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ISOLATION AND CHARACTERIZATION OF INFECTIOUS LARYNGOTRACHEITIS VIRUS IN LAYER CHICKENS

M. S. Islam[*1], M. S. R. Khan[1], M.A. Islam[1], J. Hassan[1], S. affroze[2] and M. A. Islam[3]

[1]Department of Microbiology and Hygiene, [2]Department of Parasitology and [3]Department of Medicine, Faculty of Veterinary Science, Bangladesh Agricultural University, Mymensingh-2202, Bangladesh

ABSTRACT

The present research work was conducted for the isolation and characterization of infectious laryngotracheitis (ILT) virus in layer chickens from commercial farms of Gazipur District. A total of 25 field samples were collected from suspected layer chickens of five commercial farms and were cultivated into 10-12 days old embryonated chicken eggs through chorioallantoic membrane (CAM) route for isolation of field virus. The field viruses were characterized by physico-chemical properties against p^H, heat, ether and chloroform, serological test such as virus neutralization test (VNT) and passive haemagglutination (PHA) test and pathogenicity testing. In the embryonated chicken eggs, virus produced discrete pock lesions as early as 2 days of post inoculation and embryo death was recorded within 4-6 days of inoculation. The viruses could be inactivated by p^H 4 within 2 hours. Inactivation of viruses was observed at 60^0C for 6 minutes, 55^0C for 15 minutes and 38^0C for 2 days. Ether-chloroform treatment also inactivated the viruses. Virus neutralization test revealed that all the virus isolates were neutralized by antiserum to ILT vaccine. Passive haemagglutination test showed that the tanned sheep RBC sensitized with the virus isolates were agglutinated in presence of the antiserum to ILT vaccine. The pathogenicity test recorded 100% mortality in experimental chickens. Data of this study suggest that the field isolates might be infectious laryngotracheitis virus.

Key words: Isolation, characterization, infectious laryngotracheitis virus, layer chickens

INTRODUCTION

Infectious Laryngotrachitis (ILT) is an important respiratory disease of chicken caused by gallid herpes virus-I of the family *Herpesviridae,* subfamily *Alphaherpesvirinae*, genus *Iltovirus*. It is an enveloped, non segmented and linear double-stranded DNA virus (Murphy *et al.,* 2000). Although Infectious Laryngotrachitis virus (ILTV) strains are antigenically homogenous, ILTV strains naturally vary in virulence, from highly virulent strains, causing high morbidity and mortality, to strains with low virulence, which that produce mild-to-unapparent infection (Bauer *et al.,* 1999; Guy & Bagust, 2003). Clinical signs associated with the severe form of the disease include gasping, depression, nasal discharge, conjunctivitis, and expectoration of bloody mucus. Upon gross examination of the trachea, characteristic severe hemorrhages and mucus plugs are observed (Cover, 1996; Sellers *et al.,* 2004). The clinical signs associated with less severe forms of the disease include conjunctivitis, swelling of the infraorbital sinuses, closed eyes, persistent nasal discharge and mild tracheitis (Timurkaan *et al.,* 2003). This disease is common in areas of intensive poultry production and its outbreaks result in high economic losses due to increased mortality, decreased growth rates, and lower egg production (Guy & Bagust, 2003; Humberd *et al.,* 2002). Many laboratory diagnostic techniques have been used for the detection of ILTV. The detection of antibodies by serum neutralization or enzyme-linked immunosorbent assay (ELISA) can be used (Bauer *et al.,* 1999; Sander & Thayer, 1997). The virus can be isolated from field material in specific-pathogen-free (SPF) chicken embryos inoculated via the chorioallantoic membrane (CAM), or by isolation in primary chicken embryo kidney (CEK) cells, chicken embryo liver (CELi) cells, or chicken kidney cells (Hughes *et al.,* 1991; Schnitzlein *et al.,* 1994). Several investigators studied the prevalence and incidence, pathology, immunity, propagation and diagnosis of ILTV (Oldoni *et al.,* 2009; Pavlova *et al.,* 2009; Crespo *et al.,* 2007; Bagust *et al.* 2000 and Hughes *et al.,* 1991). To the best of our knowledge no study has been conducted yet on the detection of ILTV in chickens in Bangladesh. Considering the prevalence of the disease and losses to the commercial poultry raisers it is felt that there is a national need to identify the disease quickly and to protect the chicken population against the disease. In Bangladesh, for controlling ILT, commercial poultry raisers are using ILT vaccine imported from abroad without any concern of the local isolates/serotypes of ILTV. The poultry raisers of Bangladesh are using commercial ILT vaccines to immune their chickens against ILTV without confirming the causal agent of the disease. The present study was conducted for the isolation and characterization of the ILTV in layer chickens manifested the clinical signs characteristics of ILT.

