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CHARACTERIZATION OF SYRIAN ISOLATES OF AVIAN INFECTIOUS LARYNGOTRACHEITIS VIRUS BY RESTRICTION FRAGMENT LENGTH POLYMORPHISM

(With 2 Tables and 4 Figures)

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(Received at 15/9/2010)

صفات العزولات السورية لفيروس التهاب الحنجرة والرغامى المعدي الطيري عن طريق التعددية الشكليائية لأطوال شدف الحصر

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عرّف مرض التهاب الحنجرة والرغامى المعدي في معظم بلدان العالم ولا يزال يشكل تهديداً لصناعة الدواجن المكثفة. استخدم في هذه الدراسة اختبار التعددية الشكليائية لأطوال شدف الحصر (RFLP) المعتمد على تفاعل البلمرة المتسلسل لمورث الثيميدين كيناز بواسطة أنزيمي حصر النوكلياز الداخليَّة HaeIII و MspI ، وذلك لوصف الخصائص الجزيئية لـــ ٢٠ عزولة حقلية من فيروس التهاب الحنجرة والرغامى المعدي تم الحصول عليها خلال أوبئة المرض في سوريا خلال الفترة بين أعوام ٢٠٠٦ و٢٠٠٩. صنّفت نتائج اختبار RFLP عزولات الفيروس إلى ثلاث مجموعات: ٥ عزولات ضمن المجموعة الأولى وكانت مطابقة لنموذج عترات اللقاح المحضرة على أجنة بيض الدجاج CEO ، و٩ عزولات ضمن المجموعة الثانية والتي اختلفت بنموذجين RFLP عن المجموعة الأولى، و ٦ عزولات ضمن المجموعة الثالثة وهي النمط المشترك الحاوي على نمطي المجموعة الأولى والثانية معاً. أشارت نتائج هذه الدراسة إلى أن فيروسات النمط الحقلي الضاري والفيروسات ذات المنشأ اللقاحي قد ساهمت في حدوث أوبئة مرض التهاب الحنجرة والرغامى المعدي في قطعان الدجاج في سوريا.

SUMMARY

Avian Infectious Laryngotracheitis (ILT) has been identified in most countries around the world and remains a threat to the intensive poultry industry (Guy and Bagust, 2003). In this study, polymerase chain reaction and restriction fragment length polymorphism (PCR-RFLP) of thymidine kinase (TK) gene using restriction endonucleases HaeIII and MspI was utilized to characterize 20 field isolates of ILTV obtained from different chicken flocks during ILT outbreaks in Syria between 2006 and 2009. Combinations of PCR-RFLP patterns classified the ILT virus isolates into three groups. Five isolates were categorized as group I that had identical pattern to the two chicken embryo origin CEO vaccine strains. Nine isolates were categorized as group II that differed in two RFLP patterns from CEO vaccine strains, whereas six isolates categorized as group III that contained a mixture pattern of group I and group II patterns. The results of this study indicated that both wild type and vaccine derived viruses were involved in ILT field cases in Syria.

Key words: *Infectious Laryngotracheitis virus, Polymerase chain reaction, Restriction fragment length polymorphism.*

INTRODUCTION

Avian infectious laryngotracheitis (ILT) is a highly contagious acute respiratory disease of chickens that is caused by Gallid herpesvirus 1 (family Herpesviridae). Sever forms of infections are characterized by severe respiratory signs, expectoration of bloody mucus and high mortality, while mild respiratory signs, mucoid tracheitis and low mortality are described in mild forms of infections (Guy and Bagust, 2003). Laryngotracheitis virus has been identified in most countries and remains a serious disease wherever susceptible poultry populations occur, especially in large numbers (Biggs, 1982).

More recently, DNA detection by conventional polymerase chain reaction (PCR) or real-time PCR have become the methods of choice for virus diagnosis (Villarreal *et al.*, 2004; Creelan *et al.*, 2006; Callison *et al.*, 2007; Chacon *et al.*, 2007; Crespo *et al.*, 2007).

