swollen eyes. The main gross lesions were catarrhal, caseous and / or haemorrhagic inflammation of the tracheal mucosa, conjunctivitis and a blood tinged mucous exudate or yellowish caseous materials were present in some cases. In some farms, the affected birds showed severe dyspnea characterized by obvious extension of the head and neck during inspiration, coughing and expectoration of blood which stained the walls and equipments of the houses. The main gross lesions were haemorrhagic tracheitis, with clotted blood present in the tracheal lumen. (Fig. 1 and 2).

In all investigated outbreaks the flocks had no history of LTV vaccinations except farms no.8 and 9 that were vaccinated against ILT at 10 week- old with tissue culture origin (TCO) vaccine. The affected birds in farms no. 8 and 9 showed lacrimation, nasal discharge, coughing and swollen eyes and the main gross lesion was the presence of yellowish caseous materials in the tracheal lumen. It is obvious that hygienic measures and biosecurity were suboptimal in the majority of the investigated farms.

LTV isolation and identification:

LTV was isolated from 4 outbreaks (farm no 1, 2, 8, and 9) following CAM inoculation of SPF chicken embryos. All isolates gave pock - like lesions by the first passage (Fig. 3) except isolate no. 8 by the second passage. Pock like lesions were characterized by having an opaque raised edge and grey central area of necrosis. CAMs were edematous and haemorrhagic in some cases. Embryo deaths occurred 2- 7 days PI and characterized by stunted growth. Rapid HA test with AAF from inoculated eggs were negative. AGPT on the harvested CAM using reference anti-ILT antisera revealed positive results confirming the success of isolation.

Pathogenicity study on selected field isolate (No 1) of LTV

As shown in table (3) the MICE of local isolate no. 1 was 0.40 while LTV vaccines (CEO and TCO) the MICE were 0.11 and 0.02, respectively. Tables 4, 5 and 6 present the cumulative clinical signs and mortalities as well as the intratracheal pathogenicity index during the 8 days observation period of the experimentally infected cross-bred chickens.

As shown in table (7) group I chickens, which inoculated intratracheally by isolate no. 1 showed coughing, sneezing, gasping and lacrimation. Mortalities began 5 days PI. Morbidity and mortality rates reached 80% and 13.3% respectively. Gross lesions in chickens which died were characterized by blood - tinged mucous to mucopurulent exudate in the tracheal lumen and severely congested tracheal mucosa. While group II chickens, which inoculated intratracheally with CEO vaccine, showed mild respiratory manifestations, including coughing and sneezing. Morbidity rate

reached 13.3% on the 5th day PI while mortality rate was 0.03%. However, group III chickens, which inoculated intratracheally by TCO vaccine, showed neither morbidity nor mortality during all observing period. In addition, group IV chickens, which inoculated intratracheally with AAF of normal eggs showed neither clinical signs nor mortalities during all observing period.

LTV was re-isolated from pooled tracheal swabs at 4 days PI. Moreover, LTV precipitating antibodies in survived chickens infected with isolate no.1, CEO vaccine and TCO vaccine were revealed seroconversion at 15 days PI by AGPT (4/5, 2/5, 2/5) and by ELISA (5/5, 5/5, 5/5), respectively .

The histopathological findings of chickens inoculated intratracheally by isolate no.1 were characterized by desquamation of tracheal epithelium, haemorrhages and inflammatory cell infiltrations. In could be detected in some epithelial cells 5- 7 days PI. (Fig. 4. 5 and 6), However, tracheal lesions of chickens inoculated intratracheally with TCO vaccine were characterized by mild congestion of blood vessels, slight desquamated epithelial cells with hypertrophy of mucosal glands and mild inflammatory cells infiltration (Fig. 7), and revealed Mean lesion score (MLS) of 1.5. Furthermore, chickens inoculated intratracheally with CEO vaccine revealed MLS of 2.3. (Table 7).

Results of laboratory vaccination trials:

As shown in table (8) the mortality percentages in vaccinated-challenged chickens, regardless the frequency of vaccination and type of vaccine were zero percentage during 15 days post challenge. While the mortality percentages in challenged non-vaccinated chickens were 13.5% and 15%, for chickens challenged at 50 and 65 days, respectively. The gross lesions in dead chickens were confined to the trachea and characterized by blood tinged mucous to muco- purulent exudates in the tracheal lumen. The results of antibody response as measured by AGPT after 15 days PV was 3/5 and ELISA titers ranged from 1064-1335, for chickens vaccinated once with TCO vaccine. While in challenged non vaccinated chickens the result of AGPT was 2/5 and ELISA titers ranged from 2556- 2965 when challenged at 50 days of age.

