



## Isolation and Identification of Avian Infectious Laryngotracheitis Virus in Sudan

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Article history: Received: 18.08.2014

Accepted: 10.09.2014

### Abstract

Three outbreaks of mild respiratory disease of chickens were occurred in Sudan during winter, one of which at Halfaya in Khartoum State on December 2010 and two other at Atbara on January 2011. The main respiratory signs of these outbreaks were rales, coughing with nasal and oral discharge, depression, conjunctivitis and swollen head and sinuses in some cases. The affected flocks were varied in age from 5 to 18 months, and they were from different layer breeds (Bovans, Hyline 98, Lohman and Hisex). Also, birds showing respiratory signs were obtained from different farms in Khartoum State at Shambat and Dekheinat. Using embryonated chicken eggs and chicken embryo liver (CELi) cell culture, 5 Infectious Laryngotracheitis (ILT) virus field isolates (At1, At2, Hf, Sh1 and D2) were isolated from organs such as tracheas, larynx, lungs and tracheal swabs. The isolates were identified as an ILT virus by means of agar gel immunodiffusion (AGID) test and histopathological examination of infected chorioallantoic membrane segments. This is the first report of the isolation of ILT virus in Sudan.

**Keywords:** Infectious Laryngotracheitis, Poultry, Sudan, Isolation.

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### Introduction

Infectious Laryngotracheitis (ILT) is an important worldwide occurring respiratory disease of chickens (Bagust *et al.*, 2000). The causative agent is a virus, belongs to the *Herpesviridae* family, *Alpha herpesvirinae* subfamily, *Iltovirus* genus, and the *Gallid herpesvirus 1* species (Mc Geoch *et al.*, 2000). Chickens are the primary natural host, and the most characteristic signs are observed in adult birds (Guy and Garcia, 2008). The disease is characterized by respiratory signs, decreased egg production and/or decreased weight gain. Like other *Alpha herpesvirinae* viruses, ILT

virus produces latency in nervous or respiratory tissue of infected individuals (Williams *et al.*, 1992), followed by episodes of intermittent virus shedding, which may be associated with stresses such as re-housing and start of laying (Hughes *et al.*, 1989).

The severity of clinical symptoms depends on the virulence of a particular strain, and mortality rates vary between 0 to more than 70%. Symptoms of milder form of ILT are nasal discharge, conjunctivitis, and reduced egg production, where as severe forms are additionally characterized by gasping, coughing, expectoration of bloody mucous

and marked dyspnoea which may lead to suffocation (Fuchs *et al.*, 2007).

Based on serosurveillance study on the prevalence of ILT virus antibodies, which was done by Shaza *et al.*, (2011) samples for virus isolation were collected from the three outbreaks and clinically affected birds from different farms in Khartoum state and Atbara. The flocks were suffering from respiratory symptoms, drop in egg production, and sometimes deaths.

This study was designed in the following objectives: to isolate Infectious Laryngotracheitis virus circulating in the field and to identify the isolates using conventional techniques.

## Materials and Methods

### Samples:

Tracheal swabs from chickens with respiratory signs were obtained from different farms in Khartoum state (Shambat and Dekheinat). In addition to three field outbreaks of mild respiratory disease of chickens that were occurred during winter; one of which in Khartoum State at Halfaya, on December 2010 and the two other at Atbara, on January 2011. The swabs were pooled in Tryptose Phosphate Broth (TPB) agitated vigorously and centrifuged at low speed to remove debris. Tracheas, larynges, and lungs samples from suspected farms were pooled, cut into small pieces with sterile scissors and forceps, and then prepared as 10% homogenate in sterile phosphate buffer saline (PBS) containing  $10^3$  I.U penicillin,  $10^3$   $\mu$ g streptomycin sulphate, and 50 units fungizone. The homogenate suspension was centrifuged in refrigerated centrifuge at 3000 rpm for 10 minutes. All supernatants of swabs and tissues were collected and designated as At1, At2, Hf, Sh1, D2, for all samples from Atbara1, Atbara2, Halfaya, Shambat1, Dekheinat1 respectively, then kept at  $-20^\circ\text{C}$  till used for virus isolation in chicken embryos.