Differentiation of LTV strains of varying virulence, particularly wild-type and modified live-vaccine viruses, is an important practical problem (Guy and Bagust, 2003). The differentiation between field and vaccine strains has been demonstrated through the use of PCR coupled with restriction fragment length polymorphism (RFLP) of the viral genes gG, TK, ICP4, gC and gM/UL9 and have been described for epidemiological investigations (Graham *et al.*, 2000; Kirkpatrick *et al.*, 2006; Neff *et al.*, 2008; Oldoni *et al.*, 2008). In Syria, numerous cases of ILTV were observed and diagnosed (Arnaout, 2005; Arnaout *et al.*, 2009), but it doesn't know whether such cases are related to vaccine strains or wild type viruses of ILTV. The objective of the present study was to characterize 20 field isolates of ILTV collected from different poultry farms in Syria by using RFLP-PCR of Thymidine kinase (TK) gene to investigate the variety of the strains.

MATERIALS and METHODS

Viruses

A total of 20 field isolates of ILTV collected between February 2006 and June 2009 were analyzed. Four field isolates were obtained from previous study (Arnaout *et al.*, 2009) and the other isolates were obtained during the course of this study. Nineteen out of 20 isolates were obtained from different commercial chicken flocks and one isolate were from a backyard flock. The viruses compared in present study were isolated from natural outbreaks of ILT in Syria. All isolates were propagated during acute phase of the disease by inoculation on the chorioallantoic membrane (CAM) of SPF embryonated chicken eggs according to OIE virus isolation procedures of ILTV (OIE, 2008). Two commercial ILT CEO vaccines, Nobilis ILTV Serva strain (Lot/A011BJ01, Intervet, Boxmeer, The Netherlands) and Fowl Laryngotracheitis Vaccine (Lot/54354, Lohmann, Animal Health, Winslow, Maine, USA), were used in this study.

Table 1: Details of ILTV isolates used in this study.

Isolate ID	Date of field case	Vaccination	Origin	Mortality %
BH	26/02/2006	Non-vaccinated	Layer	10
A2	05/03/2006	Non-vaccinated	Layer	5
A1	12/04/2006	CEO vaccine	Layer	29
TW	08/06/2006	Non-vaccinated	Layer	5
B1	16/05/2007	Non-vaccinated	Layer	16
GZ	25/09/2007	Non-vaccinated	Broiler Breeder	10
ZZ	23/11/2007	CEO vaccine	Broiler Breeder	2
A001	02/01/2008	Non-vaccinated	Broiler Breeder	30.77
D3311	20/01/2008	Non-vaccinated	Broiler Breeder	25
CM991	12/02/2008	Non-vaccinated	Broiler Breeder	1.5
F25-3	25/03/2008	Non-vaccinated	Broiler	*
Backyard 1	25/03/2008	Non-vaccinated	Backyard	*
F1-4	01/04/2008	CEO vaccine	Broiler	*
L921	25/01/2009	Non-vaccinated	Layer	24
F4-2	04/02/2009	Non-vaccinated	Broiler	*
PX1	04/02/2009	CEO vaccine	Layer	1
CC2	17/03/2009	CEO vaccine	Layer	1
MJ1	19/03/2009	CEO vaccine	Layer	1
MJ2	19/03/2009	CEO vaccine	Layer	1
XLM	05/06/2009	CEO vaccine	Layer	1.5

* Increased mortality but the data is not available accurately

Extraction of viral DNA

DNA was extracted using commercial DNA extraction kit (PeqGOLD DNA Tissue Mini, lot/07016, Germany) according to the manufacturer. Briefly, 25 ul of protease was added to 250 ul aliquots of supernatants from homogenized CAMs, then 250 ul of lysis buffer BL was added, and the mixture incubated at 70 °C for 10 min. The protein free DNA was precipitated with 260 ul of 100% ethanol. The solution was transferred to HiBind®-DNA column and centrifuged at 8000 rpm for 1 min. Two washings steps were performed, and the DNA was eluted with 50 ul elution buffer and stored at 70- C.

Polymerase Chain Reaction of TK gene

PCR was performed using PCR commercial kit (Lot/133194470, HotStarTaq Master mix kit, Qiagen, Germany). Each amplification reaction was performed in a 50 ul volume, containing 25 ul HotStarTaq® Master mix 2x, 0.5 um of each primer, 15 ul RNase free water and 5ul of DNA template. The primers utilized in this study were selected from previously published work (Neff *et al.,* 2008). The primers were used to amplify a 2.1 kb fragment of the ILTV genome containing the TK gene. The primers were TK gene (sense) 5-GCTGGGCTAAATCATCCAAG-3 and TK gene (antisense) 5-GGAAGCGGAACATTACGAAC-3. The reaction mixture was incubated in thermal cycler (Techne, England) at 95 °C for 15 min, then subjected to 35 cycles of 94 °C for 1 min, 56 °C for 1 min, and 72 °C for 2 min, then finally incubated at 72 °C for 10 min. PCR products were separated by electrophoresis in 1.5 % agarose gel (Agarose, Genkam, Germany) in 1X TAE buffer stained with ethidium bromide (5 ul/100ml) and visualized under UV illuminator.