Antibody response as measured by AGPT were 3/5 and 2/5 and ELISA titers ranged from 1243-1489 and 1222- 1749 after first and second vaccinations, respectively, for chickens vaccinated twice with TCO vaccine. While results of AGPT of challenged non-vaccinated birds revealed 2/5 and ELISA titers were 1915- 2229 when challenged at 65 days of age. On the other hand antibody response as measured by AGPT were 2/5 and 3/5 and ELISA titers ranged from 1232-1400 and 1780- 2149 after first and second vaccinations, respectively, for chickens vaccinated twice by TCO vaccine followed by CEO vaccines.

While results of AGPT of challenged non-vaccinated birds revealed 2/5 and ELISA titers were 1915- 2229 when challenged at 65 days of age. Non- treated chickens (non vaccinated and non-challenged) revealed no seroconversion as measured by AGPT and ELISA neither at 50 nor 65 days of age.

The MLS of vaccinated non-challenged chickens with TCO vaccine at 35 days of age was 1.3 at 7 days PV. While it was 2.5 at 7 days Pch in chickens vaccinated with TCO vaccine at 35 days of age and challenged 15 days PV. Moreover, the MLSs of chickens non-challenged and vaccinated at 35 - days of age with TCO and revaccinated with TCO or CEO vaccines were 0.8 and 1.0, respectively, at 7 days after 2nd vaccination. In addition, the MLSs in chickens vaccinated at 35 days of age with TCO vaccine, revaccinated with TCO vaccine or CEO vaccine and challenged after 15 days after 2nd vaccination was 0.1 at 7 days Pch. Furthermore, the MLSs of non- vaccinated challenged chickens at 50 and 65 days of age were 4.0.

Table (1) : History of examined chicken farms:

Code No.	Date	Governorate	Bird type	Bird variety	House capacity	Age/ day at disease onset	* Mortality		Vaccination schedule
							total	%	
1	Feb. 01	Behera			6,000	65	520	8.7	ND, IBD, MD
2	Mars. 01	Behera			5,000	54	300	6	ND, IBD, MD
3	Apr. 02	Behera			20,000	64	2500	12.5	ND, IB, IBD
4	May. 02	Behera			5,000	60	220	4.4	ND, IBD, MD
5	May. 02	Behera	** Cross-bred		5,000	39	450	9	ND, IBD, MD
6	Feb. 03	Behera			5,000	51	600	12	ND, IBD, MD
7	Feb. 03	Behera			5,000	42	370	7.4	ND, IBD,
8	May. 03	Giza	Broiler	Cobb	4,000	1430	80	2	MD,ND, IB, IBD,
9	May. 03	Kalyobia	breeders	Cobb	5,000	135	75	1.5	CAV,ILT MD,ND, IB,IBD, CAV,ILT

** Cross-bred produced from native X Sasso imported bred.
* Recorded number from onset of ILT mortality.
ND: Newcastle disease
IB: Infectious bronchitis
ILT: Infectious laryngotracheitis.
MD: Mareks disease.
IBD: Infectious bursal disease

Table (2) : Characteristics of LTV outbreaks in examined chicken farms

Flock type	Age incidence		Mortality %	
	Range/ day	Mean ± Sd	Range	Mean ± Sd
Cross- bred N =7	39-65	53.6 ± 6.44	4.4 - 12.5	8.6 ± 4.14
Broiler breeders N=2	135-140	137.5 ± 2.17	1.5 - 2	1.75 ± 0.27

N = Number of farm examined

Table (3) : Results of mortality index for chicken embryos (MICE):

Inoculum	Dose	Status of embryo	No. of embryos on days PI							Cumulative no. of embryos	MICE
			1	2	3	4	5	6	7		
Behera/2001 Isolate	$10^{3.2}$ EID_{50}	Dead	0	0	2	6	8	12	12	40	0.40
		Live	20	20	18	14	12	8	8	100	
CEO vaccine	$10^{3.2}$ EID_{50}	Dead	0	20	0	4	4	4	4	14	0.11
		Live	20	20	20	18	17	17	17	129	
TCO vaccine	$10^{3.2}$ EID_{50}	Dead	0	0	0	0	1	1	1	0	0.02
		Live	20	20	20	20	19	19	19	137	

MICE is calculated according to Isuchi and Hasegawa (1982) by dividing the cumulative no. of dead embryos for 7 days PI by the cumulative no of live embryos for 7 days PI.