*Corresponding author: e-mail: shafiqdvm@yahoo.com

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MATERIALS AND METHODS

Study areas and period

The study was conducted in layer chickens belonged to Paragon, Phenix, Kazi, Adunik and S-R Poultry Farm of Gazipur District. The laboratory works were performed in the Department of Microbiology and Hygiene, Faculty of Veterinary Science, Bangladesh Agricultural University (BAU), Mymensingh during the period from January, 2009 to October, 2009.

Collection of samples

A total of 25 field samples comprising of trachea, larynx, lungs and eyelid from chickens manifested the clinical signs of difficult respiration with loud coughing and gasping, discharges from the eyes and nostril, swelling of the infra-orbital sinus and closed eye were aseptically collected. The samples were placed in 50% buffered glycerine and then brought to the laboratory of the Department of Microbiology and Hygiene, BAU, Mymensingh and stored at -20^{0}C until use.

Processing of samples

The preserved field samples were thawed and macerated separately using sterilized pestle and mortar to prepare a 10-20% (w/v) suspension in sterile PBS. The suspension was centrifuged at 3000 rpm for 30 minute for clarification. Supernatant fluid was treated with a broad-spectrum antibiotic (Gentamycin 50µg/ml) at room temperature for one hour. The sterility of the inoculum was checked on blood agar.

Isolation of virus in the embryonated chicken eggs

The inoculum was inoculated in the 10-12 days-old embryonated chicken eggs through CAM route. The positive samples were allowed for five subsequent passages in chicken embryos using same route. The virus suspensions were prepared from infected CAM using standard procedure.

Rearing of chicks

A total of 15 day-old chicks were purchased from Sutiakhali, Mymensingh. Each group consisted of five chicks. All these chicks were kept in a well-ventilated poultry shed of the Department of Microbiology and Hygiene, BAU, Mymensingh. The chicks were provided with feed and water adlibitum.

Raising antibody against ILTV

Twenty eight days old five chickens were vaccinated with a commercial ILT vaccine (Gallivac LT®) through intraocular route using one drop of vaccine in each eye. Sera were collected at 0, 7, 14, 21, 28 and 35 days post vaccination. The sera were kept at -20^{0}C until use for VNT and PHA test.

Physico-chemical characterization of field virus isolates

Physico-chemical characterization was performed by inactivation of virus through exposure of virus suspension at different p^H, temperature and lipid solvents such as ether and chloroform. The inactivation of virus was performed by following methods:

p^H treatment

This was carried out to study the sensitivity or resistance of the virus isolates at p^H 4 and p^H 5 for varying period of time. After p^H treatment each virus isolate was immediately inoculated into five 10 to 12 days old chicken embryo through CAM route. Five chicken embryos were also inoculated with untreated virus to serve as control. The inoculated eggs were incubated at 37^{0}C in an incubator and were observed daily for 4-6 days. The inoculated chicken embryos were observed daily for recording death or any lesion.

Thermal treatment

This was carried out to study the sensitivity or resistance of the virus isolates at different temperature following the procedures described by Jordan (1966).

Each isolate was treated at 60^0C for 4, 6 and 10 minutes; at 55^0C for 10, 15 and 20 minutes; at 38^0C for 1, 2 and 3 days respectively. After heat treatment, the inoculum was inoculated into five 10 to 12 days old embryonated chicken eggs through CAM route. Five embryos were also inoculated with untreated virus to serve as control. Each embryo received 0.1 ml of inoculum. The inoculated eggs were incubated at 37^0C in the incubator and were observed daily for 4-6 days after inoculation. The deaths of embryos and / or lesions in embryonic tissues were recorded daily.

