Dept of Animal Disease,
Faculty of Veterinary Medicine, Hama, Al-Baath University, Syria.

ISOLATION AND MOLECULAR DETECTION OF INFECTIOUS LARYNGOTRACHEITIS VIRUS IN LAYERS AND BROILER BREEDERS IN SYRIA

(With One Table and 3 Figures)

By

M.Y. ARNAOUT; M.M. FADEL and I.M. MOHRA*

* Dept. Animal Production, Fac. of Agriculture, Damascus University, Syria.

(Received at 7/9/2009)

العزل والكشف الجزيئي عن فيروس مرض التهاب الحنجرة والرغامى المعدي في دجاج بيض المائدة وأمات الفروج في سوريا

ماهر ياسين الارناؤوط ، محمد مصطفى فاضل ، إبراهيم محمد مهرة

شُخص في دراستنا مرض التهاب الحنجرة والرغامى المعدي في مزارع دجاج بيض المائدة وأمات اللحم في سوريا في الفترة بين أعوام ٢٠٠٦ و ٢٠٠٨ وكانت الأعراض السريرية والآفات التشريحية نموذجية لمرض التهاب الحنجرة والرغامى المعدي الحاد ووجد عند تشريح الطيور المصابة نزف دموي وتغيرات دفتريائية ومواد متجبنة في الحنجرة والرغامى وتراوحت نسبة النفوق من ٥% إلى ٢٩%. جمعت عينات من أنسجة الحنجرة والرغامى من كل حالة مرضية وحضرت للعزل الفيروسي وأنجز العزل بالحقن في أجنة بيض الدجاج الخالي من المسببات المرضية النوعية، حيث لوحظ توضع للويحات عاتمة على الغشاء المشيمي السقائي بعد ٧ أيام من الحقن. استخدم اختبار تفاعل البلمرة المتسلسل لإثبات وجود DNA فيروس التهاب الحنجرة والرغامى المعدي ولوحظت النتائج الإيجابية عند ٤٤٣ (زوج قاعدي) لعزولات الفيروس التي تم الحصول عليها من العينات السريرية الحقلية ، في حين لم تكتشف أي عُصَابة (حزمة) في الشواهد السلبية. أشارت نتائج دراستنا الحالية إلى وجود ودوران فيروس التهاب الحنجرة والرغامى في مزارع الدجاج في سوريا، ومن المحتمل أنه يتصف بإمراضية عالية في الدجاج، لذلك يجب أن تُجرى دراسات أخرى عن المرض وتُتخذ إجراءات الوقاية للحد من خطورة المرض في سوريا.

SUMMARY

In this study, Avian Infectious Laryngotracheitis (ILT) was diagnosed in layer and broiler breeder farms in Syria between 2006 and 2008. Clinical signs and gross lesions were typical to acute ILT infections. At necropsy of affected birds, haemorrhage, diphtheritic changes and caseous material were found in trachea and larynx. Mortality rates varied from

5% to 29%. Laryngotracheal tissue samples from each field case were collected and prepared for virus isolation. Virus isolation was carried out by inoculation of embryonated specific pathogen free eggs (SPF). Several opaque plaques on chorioallantoic membrane (CAM) were observed after seven days of inoculation. The polymerase chain reaction PCR was utilized to confirm the presence of ILTV DNA. Positive results by PCR were observed in ILTV isolates obtained from field specimens. No bands were detected in negative controls. Our results indicate the presence and circulation of ILTV in chicken Syrian farms, which probably have a high pathogenicity in the field. Further studies and prevention strategies on avian infectious Laryngotracheitis in Syria should be carried out.

Key words: *Infectious Laryngotracheitis virus, Polymerase chain reaction, Virus isolation.*

INTRODUCTION

Infectious Laryngotracheitis (ILT) has been identified in many countries of the world, and still remains a threat to the intensive poultry industry. Recently, ILT infections have occurred in USA and Brazil (Crespo *et al*., 2007; Chacon *et al*., 2008). The disease is characterized by depression, conjunctivitis, sneezing, coughing, anorexia, nasal exudates, gasping, and decreases in eggs production. In severe forms of the disease, expectoration of blood–stained mucus and high mortality rate are described (Guy and Bagust, 2003). Mild enzootic forms generally result in low mortality (0.1%-2%).

Causative agent belongs to the family Herpesviridae viruses, subfamily of Alphaherpesvirinae, Iltovirus genus, and called Infectious Laryngotracheitis virus (ILT) (Davison *et al*., 2005).