Table (4) :Cumulative clinical signs and mortality as well as calculation of intratracheal pathogenicity index during 8 days observation of cross- bred chickens experimentally infected intratracheally with 100µl ($10^{3.4}$ EID_{50}/0.1ml) with field isolate Behera/2001 at 50 days of age.

Criteria	Days of observation								Total	Score	Total score	ITPI*
	1	2	3	4	5	6	7	8				
Cumulative Signs	0	0	0	13	17	24	19	11	84	1	84	0.44
Cumulative Mortality	0	0	0	0	2	2	3	4	11	2	22	
Normal	30	30	30	17	11	4	8	15	145	0	0	
Total No. of birds	30	30	30	30	30	30	30	30	240	-	106	

* Intratracheal pathogenicity index determined after Hanson (1980) by dividing the sum of scores by total number of obsevations

Table (5) :Cumulative clinical signs and mortality as well as calculation of intratracheal pathogenicity index during 8 days observation of cross- bred chickens experimentally infected intratracheally with 100µl ($10^{3.4}$ EID_{50}/0.1 mml) with chicken embryo origin vaccine at 50 days of age.

Criteria	Days of observation								Total	Score	Total score	ITPI*
	1	2	3	4	5	6	7	8				
Cumulative Signs	0	0	0	3	4	4	2	1	14	1	14	0.08
Cumulative Mortality	0	0	0	0	0	1	1	1	3	2	6	
Normal	30	30	30	27	26	25	27	28	223	-	0	
Total No. of birds	30	30	30	30	30	30	30	30	240	-	20	

* Intratracheal pathogenicity index determined after Hanson (1980) by dividing the sum of scores by total number of obsevations.

Table (6) :Cumulative clinical signs and mortality as well as calculation of intratracheal pathogenicity index during 8 days observation of cross- bred chickens experimentally infected intratracheally with 100µl ($10^{3.4}$ EID_{50}/0.1 mml) with tissue culture origin vaccine at 50 days of age.

Criteria	Days of observation								Total	Score	Total score	ITPI*
	1	2	3	4	5	6	7	8				
Cumulative Signs	0	0	0	0	0	0	0	0	0	1	0	0
Cumulative Mortality	0	0	0	0	0	0	0	0	0	2	0	
Normal	30	30	30	30	30	30	30	30	0	0	0	
Total No. of birds	30	30	30	30	30	30	30	30	240	-	-	

* Intratracheal pathogenicity index determined after Hanson (1980) by dividing the sum of scores by total number of obsevations.

Table (7): Summary results of intratracheal pathogenicity indices and seroconversion of experimental infection of cross-bred chickens with LTV local field isolates (Behera/2001), chicken embryo origin vaccine and tissue culture origin vaccines.

Group no.	Birds		Virus infection	Antibody detection at time of vaccination				Observation for 8 dpi.					Seroconversion at 15 dpi				MICE	Mean lesion score
				AGPT		ELISA		Morbidity		mortality		ITPI*	AGPT		ELISA			
	Age/ day	No.		Pos.no. / Exam. no.	%	Pos.no. / Exam. no.	%	Total	%	Total	%		Total no.	%	Pos.no. / Exam. no.	%		
I	50	30	local isolate Behera/2001	0/30	0.0	0.5	0.0	24	80	4	13.3	0.44	4.5	80	5/5	100	0.40	4
II	50	30	CEO vaccine	0/30	0.0	0.5	0.0	4	13.3	1	0.03	0.08	2.5	40	5/5	100	0.11	2.4
III	50	30	TCO vaccine	030	0.0	0.5	0.0	0	0	0	0	0	2.5	40	5/5	100	0.02	1.5
IV	50	30	Non infected	0/30	0.0	0.5	0.0	0	0	0	0	0	0.5	0.0	0/5	0.0	--	0.2

AGP=agar gel precipitation test. ELISA=enzyme liked immunosorbant assay. * ITPI=intratracheal pathogenicity index.

Exp.No=experiment number. CEO = chicken embryo origin vaccine. TCO=tissue culture origin vaccine.