Ether treatment

This was carried out to study the sensitivity or resistance of the virus isolates to diethyl ether following the method described by Andrewes and Horstmann (1947).

Pure Diethyl ether was added to each tube of virus suspension at a concentration of twenty percent by volume. An adhesive tape was bound round the cap to reduce any risk of loss of ether. After being shaken, the tube was held at $+4^0$C for 24 hours. A tube containing only a volume of virus suspension was used as control. After 24 hours of treatment, the specimen was poured into an uncovered petridishes and allowed the ether to evaporate at room temperature for 10 minutes. The treated virus and the control specimen were then inoculated into five 10 to 12 days old embryonated chicken eggs separately by CAM route. The inoculated eggs were incubated at 37^0C in the incubator for 4-6 days and were observed twice daily. The deaths of embryos and / or lesions in embryonic tissues were recorded daily.

Chloroform treatment

This was carried out to study the sensitivity or resistance of the virus isolates to Chloroform treatment following the method described by Feldman and Wang (1961).

Twenty percent suspension of the CAM infected with virus isolates were used in this study. The test was carried out by mixing 0.25 ml of reagent grade chloroform with 5 ml of virus suspension. The mixture was shaken for 10 minutes at $+4^0$C. Immediately after this treatment the mixture was centrifuged at 500 rpm for 5 minutes. The chloroform then settled at the bottom of the tube leaving supernatant clear fluid containing the virus interphased by an opaque layer. The clear supernatant fluid was removed by sterile pipette and then inoculated into five, 10-12 days old embryonated chicken eggs by CAM route at the rate of 0.1 ml per egg. The untreated or control specimen of each isolate was similarly inoculated into another group of five embryos. The inoculated embryos were incubated at 37^0C in incubator and observed daily for 4-6 days for embryopathy. The deaths of the embryos were recorded daily and the lesions in embryonic tissues were observed. Similar procedure was adapted for each isolate of virus.

Serological characterization of field virus isolates

The field virus isolates were characterized serologically by PHA test and VNT using inactivated sera collected from 21 days of post vaccination.

Passive haemagglutination test

The test was used to detect the ILTV isolates as per method describe by Tripathy *et al.* (1970). The test antiserum against ILTV vaccine was diluted with PBS to prepare two fold dilutions ranging from 1:2 to 1:256. A volume of 50 microlitre of each dilution of serum was transferred to each well of the 4 rows (2^{nd}, 4^{th}, 6^{th} and 8^{th}) of 96 well Microtitre plate. Fifty microlitre of 0.5% sensitized tanned RBC of one type was added to each well of one row containing 50 microlitre of serum dilution. Three types sensitized tanned RBC were similarly added to each well of other 3 rows respectively. These were then mixed thoroughly with the help of microtitre plate shaker. The antigen, tanned RBC, normal rabbit serum and sensitized sheep RBC control system were also kept during the test procedure. The microtitre plate was then incubated at room temperature for an hour and observed carefully for agglutination. The deposit of a diffuse thin layer of clumping RBC on the bottom of the well indicated HA positive and a compact buttoning with clear zone around indicated HA negative. The end point of serum was calculated as the highest dilution of serum causing agglutination of sensitized tanned RBC. The reciprocal of the highest dilution of serum causing agglutination of sensitized tanned RBC was considered as titre of the serum. Triplicate tests were conducted to make final observation for the test.

Virus neutralization test

The neutralization test was carried out in 10-12 days old embryonated chicken eggs by CAM route of inoculation. Ten fold dilutions of the suspension of virus isolates ranging from 10^{-1} to 10^{-8} were prepared in Nutrient broth. Equal volume of diluted virus and the serum to be tested were mixed in separate series of test tube. The virus dilutions and serum virus mixture were incubated at room temperature for 1 hr. A 0.1 ml of each dilution of virus and 0.2 ml of serum virus mixture was inoculated to each of the embryonated chicken eggs separately using CAM route of inoculation. Five embryonated chicken eggs were inoculated by each dilution of virus and serum-virus mixture separately. The inoculated eggs were incubated at 37^0C for 4-6 days and candled twice daily. The deaths of the embryos were recorded daily. The Chicken Embryo Lethal Dose Fifty ($CELD_{50}$) for virus suspension and serum-virus mixture were calculated separately by using the method of Reed and Muench (1938). The neutralizing dose of serum in relation to $CELD_{50}$ was calculated as the antilogarithm of the difference of $\log_{10}$ $CELD_{50}$ of virus and the $\log_{10}$ $CELD_{50}$ of serum-virus mixture. The same technique was applied for all the virus isolates using antiserum to ILT vaccine.