RFLP

PCR products were purified post-amplification using the QIAquick PCR purification Kit (Lot/133198423, Qiagen, Germany) according to manufacturer. Ten ul of purified PCR products were digested separately with 10 unit of restriction enzyme HaeIII and MspI (NewEngland biolab, USA), 2 ul of enzyme buffer and 2 ul of distilled water. DNA fragments were separated by electrophoresis (3V/cm for 2 hr) in 2% agarose gel.

RESULTS

PCR and RFLP

2.1 kbp PCR product was successfully amplified from all field viruses and the two commercial ILT vaccine. Some of positive samples are presented in Fig 1. No bands were seen in negative controls.

Restriction digestion of TK using HaeIII gave pattern A and pattern B and mixture pattern A+B (Fig 3 and 4b). Pattern A consisted of four bands approximately of 104, 146, 851, 954 bp while pattern B consisted of five bands 104, 146, 367, 484, 954.

Restriction digestion of TK using MspI gave pattern A and pattern B and mixture pattern A+B (Fig 3 and 4a). Pattern A consisted of five patterns 119, 140, 250, 588, 966 bp while pattern B consisted of six bands 119, 140, 192, 255, 390, 966.

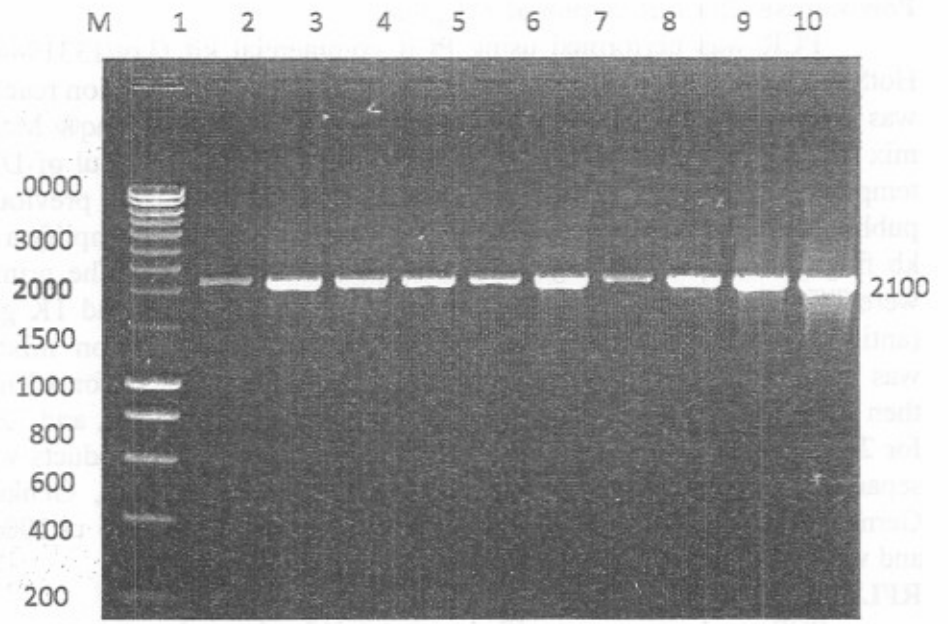

Fig. 1: M= Smart Ladder 200-10000 bp, 1= L921, 2=TW, 3=GZ, 4= A2, 5= CM991, 6= D3311, 7= ZZ, 8= BH, 9= MJ2, 10= A001.