MICE: Mortality index for chicken embryos according to Isuchi and Hasegawa (1982) Mean lesion scores according to Guy et al., (1990)

Table (8): Results of serological response and degree of protection following LTV challenge of vaccinated cross- bred chickens with tissue culture origin and/ or chicken embryo origin vaccines.

Treatment.	Birds no.	Vaccination regime			ILIV# Challenge Age/day	Seroconversion				
		Freq.	Age/ day	Vaccine type		Mortality %*	ELISA			MLS**
							AGPT	Mean ± sd	Range	
Challenged vaccinated	20	1X*	35	TCO	50	0.0	2/5	1629b ± 1200	1215 - 1883	2.5 b
Challenged non vaccinated	20	--	--	--	50	13.5	2/5	2820.3 ^{a} ± 1750	2556 - 2962	4.0 ^{a}
Vaccinated non challenged	20	1X	35	TCO	--	0.0	3/5	1199.7 ^{bc} ± 908	1064 - 1335	1.3 ^{c}
Non treated	20	--	--	--	--	0.0	0/5	277^{cd} ± 508	108 - 523	0.2 ^{d}
Challenged vaccinated	20	2X	35	TCO	--	--	--	--	--	--
			50	TCO	65	0.0	2/5	2843.7 ^{a} ± 1780	1625 - 5189	1.0 ^{cd}
Challenged non vaccinated	20	--	--	--	65	15	2/5	2060.3^{ab} ± 1400	1915 - 2229	4.0 ^{a}
Vaccinated non challenged	20	2X	35	TCO	--	0.0	3/5	1290.7^{bc} ± 980	1243 - 1489	--
			50	TCO	--	0.0	2/5	1557.7b ± 1230	1222 - 1749	0.8 ^{cd}
Non treated	20	--	--	--	--	0.0	0/5	128.7^{d} ± 203	96 - 190	0.2 ^{d}
Challenged vaccinated	20	2X	35	TCO	--	--	--			--
			50	CEO	65	0.0	3/5	2304^{b} ± 2050	1827 - 2779	1.0 ^{cd}
Challenged non vaccinated	20	--	--	--	65	15	2/5	2060.3^{ab} ± 1720	1915 - 2229	4.0 ^{a}
Vaccinated non challenged			35	TCO	--	0.0	2/5	1290.7^{bc} ± 1100	1232 - 1400	--
	20	2X	50	CEO	--	0.0	3/5	1984.7^{ab} ± 1780	1780 - 2149	1.0 ^{cd}
Non treated	20	--	---	--	--	0.0	0/5	110^{d} ± 180	90 -140	0.2 ^{d}

X= Number of vaccination TCO=Tissue culture origin vaccine. Mortality for 15 days observation

ELISA= Enzyme linked immunosorbent assay. Pch= Post-challenge CEO=Chicken embryo origin vaccine.

**MLS= Mean lesion score calculated by dividing the sum of total scores by total examined number (Guy et al. 1990).

Means with different lowercase superscripts indicate different statistical differences ($p< 0.05$).