Pathogenicity test to Infectious laryngotracheitis virus

This was performed in two months of local chickens using the calculated Chicken embryo lethal dose fifty ($CELD_{50}$) of ILTV. Briefly $CELD_{50}$ was calculated according to Reed and Muench (1938) method. 1.0 ml of 1000 $CELD_{50}$ of ILTV was used for pathogenicity study. Sixty five days old five chickens were inoculated with 1 ml of virulent suspected field ILTV isolate through tracheal route. The survivability and the mortality of chickens were recorded. Reisolation of ILTV was conducted from death chickens.

RESULTS AND DISCUSSION

Table 1. Isolation of Infectious laryngotracheitis virus from suspected field samples using day old chicken embryos

SL. No.	Name of the Farms	Name of samples	Number of samples cultivated	Number of samples		% positive to ILTV
				Positive to ILTV	Negative to ILTV	
1.	Paragon poultry farm	Trachea and larynx	3	1	2	
		Lung	2	0	2	
		Eyelid	2	0	2	14.28
2.	Phenix poultry farm	Trachea and larynx	2	1	1	
		Lung	2	0	2	
		Eyelid	2	0	2	16.67
3.	Kazi farm	Trachea and larynx	2	1	1	
		Lung	1	0	1	
		Eyelid	1	0	1	25.00
4.	Adunik poultry farm	Trachea and larynx	2	1	1	
		Lung	1	0	1	
		Eyelid	2	0	2	20.00
5.	S-R poultry farm	Trachea and larynx	1	0	1	
		Lung	1	0	1	
		Eyelid	1	0	1	_
Total			25	4	21	75.95

ILTV= Infectious laryngotracheitis virus, S-R=S-R poultry farm

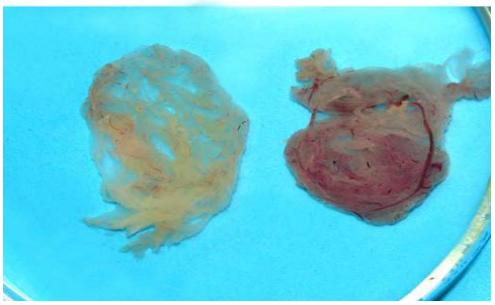

Fig. 1. Left one showing infected CAM and right one normal CAM

Fig. 2. Left one showing slunted growth and right one normal growth of embryo

Out of 25 samples a total of four samples were found to be positive to ILTV infection. Positive or negative infection was determined through embryo mortality or embryo survivability after inoculation of ILTV suspected field samples through CAM route. The typical gross pathological lesions were visually observed as discrete pock formation with opaque edges and central depressed area of necrosis in the CAM and slunting growth of the ILTV affected embryo after 2 days post inoculation with field strains at passage number four (Fig. 1 and 2). Burnet (1934) stated that embryo mortality due to ILTV occurs within 2-12 days post inoculation. The gross lesions recorded in this study were similar to Calnek *et al.* (1997). The overall incidence of ILTV infection in five commercial farms was recorded as 75.95%. The highest incidence rate was recorded as 25.00% in layer chickens of Kazi farm and the lowest incidence rate was recorded as 14.28 % in layer chickens of Paragon poultry farm. The pathogenicity test showed 100% mortality of chickens. The same observation was also recorded by Andrease *et al.* (1989).

Table 2. Results of p^H treatment of the virus isolates

Virus isolates	Treatment at p^H 4 for			Treatment at p^H 5 for			Untreated control
	1 hr.	2 hrs.	2.5 hrs.	3 hrs.	3.5 hrs.	4 hrs.	
ILT-1	5/5	0/5	0/5	5/5	5/5	5/5	5/5
ILT-2	5/5	0/5	0/5	5/5	5/5	5/5	5/5
ILT-3	5/5	0/5	0/5	5/5	5/5	5/5	5/5
ILT-4	5/5	0/5	0/5	5/5	5/5	5/5	5/5

Prefix=No. of embryo dead, Suffix=No. of total embryos, ILT=Infectoius laryngotracheitis, hr=Hour, hrs=Hours.