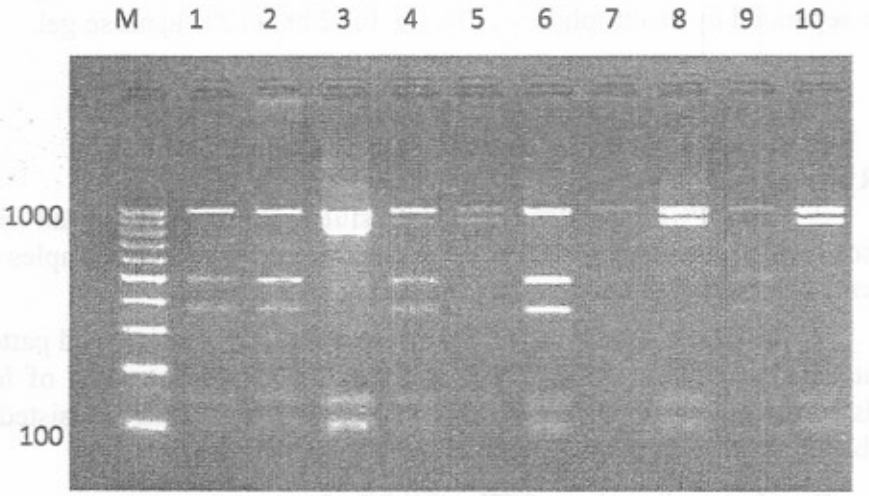

Fig. 2: (RFLP-TK-HaeIII): M= 100bp marker, 1= TW, 2= BH, 3= Lohmann Vaccine, 4= F25-3, 5= ZZ, 6= D3311, 7= Backyard1, 8= XLM, 9= L921, 10= PX1.

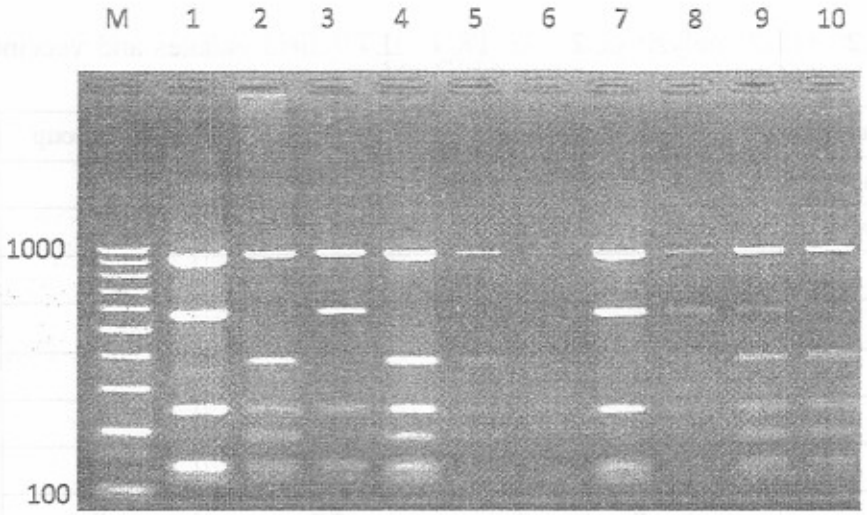

Fig. 3: (RFLP- TK-MspI): M=100 bp marker, 1= Lohmann Vaccine, 2= BH, 3= Serva vaccine , 4= D3311, 5= L921, 6= Backyard1, 7= XLM, 8= ZZ, 9= F25-3, 10= TW.