The DNA genome possesses a type D herpesvirus genome (Roizman and Pellet, 2001), consists of a linear 155-kb double-stranded molecule comprised of unique long and short regions flanked by inverted repeats (Lieb *et al*., 1987; Johnson *et al*., 1991). Differences between ILTV strains based on virulence studies and restriction fragment length polymorphism analysis were described (Graham *et al*., 2000; Han and Kim, 2001; Creelan *et al*., 2006; Ojkic *et al*., 2006).

ILT Virus was isolated successfully on embryonated chicken eggs (Hughes and Jones, 1988) and in cell culture such as chicken embryo liver (McNulty *et al*., 1985), chicken embryo kidney (Chang *et al*., 1960) and chicken kidney (Van Kamr .en and Spadbrow, 1976). Viral antigens and antibodies may be demonstrated by immunohistochemistry (IHC)

(Timurkaan *et al.*, 2003), immunofluorescence (IF) (Braune and Gentry, 1985), agar gel precipitation test (AGPT), virus neutralization test (VN) and enzyme linked immunosorbent assay (ELISA) (Bauer *et al.*, 1999).

Several molecular methods including PCR for identifying ILTV DNA in clinical samples have been reported (Keam *et al.*, 1991 and Key *et al.*, 1994). The polymerase chain reaction (PCR) has been used to detect nucleic acid of ILTV in the trachea and other tissues and it is very sensitive in ILT diagnosis (Williams *et al.*, 1992; Abbas *et al.*, 1996; Alexander and Nagy, 1997, and Alexander *et al.*, 1998; Humberd *et al.*, 2002). PCR allows detecting DNA in samples contaminated with other microorganisms, such as adenoviruses, that may prevent ILTV isolation due to overgrowth in culture (William *et al.*, 1994). In current time, the use of PCR for the detection of ILT DNA in clinical cases has become widespread.

In Syria, ILT infections were reported in layers and broiler breeders based on serological tests (Arnaout, 2005). Knowing that, ILT virus wasn't isolated in layers in Syria.

This article describes the diagnosis of acute infectious Laryngotracheitis in layer and broiler breeder farms, and the detection of ILTV isolates by PCR. This work reports the first isolation of ILTV in commercial layer hens in Syria.

MATERIALS and METHODS

History of examined flocks and samples collection:

Tissue samples of affected larynx and trachea were obtained from four different commercial layer and broiler breeder chicken flocks from three different regions in Syria between March 2006 and January 2008. No ILT vaccine had been used in these farms. Samples from each case were identified by case code, followed by letters representing country (Syria) and the year of sample collection as described by (Oldoni *et al.*, 2008) (Table 1).

Table 1: The summary of case history to the four ILT suspected flocks.

Case ID/(isolate)	Y/O[1]	chicken	A/D[2]	mortality	N/B[3]
A2/SY006	Mar 2006	layers	355	5%	5500
A1/SY006	Apr 2006	layers	28	29%	14000
B1/SY007	May 2007	layers	133	16%	5950
D3311/SY008	Jan 2008	Broiler breeder	21	25%	20400

1 year of occurrence, 2 age in days, 3 number of birds

Virus isolation:

Virus isolation of ILTV was carried out according to (Tripathy, 1998; OIE, 2008). Briefly, a pool of 3-4 larynx and trachea tissues from each case were homogenised with PBS (pH= 7.2) and prepared as suspension 20% (W/V). The suspension was centrifuged at 5000 rpm for 10 min in 4 °C and after filtration the supernatant was inoculated in allantoic cavity or in CAM membrane of 10 days SPF eggs (5 eggs for each) and incubated for 7 days at 37 °C. At least two passages were done. The CAMs after the first and the second passages were examined for the presence of lesions.

MA/SY004 field isolate of ILTV which isolated in previous study from the upper respiratory disease of broiler breeder chickens in Syria in 2004 (Arnaout, 2005) used as positive control, and commercially avian pox virus vaccine from (Lohmann, Germany) used as a negative control.

Extraction of viral DNA:

DNA was extracted using commercial DNA extraction kit (QiaAmp DNA Mini Kit, Qiagen, Germany) according to the manufacture with the recommendations for viral DNA. Briefly, 20 µl of proteinase K was added to 200 µl aliquots of supernatants from homogenized CAMs, then 200 µl of lysis buffer AL was added, and the mixture incubated at 56 °C for 10 min. The protein free DNA was precipitated with 260 µl of ethanol. The solution was transferred to QIAamp Mini spin column and centrifuged at 8000 rpm for 1 min. Two washings steps were performed: The first with 500 µl AW1 (centrifuged for 1 min at 8000 rpm), the second with 500 µl AW2 (centrifuged for 1 min at 14000 rpm). DNA was eluted with 100 µl AE buffer from QiaAmp column (centrifuge at 14000 rpm for 1 min). The DNA was stored at 70- °C.