The infectivity of the virus isolates remained unaffected after exposure at p^H 4 for 1 hour and at p^H 5 for 3 hours since 100% embryo mortality was recorded. The virus isolates were inactivated by treatment at p^H 4 for 2 hours as was evident by the survivability of chicken embryos. The findings of this study are in agreement with the findings of Meulemans & Halen (1978b) who reported that 90% infectivity of ILTV lost at p^H 9 for 2 hours or at p^H 4 for 2 hours. From this study it was evident that the virus isolates were P^H labile.

Table 3. Results of thermal treatment of the virus isolates

Virus isolates	Survivability/mortality of chicken embryos at 60^0C for			Survivability/mortality of chicken embryos at 55^0C for			Survivability/mortality of chicken embryos at 38^0C for			Control
	4 min.	6 min.	10 min.	10 min.	15 min.	20 min.	1 day	2 days	3 days	
ILT-1	5/5	0/5	0/5	5/5	0/5	0/5	5/5	0/5	0/5	5/5
ILT-2	5/5	0/5	0/5	5/5	0/5	0/5	5/5	0/5	0/5	5/5
ILT-3	5/5	0/5	0/5	5/5	0/5	0/5	5/5	0/5	0/5	5/5
ILT-4	5/5	0/5	0/5	5/5	0/5	0/5	5/5	0/5	0/5	5/5

Prefix= No. of embryo dead, Suffix= No. of total embryo, min. =minutes, ILT=Infectoius laryngotracheitis

The infectivities of the virus isolates remained unaffected by heating at 60^0 C for 4 minutes, at 55^0C for 10 minutes and at 38^0C for one day as was evident by the presence of lesions in embryonic tissues and the mortality of chicken embryos. The infectivities of the virus isolates were destroyed by heating at 60^0C for 6 minutes, at 55^0C for 15 minutes and at 38^0C for 2 days since no embryo mortality or lesions in embryonic tissues were recorded.

All the virus isolates in this study demonstrated similar thermal characteristics indicating that they were belonged to same species. The results of this study support the findings of Jordan (1966) who reported that ILTV is inactivated by heating at 60^0C for 6 minutes, 55^0C for 15 minutes and at 38^0C for 2 days. On the basis of thermal treatment results it can be concluded that the field virus isolates were heat stable.

Table 4. Results of sensitivity of the virus isolates to lipid solvents

Test components	Lipid Solvents		Untreated control
	Diethyl ether	Chloroform	
ILT-1	0/5	0/5	5/5
ILT-2	0/5	0/5	5/5
ILT-3	0/5	0/5	5/5
ILT-4	0/5	0/5	5/5

Prefix=No. of embryo dead, Suffix=No. of total embryos, ILT=Infectoius laryngotracheitis

The virus isolates became inactivated after treatment with diethyl ether or chloroform which was indicated by the survivability of chicken embryo inoculated with treated virus isolates. The loss of infectivity of the virus isolates after treatment with lipid solvents indicated that the presence of lipids in their structure particularly the presence of envelope in the virus. The findings of this study are in conformity with those of Fitzgerald & Hanson (1963); Meulemans & Halen (1978a), who reported that ILT virus lost its activity when treated with lipid solvents.

Passive haemagglutination test

The virus isolates were sensitive to PHA test as was evident from the haemagglutination of virus sensitized sheep RBC with the antiserum to virus isolates upto a serum dilution of 1:32. This clearly demonstrated that the antiserum and the virus isolates were specific and homologus which resulted the haemagglutination of virus sensitized sheep RBC in presence of antiserum to the ILT vaccine. This indicated that all the isolated viruses from suspected field samples were ILTV. The results of this study co-related with those of Tripathy *et al.* (1970) who demonstrated passive haemagglutination test of ILT virus.These investigator used tanned horse red blood cells coated with partially purified ILT viral antigen. In this study tanned sheep RBC was sensitized with antigen (virus isolates) and used for the test.