### Embryonated chicken eggs:

All samples were inoculated on the dropped chorioallantoic membrane (CAM) of five (11-12 days) embryonating chicken eggs with 0.2 ml of each sample suspension, and were subsequently sealed with paraffin wax. Five eggs were left as uninoculated controls. All eggs were incubated at  $37^\circ\text{C}$  for 7 days, and candled daily. Embryos died within 24 hours of inoculation considered as non-specific and were discarded. The CAMs of dead embryos or those surviving for 7 days were examined for typical pock lesion. Allantoic fluid was collected for virus identification. Embryos were examined for any morphological changes. Up to three or more serial passages (blind passage) were done. CAMs with clear pock lesions were collected aseptically, parts of them were placed in 10% formal saline and submitted for histopathological examination, while other parts were minced and homogenized as 10% suspension in antibiotic diluent (PBS) then centrifuged at 3000 rpm for 10 min. The supernatant was kept at  $-20^\circ\text{C}$  till used for ILT virus identification by means of AGID and for virus isolation in the CELi cell culture.

### Chicken embryo liver (CELi) cells:

Supernatant of harvested CAMs were used to inoculate CELi cell culture suspension in a rate of 0.1 ml / 25  $\text{cm}^2$  tissue culture flask. Two flasks were left as uninoculated controls. All flasks were incubated at  $37^\circ\text{C}$  for 7 days, and examined daily under the inverted microscope to observe the viral Cytopathic Effect (CPE) appearance.

### Haemagglutination (HA) test:

The haemagglutinating activity of suspected virus isolates in allantoic fluid and the CAM homogenate was tested against chicken red blood cells (RBCs) according to the method described by Terregino and Capua (2009).

### Agar Gel Immunodiffusion test:

The harvested CAMs and infected cell culture were demonstrated for ILT virus by AGID

test. Reference ILT virus antigens and antisera for agar gel immunodiffusion (AGID) test were obtained from Istituto Zooprofilattico delle Venez. OIE \ FAO laboratory for Avian Influenza and New Castle disease viruses (Italy). Procedure was carried out as described in OIE (2008) manual in brief: a volume of 30  $\mu$ l of the positive control specific ILT hyper immune serum was pipette into the central wells. While surrounding wells were filled with 30  $\mu$ l of the suspect virus suspension, except two wells one was containing the positive control ILT antigen and the other containing the negative control. The plates were incubated in a humid chamber at room temperature, and examined after 24 -48 hours for clear lines of precipitation which were recorded as a positive result.

#### **Histopathology:**

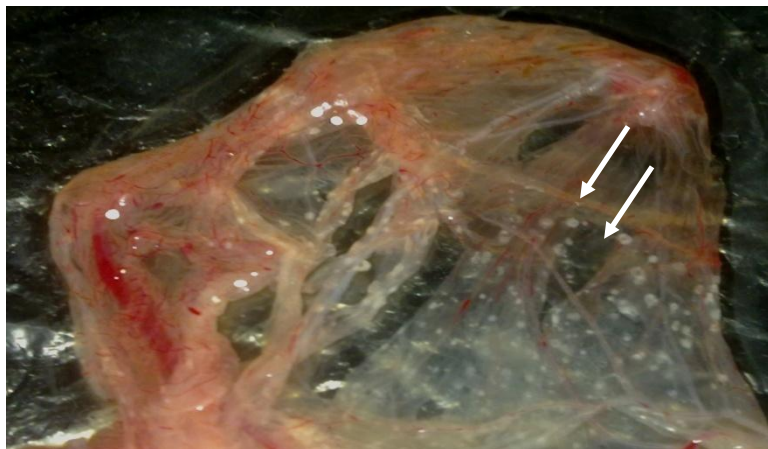
Segments of the chorioallantoic membranes which were inoculated with suspected ILT virus field isolates were placed 10% formal saline and submitted for routine processing. Sections were stained with haematoxylin and eosin (H & E).

#### **Results**

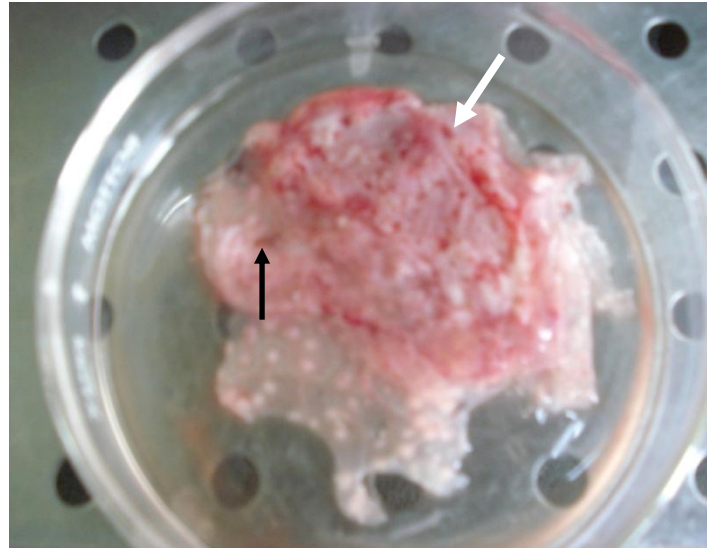
The postmortem findings of the affected chickens showed congestion in addition to mucoid exudates in tracheas and larynges. The infraorbital sinuses of some birds were oedematous and congested, and some of them showed caseous materials.