Polymerase Chain Reaction:

Amplification of extracted DNA was performed by one-step PCR with a commercial complete kit (Avian Infectious Laryngotracheitis kit, serial number 2918, GeneKam Biotechnology AG, Germany). The kit contains [solution A, solution B] which included PCR master mix and ILTV specific primers (forward and backward) (Alexander *et al.*, 1998). The procedure was carried out according to manufacturer. Briefly, a 20 µl final PCR reaction mixture contained 2 µl of DNA template added to 10 µl of solution B and 8 µl of solution A. The reaction mixture was incubated in theromcycler (TECHNE TC-512, England), at initial denaturation 94°C for 1 min, followed by 35 cycles at 95°C for 20

seconds, 60°C for 90 seconds, 72°C for 90 seconds, and a final extension at 72 °C for 300 seconds. PCR product was fractionated by electrophoresis using a 1.5% agarose gel in 1X TAE buffer (Agarose, 25x TAE, Biobasic, Canada) containing ethidium bromide (5µl/100ml). Sample that generated the expected product band at 443 bp was considered positive for ILTV according to manufacturer instructions.

RESULTS

Clinical signs and post mortem lesions:

The affected birds had shown severe respiratory signs including gasping, coughing, gurgling, open mouth and extended neck, asphyxia, and blood-stained mucus which observed on the wall of poultry houses, accompanied by increased mortality and high morbidity.

At necropsy of affected birds, the gross lesions were located mainly in the upper respiratory tract, while other tissues were normal in euthanized birds. These lesions consisted of mild-sever congestion with haemorrhage in trachea, mucus mixed with blood along trachea, a plug of caseous material in the entryway of larynx and diphtheritic membranes adherent to the larynx and trachea.

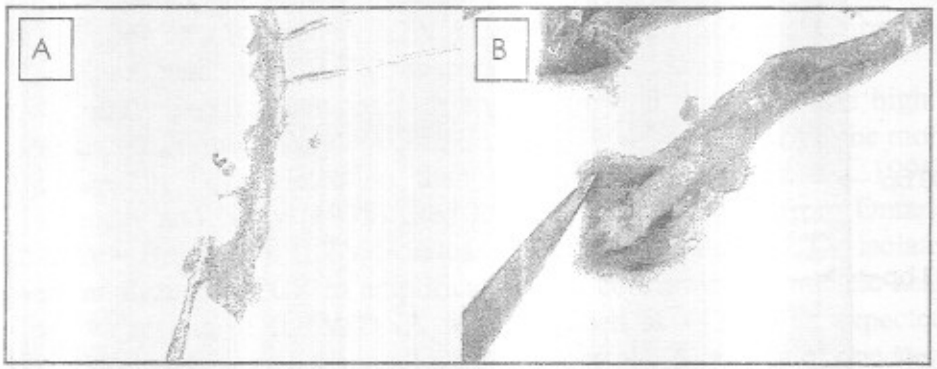

Fig. 1: Gross lesions observed in (D3311/SY008) ILT field case A; haemorrhage and congestion in trachea, B; a plug of caseous material in the larynx.

Virus Isolation:

ILTV was isolated from each case when inoculated via chorioallantoic membrane (CAM). In the first and the second passage, dispersed opaque plaques (pocks) and edema on the CAMs were observed seven days post-inoculation (Figure 2).

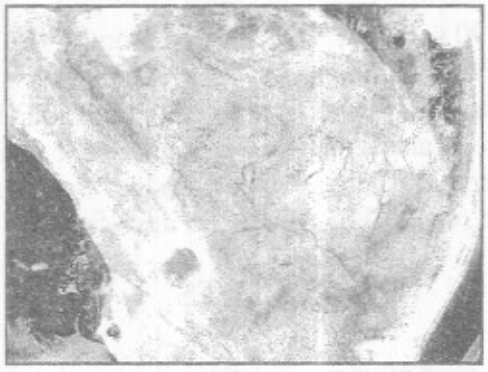

Fig. 2: Opaque plaques on CAM of chicken embryos inoculated with ILTV.