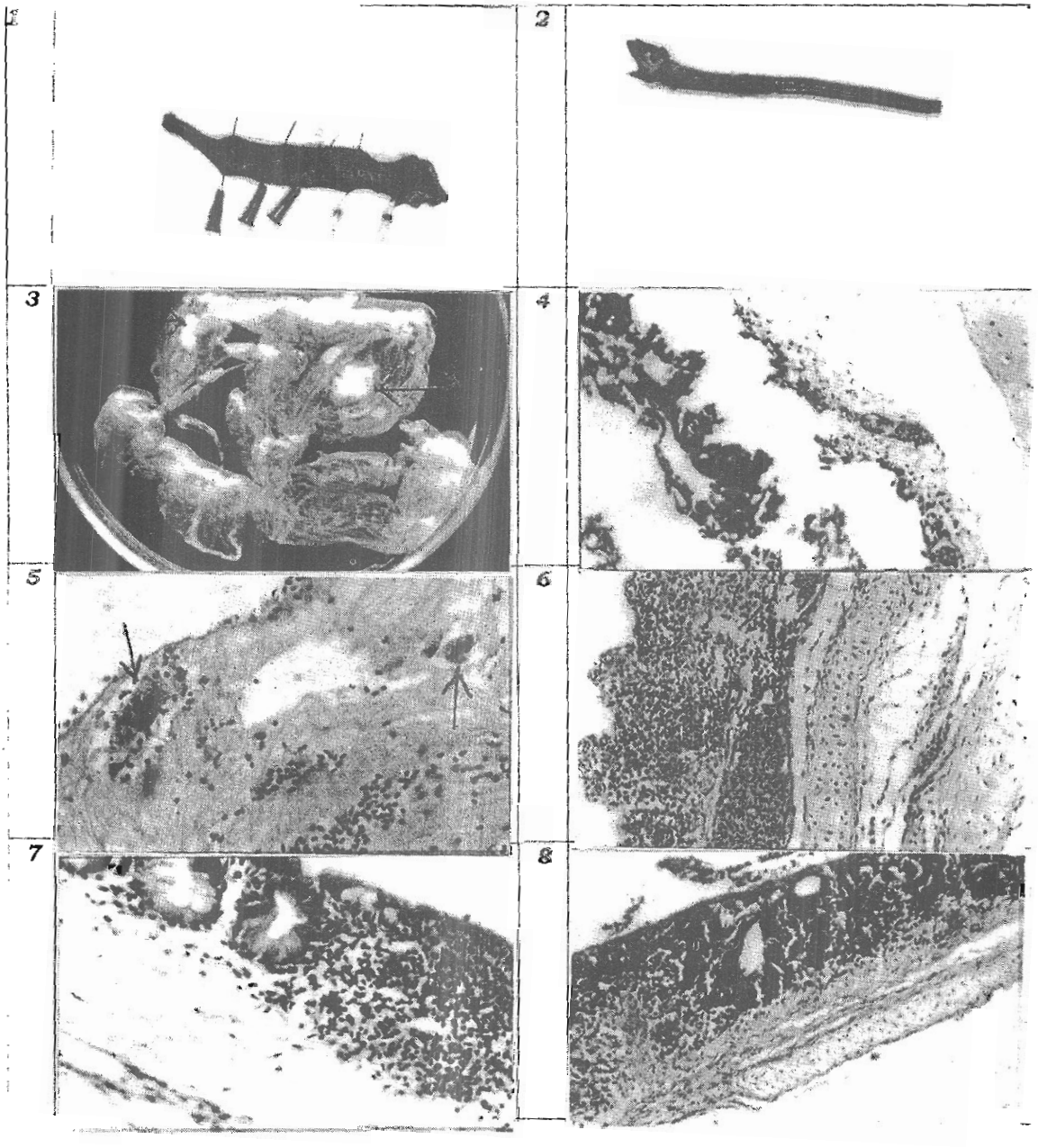

Fig. (1, 2): Haemorrhagic tracheitis with clotted blood in 65-day-old cross- bred chicken naturally infected with ILT.

Fig. (3): Chorioallantoic membrane showing pock lesions at 4 days post inoculation of 10 - day - old specific pathogen free chicken eggs with Behera/2001 isolate of ILT.

Fig (4): Trachea of 55- day- old cross- bred chicken experimentally infected with isolate Behera/2001 showing detachment of epithelium, haemorrhages and lymphocytic infiltrations at 5 days PI (H& E, X 10).

Fig. (5): Trachea of 55- day- old cross- bred chicken experimentally infected with isolate Behera/2001 , showing detachment of the epithelium, haemorrhages, INI bodies and lymphocytic infiltration at 5 days PI (H& E, X 40).

Fig. (6): Trachea of 58 - day- old cross- bred chicken experimentally infected with isolate Behera/2001 , showing detachment of epithelium, congested submucosal blood vessels and lymphocytic infiltration at 8- days PI. (H& E, X 40).

Fig. (7): Trachea of 57- day- old cross- bred chicken experimentally infected with tissue culture origin vaccine, showing congested submucosal blood vessels and lymphocytic infiltration at 7- days PI. (H& E, X 20).

Fig. (8): Trachea of 42 - day- old cross- bred chicken, vaccinated by TCO vaccine, showing thickened mucosa with lymphocytic infiltration, hyperemia and slight detachment of epithelium at 7 days after, first vaccination (H&E, X 20).