#### **Virus isolation:**

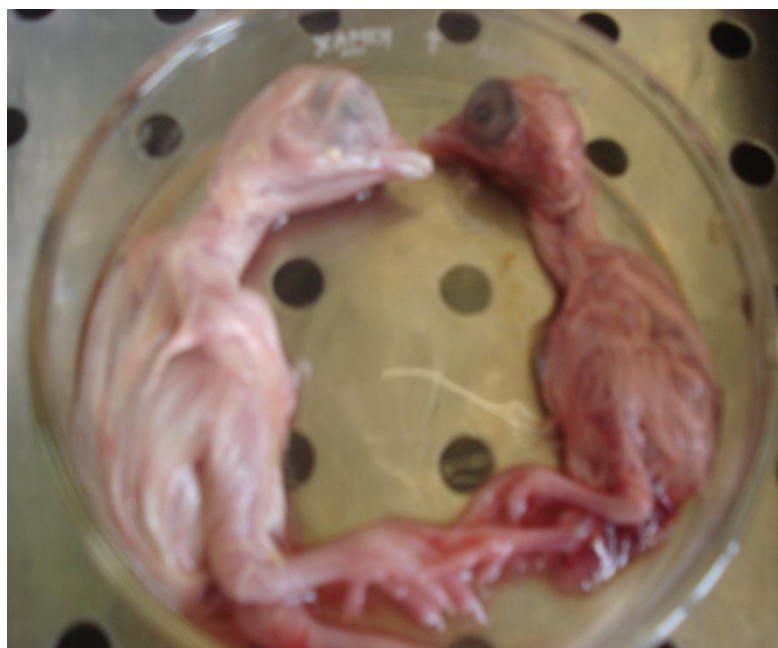
During the incubation period, the inoculated embryos when candled showed weak movement and when opened the embryos were stunted and congested. CAMs with pock lesions were harvested from these embryos. All isolates caused formation of pock lesion which varied from small white foci, generally these has an opaque edge with central depressed area of necrosis (Figure, 1), to large yellowish white pock lesions which were distributed throughout the CAM (Figure, 2), some of these lesions appeared with haemorrhagic centers, and the CAMs showed thickening at the inoculation sites, beside generalized oedema. The uninoculated control embryo showed normal growth (Figure, 3).



**Figure 1: Chorioallantoic membrane inoculated with P2 of suspected ILT virusfield isolate, small white pock lesions with depressed central area are present (arrows).**



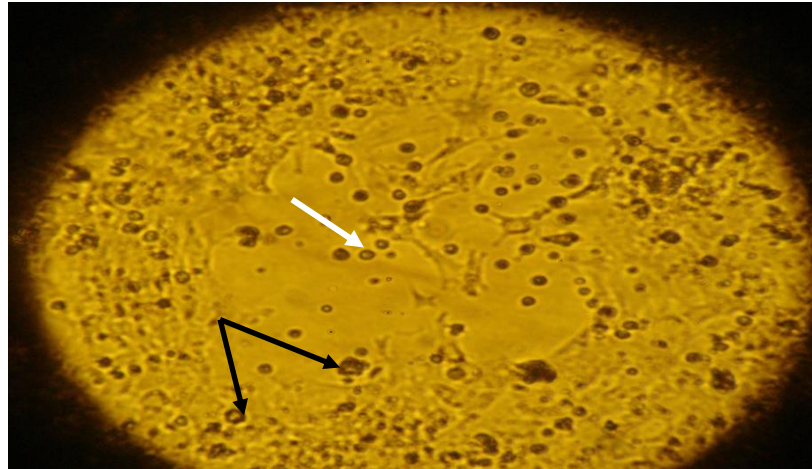
**Figure 2: Chorioallantoic membrane inoculated with P2 of suspected ILT virus field isolate, large pock lesions (black arrow), haemorrhagic centers and thickening (white arrow).**



**Figure 3: Chicken embryos at 17 days of age, normal embryo (left). ILT virus infected embryo (stunted and congested) (right).**

Regarding inoculated CELi cells, within 24 - 48 hours post inoculation, the CPE was observed, and well developed within 7 days. The CPE consist of round refractile single cells or small syncytia in focal arrangement.