Polymerase Chain Reaction:

As shown in Figure 3, the expected fragment of 443 bp of ILTV DNA was detected in A1/SY006, A2/SY006, B1/SY007 and D3310/SY008 ILTV isolates, as well as in the two positive controls. No bands were detected in negative control, dH2O, normal CAM, AP vaccine, normal

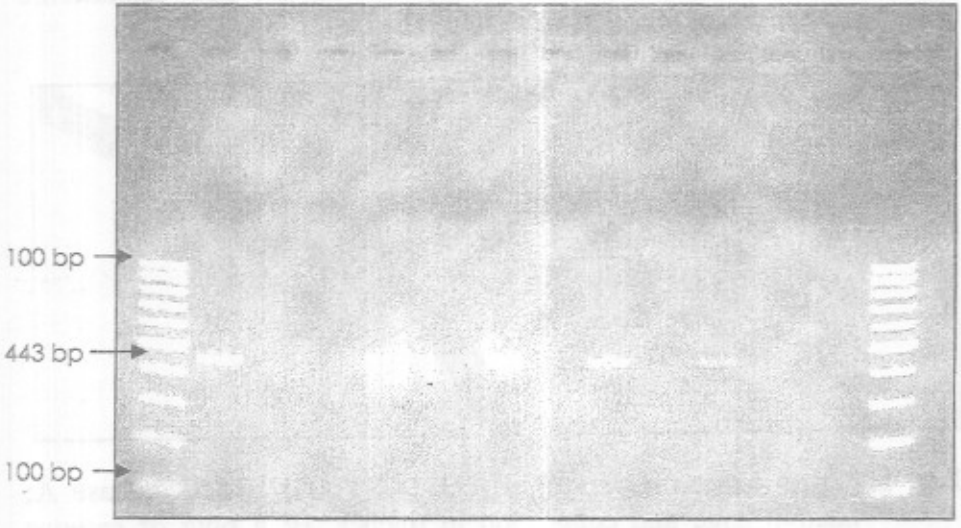

Fig. 3: 01- Molecular Ladder 100bp (Genekam), 02- Positive control (GeneKam), 03- Negative control (GeneKam), 04- dH2O, 05- A2/SY006 isolate, 06- MA/SY004, isolate 07- B1/SY007 isolate, 08- normal CAM from SPF egg, 09- D3310/SY008 isolate, 10- Avian Pox vaccine, 11- A1/SY006 isolate, 12-trachea from uninfected bird, 13- PBS pH = 7.2, 14- Molecular ladder 100 bp.

DISCUSSION

Laryngotracheitis still threats poultry industry around the world. In Syria, previous serological screening on ILT in chicken farms indicated to presence of ILTV antibodies in layers and broiler breeders, and one ILT virus isolate was obtained from a broiler breeder flock (Arnaout, 2005).

In the present study, acute infectious Laryngotracheitis was diagnosed in three field cases in layers and one field case in broiler breeders by clinical signs, gross lesions and virus isolation and PCR.

In our study, clinical signs and lesions in the field cases such as dyspnea, expectoration of bloody mucus and haemorrhage in trachea were observed, which associated with high mortality rates varied from 5% to 29%. These findings are typical to the ILT disease (Guy and Bagust, 2003), Similar findings were described for severe epizootic forms of ILT infections (Jordan, 1958).

ILT virus was isolated in each cases by inoculation in SPF chicken eggs. The virus caused pocks formation on CAM after inoculation, which is characteristic of ILT virus (Tripathy, 1998). The virus isolates obtained from these cases were easily propagated in embryonating chicken eggs, and that refer to the virulence of the strain. Hughes *et al*. (1991) and Garcia and Riblet (2001) explained the difficult of isolation of low virulent ILTV, also Sellers *et al*. (2004) described the limited propagation of ILT virus isolated from mild cases.

Polymerase chain reaction applied to viral diagnosis is a highly sensitive technique (Forghani and Erdman, 1994), and shown to be more sensitive in DNA detection than other tests (Abbas *et al*., 1996). Alexander and Nagy (1998) used PCR to amplify DNA from Ontario and New Brunswick ILTV isolates. In the present study, ILTV isolates were analyzed by PCR to amplification and confirming of nucleic acid. Positive results of ILTV DNA were obtained at 443 bp as expected, while no bands were seen in all negative controls. A benefit of one step-PCR using the PCR kit mentioned herein is reducing pipetting steps, which decreases the risk of contamination.

Our results of virus isolation and PCR are in agreement with the characteristic symptoms and lesions of avian infectious Laryngotracheitis virus observed in the field. These correlations are very significant in diagnosis of ILTV (Chacon *et al*., 2007). In this work, we conclude the presence of ILT virus in layer and broiler breeder chickens in Syria, and it is may be responsible for significant economical losses in

chicken farms. The circulating virus strain probably has a high virulence in chickens. Therefore, adequate applications for controlling of ILT should be done. However further pathogenicity, molecular and epidemiological studies are still carrying out on Laryngotracheitis virus isolates in Syria.

ACKNOWLEDGMENTS

The authors thank Dr. Zohair AL Ahmad, Faculty of Veterinary Medicine, University of AL Baath, Syria, for his kind scientific support about polymerase chain reaction.

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