Virus neutralization test

The results of VNT using immunized sera collected from 21 days of post vaccination are presented in the Table 4. All virus isolates were neutralized by the antiserum to ILT vaccine. The NI (Neutralization Index) in relation to $\log_{10}$ $CELD_{50}$ with ILT vaccine antiserum range from 3.00 to 3.54 and the mean NI was 3.18. These results are in conformity with those of Andreasen *et al.* (1988) who stated that the ILT virus NI of 1.75 $\log_{10}$ $CELD_{50}$ or more indicated the presence of specific ILT virus antiserum and the NI of 0.0 to 1.5 $\log_{10}$ $CELD_{50}$ indicated normal titre of chicken serum.

Table 5. Results of neutralization test of virus isolates using sera obtained from Infectious laryngotracheitis vaccinated chickens

Test components	Virus dilution							Log_{10} $CELD_{50}$	
	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	Titre	NI
Isolate ILT-1	5/5	5/5	5/5	5/5	2/5	0/5	0/5	4.83	
Isolate ILT-1 + Antiserum of Gallivac LT	5/5	1/5	0/5	0/5	0/5	0/5	0/5	1.63	3.20
Isolate ILT-2	5/5	5/5	5/5	5/5	1/5	0/5	0/5	4.50	
Isolate ILT-2 + Antiserum of Gallivac LT	5/5	0/5	0/5	0/5	0/5	0/5	0/5	1.50	3.00
Isolate ILT-3	5/5	5/5	5/5	5/5	3/5	0/5	0/5	5.17	
Isolate ILT-3 + Antiserum of Gallivac LT	5/5	1/5	0/5	0/5	0/5	0/5	0/5	1.63	3.54
Isolate ILT-4	5/5	5/5	5/5	4/5	1/5	0/5	0/5	4.50	
Isolate ILT-4 + Antiserum of Gallivac LT	5/5	0/5	0/5	0/5	0/5	0/5	0/5	1.50	3.00

Prefix=No. of embryo dead, Suffix=No. of total embryos, NI=Neutralization index, $CELD_{50}$=Chicken Embryo Lethal Dose fifty, ILT=Infectious laryngotracheitis

Table 6. Results of neutralization test of virus isolates using prevaccination serum of chickens

Test components	Virus dilution							Log_{10} $CELD_{50}$	
	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	Titre	NI
Isolate ILT-1	5/5	5/5	5/5	4/5	0/5	0/5	0/5	4.38	
Isolate ILT-1 + Preinoculation serum	5/5	5/5	5/5	3/5	1/5	0/5	0/5	4.32	0.08
Isolate ILT-2	5/5	5/5	5/5	5/5	3/5	0/5	0/5	5.17	
Isolate ILT-2 + Preinoculation serum	5/5	5/5	5/5	4/5	3/5	0/5	0/5	5.00	0.17
Isolate ILT-3	5/5	5/5	5/5	4/5	1/5	0/5	0/5	4.50	
Isolate ILT-3 + Preinoculation serum	5/5	5/5	5/5	4/5	0/5	0/5	0/5	4.38	0.12
Isolate ILT-4	5/5	5/5	5/5	5/5	2/5	0/5	0/5	4.83	
Isolate ILT-4 + Preinoculation serum	5/5	5/5	5/5	5/5	1/5	0/5	0/5	4.63	0.20

Prefix=No. of embryo dead, Suffix=No. of total embryos, NI=Neutralization index, $CELD_{50}$=Chicken Embryo Lethal Dose fifty, ILT=Infectious laryngotracheitis

The prevaccination serum NI range from 0.08 to 0.20 log_{10} $CELD_{50}$ (Table 5) which was similar to that reported by Andreasen *et al.* (1988) who stated that NI of 0.0 to 1.5 log_{10} $CELD_{50}$ indicative of normal titre of the chicken serum. Considering the preinoculation serum NI obtained in this study as normal titre, the data in the Table 4 to 5 clearly demonstrated that the vaccine virus antiserum neutralized all the virus isolates which explain the fact that the virus isolates were homologus to ILT vaccine virus. Data of this study indicated that the field virus isolates were infectious laryngotracheitis virus.

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