Altered cells were subsequently detached from the flask surface forming plaques and lyse (Figure, 4), compared to normal uninoculated cells (Figure, 5).



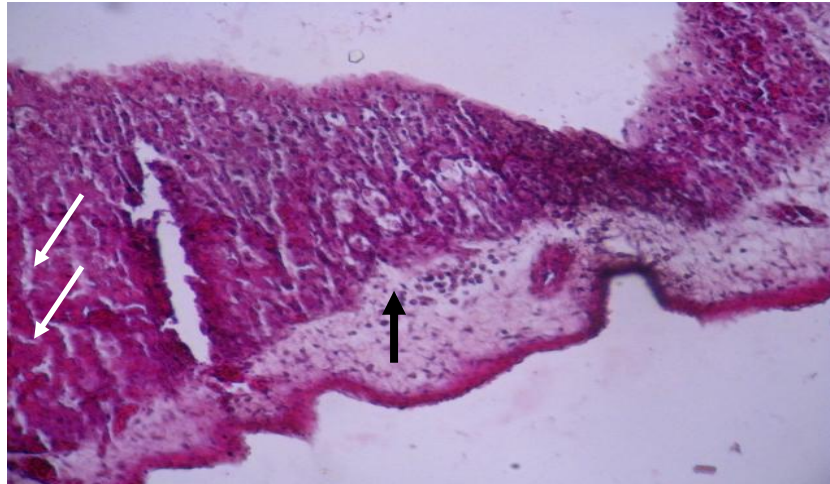
**Figure 4: The Cytopathic Effect (CPE) of ILT virus field isolates in chick embryo liver cells (on day 7 post inoculation), note round refractile single cells (white arrow), small syncytia (black arrows).**



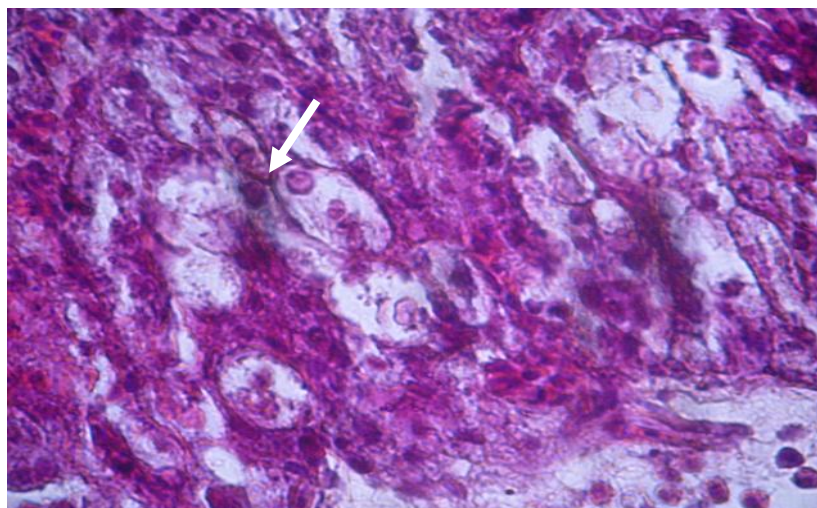
**Figure 5: Uninoculated chick embryo liver (CELi) cell culture showing normal cell growth (x 25).**

The allantoic fluid and homogenated CAMs collected from embryos inoculated with At1, At2, Hf, Sh1, and D2 did not agglutinate chicken RBCs (no haemagglutinin activity). Using AGID test, a clear precipitin line of identity (after 24 hours) was produced when homogenated CAMs and infected CELi cell culture (inoculated with At1, At2, Hf, Sh1 and D2) were used as antigens against an antiserum of known ILT virus.

The histopathological examination of H & E stained sections of inoculated CAMs revealed thickening of the three layers of the CAMs, swelling of the epithelial cells, infiltration of inflammatory cells in ectoderm and mesoderm layers (black arrows), beside odema (white arrows) (Figure, 6). As shown in (Figure, 7) in ectoderm layer, there was an epithelial cell with intranuclear inclusion body.