DISCUSSION

In the present study, 9 LTV outbreaks in cross-bred and broiler breeder chicken farms located in 3 governorates were investigated over the period Feb. 2001 - May 2003. The majority of these outbreaks occurred, at 6 - 10 weeks of age in cross-bred chickens and 19 - 20 weeks of age in broiler breeder chickens, within range of age susceptibility as previous reports (El-Zanaty and Ahmed, 1995; Sultan and El- Gohary, 1999; Aaya Amer, 2001). Like others who reported the characteristic findings of ILT infections, the affected birds showed nasal discharge, coughing, swollen eyes, and expectoration of blood which stained the walls and equipments of the houses especially in farm no.1 (Behera/2001) and no. 2. The main gross lesions in cross- bred chickens (farm no. 1 and 2) were haemorrhagic tracheitis with clotted blood present in the tracheal lumen (Fig. 1 and 2), while in broiler breeders (farm no 8 and 9) were the presence of a yellowish caseous materials in the tracheal lumen (Maxbrugh, 1979; Sultan and El-Gohary, 1999). Moreover, variable mortality rates ranging 4.4- 12.5% in cross- bred chickens and 1.5 - 2 % in broiler breeder chickens, were observed. Such variability may be due to variable pathogenicity of LTV strain involved, yet vaccinated flocks against LTV showed the lowest mortality rates. It seems that the benign forms of ILT associated with low mortality were the most feature of modern ILT (Guy and Bagust, 2003).

It is worthy to mention that LTV could be isolated from four outbreaks after 1- 2 passages on CAM of specific pathogen free (SPF) chicken egg embryos. Pock like lesions were characterized by having an opaque raised edges and grey central area of necrosis. CAMs were edematous and haemorrhagic in some cases (Fig. 3) (Tantawi et al., 1983; Madbouly et al., 1996). Moreover, AGPT for ILV antigen detection in CAMs of embryos that died and / or killed during 7 days PI were positive with specific LTV antiserum. In the suspected outbreaks of the disease, from which no virus was isolated, it is possible that the samples had deteriorated in transit or had been collected too late in the course of the disease. Alternatively, the ILTV may not have been involved and no attempts were made to determine other possible causes of the outbreaks of the disease except rapid hemagglutination test (Hughes et al., 1991).

The pathogenicity of field isolate no.1(Behera/2001) was 0.40 while LTV vaccines (CEO and TCO), the MICEs were 0.11 and 0.02, respectively, (Table 3). As reported by Isuchi and Hasegawa (1982) MICE less than 0.16 is low or not pathogenic for chickens, and those with MICE more than 0.27 is highly pathogenic. Hence, isolate no. 1 was considered pathogenic strain. Likewise, the pathogenicity of field isolate no.1 in 50-day- old cross-bred chickens revealed that the isolate possessed greater virulence than both LTV vaccine types based on intratracheal pathog-

enicity index (ITPI) and tracheal lesion scores (Hanson 1980) and proved to be different than CEO and TCO vaccines. Indeed, ITPI for modified life LT vaccine viruses ranged from 0.0-0.14, while those for field isolates were 0.20 - 0.82 (Guy et al. 1990).

Histopathological lesions of chickens inoculated intratracheally with isolate no. 1 were characterized by desquamation of tracheal epithelium as well as haemorrhages and inflammatory cell infiltration. Intranuclear inclusions could be detected in some epithelial cells (Figs. 4, 5 and 6). Similar findings were formerly observed (Goodwin et al., 1991; Linares et al., 1994). The histopathological lesions found with CEO inoculated chicken were characterized by slight desquamation of epithelium and moderate inflammatory cells infiltration and no inclusion body was detected. In contrast, tracheal lesions in case of TCO vaccine (Fig. 8) were less severe than chickens inoculated with CEO vaccine. Russel and Turner (1983) and Timmurkaan et al. (2003) demonstrated that chickens inoculated with low virulent ILTV showed mild inflammatory changes in the respiratory tract which were not specific for differential diagnosis.