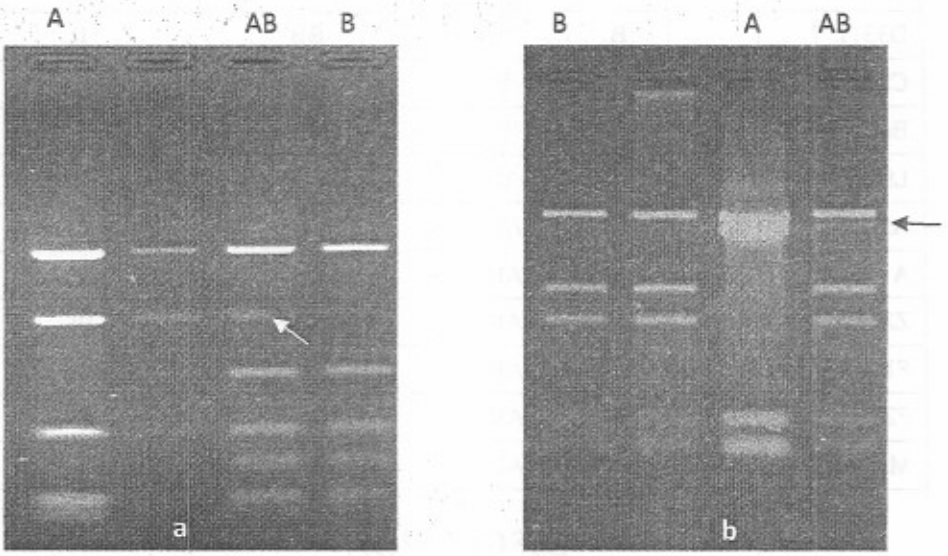

Fig. 4: (a) = TK –MspI: pattern A (CEO vaccine type), pattern B (wild type) and pattern AB (Mixture type); **(b)** = TK –HaeIII: pattern A (CEO vaccine type), pattern B (wild type) and pattern AB (Mixture type).

Table 2: RFLP analysis of 2.1 kb TK of ILTV field isolates and vaccine strains

Isolate / Strain	MspI	HaeIII	Pattern combination	RFLP Group
Lohmann Vaccine	A	A	AA	I
Intervet Vaccine	A	A	AA	I
PX1	A	A	AA	I
CC2	A	A	AA	I
MJ2	A	A	AA	I
XLM	A	A	AA	I
F4-2	A	A	AA	I
BH	B	B	BB	II
TW	B	B	BB	II
B1	B	B	BB	II
GZ	B	B	BB	II
A001	B	B	BB	II
D3311	B	B	BB	II
CM991	B	B	BB	II
Backyard1	B	B	BB	II
L921	B	B	BB	II
A2	AB	AB	ABAB	III
A1	AB	AB	ABAB	III
ZZ	AB	AB	ABAB	III
F1-4	AB	AB	ABAB	III
F25-3	AB	AB	ABAB	III
MJ1	AB	AB	ABAB	III

DISCUSSION

Several molecular assays have been developed to distinguish between wild type and vaccine viruses. Recently, RFLP based on PCR was used successfully in the differentiation of ILT viruses (Oldoni and Garcia, 2007). This approach has been utilized in several countries to characterize

circulating field strains (Graham *et al.*, 2000; Creelan *et al.*, 2006; Kirkpatrick *et al.*, 2006; Ojkic *et al.*, 2006).

The TK gene is associated with ILTV virulence, and previous studies have demonstrated that differentiation between virulent and vaccine-like field strains can be achieved through RFLP and sequence analysis of this region (Han and Kim, 2001a+b; Neff *et al.*, 2008).

In this study, PCR-RFLP of TK gene was used to examine 20 Syrian field isolates of ILTV. Previous similar study indicated that the same region of the TK gene through RFLP with HaeIII restriction enzyme enabled to distinguish between European isolates of ILTV (Neff *et al.*, 2008). In our results, RFLP of TK gene with HaeIII and MspI divided all the isolates into three groups. Five isolates (25%) belonged to group I which had identical RFLP patterns to CEO vaccine strains. These isolates except (F1-4) were obtained from vaccinated flocks that had a history of respiratory signs and low mortality with having been vaccinated approximately 5-10 days before this isolation. These findings suggests more likely that these isolates originated from re-isolation of vaccine strains.

Nine isolates (45%) belonged to group II which had different RFLP patterns from vaccine strains. All these isolates were obtained from unvaccinated flocks that had high mortality. This result indicated that wild type viruses differed from vaccine viruses of ILT have recently been circulating in poultry farms in Syria; Also the circulation of wild type viruses may explain the high mortality in unvaccinated affected chicken flocks. More comprehensive about the virulence of these wild type viruses may be obtained by pathogenicity studies in chickens.

Six isolates (30%) belonged to group III (mixture type). Three out of these 6 isolates were obtained from unvaccinated flocks, which indicate to co-infection between wild type and vaccine derived viruses. The co-infection with both types of ILTV in field cases was also reported in the USA (Oldoni *et al.*, 2008).

These results demonstrated that RFLP based on TK described herein distinguished between wild type and vaccine type of Syrian ILTV isolates. In summary, present data indicate that both "wild-type" and vaccine viruses are involved in ILT cases in Syria, but the origin of the "wild-type" virus remains unknown. Both types of viruses were detected in vaccinated and unvaccinated flocks, and although genetically different as confirmed by PCR-RFLP analysis.

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