**Figure 6: Section of CAM infected with ILT virus field isolate showing thickening of layers, infiltration of inflammatory cells in ectoderm and mesoderm layers (black arrow), and odema (white arrows) (H & E. x 40).**



**Figure 7: Infected CAM high magnification of the ectoderm epithelial cells of chick embryo inoculated with ILT virus field isolate, showing intranuclear inclusion body (white arrow), (H & E. x 100).**

### Discussion

The clinical signs recorded in some farms investigated during this study included tracheal rales, mild tracheitis, decreased egg production, low mortality, swelling of infraorbital sinuses and conjunctivitis. The postmortem findings showed congestion in addition to mucoid exudates in the tracheas and larynges, odema and congestion of infraorbital sinuses. Other respiratory

pathogens of poultry can cause similar clinical signs and lesions, so in this study the clinical diagnosis was confirmed in the laboratory by virus isolation in both embryonated chicken eggs and CELi cell culture. The isolates were identified by different serological tests such as Haemagglutination test to differentiate the virus from other respiratory viruses (New Castle disease and Avian Influenza) that agglutinated chicken red blood cells, as well

as other confirmatory laboratory diagnostic procedures including identification of ILT virus using AGID test, intranuclear inclusion bodies.

In this study, ILT virus was isolated and identified in the field samples by inoculation in embryonated chicken eggs. Five samples, At1, At2, Hf, Sh1 and D2 produced different pock lesions when inoculated on the CAMs of 11-12 days old embryonated chicken eggs. The inoculated embryos showed stunted growth and occasionally deaths. The allantoic fluid collected from these embryos did not agglutinate chicken red blood cells. Similar results were recorded by Tantawi *et al.*, (1983) and Noda *et al.*, (1990).

ILT virus was also isolated when the homogenated CAMs collected from the embryos inoculated with the field samples was re-inoculated in CELi cells, which is considered as the cell culture of choice for ILT virus isolation. In this study, the CPEs were observed within 24- 48 hours and clearly visible after 7 days of incubation. CPE showed round refractile single cells, small syncytia in focal arrangement and plaques, a similar finding was demonstrated by Williams *et al.*, (1994).

The field isolates (At1, At2, Hf, Sh1 and D2) were confirmed as ILT virus serologically by AGID test in CAM and cell culture suspensions. The test as recorded by OIE manual (2008) can be used to detect viral antigens on tracheal exudates, infected CAMs and infected cell culture materials.

Macroscopic changes such as thickening, odema and pock lesions were detected in the CAMs. The virus was also confirmed by histopathologic examination of the CAMs for intranuclear inclusion bodies and cyncytia as stated by Tripathy (1998). The histopathological lesions of the CAMs in this study revealed thickening of the three layers of the infected CAM with emphasis to ectoderm layer, swelling of the epithelial

cells, infiltration of inflammatory cells in ectoderm and mesoderm, intranuclear inclusion bodies, and odema. Rocio *et al.*, (2007) recorded that, isolation of ILT virus in the CAM was presumed by histopathology in 20% of their samples.

### Conclusion

It is to be concluded that this is the first report of the isolation of ILT virus in the Sudan.

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## عزل والتعرف علي فيروس الحنجره والرغامى المعدى للدواجن في السودان

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### المستخلص:

ثلاث تفشيات لمرض تنفسي معتدل في الدواجن تم حدوثها في السودان خلال فصل الشتاء ، إحداهما في الحلفايا بولاية الخرطوم في ديسمبر 2010 وإثنين آخرين في عطبرة في يناير 2011 . كانت العلامات التنفسية الرئيسة لهذه التفشيات هي اصوات تنفسية، سعال مع إفرازات أنفية وفمية، إكتئاب، إتهاب الملتحمة وتورم الرأس والجيوب الانفية في بعض الحالات. تفاوتت اعمار القطعان المصابة من 5 الي 18 شهر وكانت لسلاسل بياضة مختلفة (بوفان، هيلين 98، لوهمان وهايسكس). إضافة للقطعان المصابة تم الحصول علي طيور بأعراض تنفسية من مزارع مختلفة في ولاية الخرطوم من شمبات والدخينات. بإستخدام أجنة بيض الدجاج النامي والزرع الخلوي لكبد أجنة الدجاج تم عزل خمس معزولات حقلية من فيروس الحنجره والرغامى المعدى (At1,At2,Hf,Sh1,D2) من اعضاء كالقصبات الهوائية ، الحناجر والرئات وايضا من مسحات القصبات الهوائية. تم تعريف المعزولات بأنها فيروس الحنجره والرغامى المعدى عن طريق إختبار الإنتشار المناعي في هلام الأجار وبالفحص النسيجي المرضي لقطع الغشاء المشيمي السقائي. هذا هو التقرير الأول عن عزل فيروس الحنجره والرغامى المعدى في السودان.