In the present study, the MLSs were 4, 2.3 and 1.5 for Behera/2001 field isolate, CEO and TCO vaccines, respectively (Table 7). In addition, the three groups of chickens showed seroconversion to the experimental infection as detected by AGPT and ELISA test 15 days PI. (Table 7). Such results raise the possibility that the field isolate may originated from the ML vaccine viruses through reversion to parental-type virulence. Since birds-to bird and flock to flock spread of LTV modified live viruses and there subsequent reversion to virulent forms is possible, especially those of chicken embryo origin (Guy et al., 1991). It appears that under prevailing conditions of suboptimal management, hygiene and biosecurity, which permit continuous contamination of the environment and spread of pathogen, the increase incidence of LTV outbreaks in these flocks may be attributed to virulence reversion (Kotiw, 1995). Reversion to wild-type virulence has been demonstrated for several ML vaccine viruses, including: infectious bronchitis virus (Hopkins et al., 1984), polio virus (Oxford and Oberg, 1985). Furthermore, using restriction enzyme analysis of the ILT outbreak-related isolates revealed that 75% of the isolates from different geographic areas, where CEO vaccine is common, are CEO-like viruses (Garcia and Riblet, 2001). Moreover, the presence of the viral subpopulations within the vaccine preparations have been documented and might these population reveret to virulence during circulation in birds (Garcia and Riblet, 2001). Recent studies suggested that both vaccine-like and vaccine- unlike ILT viruses were involved in the field ILT outbreak (Chang et al., 2000 and Sellers et al., 2004).

The isolated ILTV from vaccinated broiler breeder chickens may be explained by failure in the vaccination program, the birds not received sufficient dose of the vaccine or speculation that live attenuated vaccine strains of ILTV occasionally reversed to parental virulence. (Andresen et al., 1990) explained the appearance of ILT outbreaks among vaccinated birds by the virus perhaps gaining virulence as a result of its passage from bird to bird. Evidence for involvement of modified live ILT vaccine viruses in recent ILT outbreaks was provided by (Guy et al., 1989) after restriction endonuclease analysis on modified live virus vaccine and field isolate. Also, Guy et al., (1990) demonstrated an increase in virulence of embryo propagated vaccine virus after 15 serial bird to bird passages, moreover, Keeler et al. (1992) explained reversion to virulence to the possibility that slow mutation of ILTV vaccines could be contributing to outbreaks of ILT virus.

The second experiment was designated to determine if there is a difference in protection against ILT in chickens vaccinated once and those vaccinated twice with available commercial LT vaccines, according to the Code of Federal Regulations (CFR) of the U.S.A. and European Pharmacopoeia, the efficacy of commercial ILTV live vaccines can be assessed by the survivability of vaccinated chickens at 14 days or 21 days after challenge with a virulent strain of ILTV (Han and Kim, 2003). In the present study, the assessment of protection was based on mortality rate, seroconversion (AGPT and ELISA) and tracheal lesion scores. The mortality percentages in vaccinated challenged chickens, regardless the frequency of vaccination and type of vaccine were zero percentages during 15- days post challenge. Han et al., (2002) demonstrated that protection rate determined by clinical signs and mortality was 100% during 14 days post challenge in vaccinated chickens with available commercial vaccines by ocular route. In addition, the antibody response after 15 days PV was measured by AGPT revealed seroconversion in some vaccinated groups (Table 8), it is obvious that AGPT is considered less sensitive test for detection of ILT antibodies (Fuchs et al., 1985)

In these contexts, after one vaccination, the mean ELISA titers were higher for vaccinated groups than unvaccinated ($p < 0.05$). The ELISA titers after second vaccination were higher than non vaccinated control and those receiving only one vaccination ($p < 0.05$). In addition, ELISA titers levels were higher after challenge. The results are in accord with those obtained by Fulton et al. (2000) who demonstrated that ELISA titers were much higher in chickens vaccinated twice by ML LTV vaccines than those vaccinated once.

Moreover, tracheal lesion scores proved that chickens receiving two vaccinations before challenge had lower lesion scores ($p < 0.05$) than unvaccinated and those receiving only one dose of LTV vaccine before challenge.

These studies indicated a high pathogenicity for examined isolate (Behera/2001) and differ from that of vaccinal viruses however it remains uncertain the origin of such field isolate as wild type or as virulence reversed modified vaccinal virus. Restriction endonuclease-polymerase chain reaction (RE-PCR) may help to solve this problem (Halina et al., 1996). Moreover, all commercially available vaccines should provide better protections if two separate doses are given to all birds within a flock. ELISA is available for ILT serology and will help in detection of ILT exposure by vaccine or field challenge but will not be a usable tool to identify the antibody response of vaccine from challenge. However, assessment of protection by these parameters alone can not be a caliber for measuring the latent infection of challenge virus in chickens receiving vaccines to ILTV (Han and Kim, 2003). Therefore, the assessment of latent infection in vaccinated chickens would be a more reliable method to determine vaccine efficacy.

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