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Full Length Research Paper

Differentiation of field isolates and vaccine strains of infectious laryngotracheitis virus by DNA sequencing

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Avian infectious laryngotracheitis (ILT) is a severe clinical respiratory disease of chickens and causes the clinical symptoms of difficulty in breathing and bloody coughing and as if involves laying hens affect the egg production. Two different regions of the infected cell protein 4 (ICP4) gene of infectious laryngotrucheitis virus (ILTV) were amplified and sequenced for characterization of field isolates and tissue culture-origin (TCO) and chicken embryo-origin (CEO) vaccine strains. Phylogenetic analysis of the two regions showed no differences in nucleotide and amino acid sequences between Iranian field isolates with high morbidity and nearly 30% mortality and CEO attenuated vaccines. These findings suggest that modified-Live (ML) ILT vaccine viruses may increase in virulence after bird-to-bird passages.

Key words: Avian infectious laryngotracheitis, infected cell protein 4, phylogenetic analysis.

INTRODUCTION

The infectious laryngotracheitis (ILT) is an acute and highly contagious disease of chickens which is caused by a member of the herpesviridae family called gallid herpes virus 1. ILT disease leads to serious economic losses due to decreased growth rates, reduced egg production and varying levels of mortality (Fuchs et al., 2007). The ILT virus (ILTV) can establish latent infections, mainly in the trigeminal ganglion, which makes control of the disease difficult (Williams et al., 1992). Two types of ILTV live ate-nuated vaccines have been widely utilized to control the disease: the vaccines attenuated by serial pas-sages in embryonated eggs-chicken embryo-origin CEO) (Samberg et al., 1971); and the vaccine generated by multiple passages in tissue culture-tissue culture origin (TCO) (Gelenczei and Marty, 1964). In many countries, the attenuated vaccines are associated with some negative effects, such as the capacity to infect non vaccinated birds, reversion of virulence and production of latently infected carrier birds (Guy et al., 1991; Hughes et al., 1991; Kotiw et al., 1995). In addition, CEO vaccine related isolates are frequently isolated from severe ILT outbreaks (Neff et al., 2008; Oldoni and Garcia, 2007; Oldoni et al., 2008). Recently, a fowl pox-vector ILTV vaccine has been utilized mainly by commercial layers and breeders for ILTV control (Davison et al., 2006).

Epidemiological studies are directed towards characterizing and differentiating the ILTV strains involved in severe and mild outbreaks and the virus circulating in poultry flocks. However, the discrimination between vaccine strains and field isolates of ILTV is further complicated by antigenic and genetic homogeneity. The first studies included the use of the

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restriction fragment length polymorphism (RFLP) analysis of the viral genome (Andreasen et al., 1990; Guy et al., 1989; Keeler et al., 1993; Keller et al., 1992; Leib et al., 1986). Subsequently, RFLP of polymerase chain reaction products (PCR-RFLP) of multiple genes and regions has permitted the differen-tiation of ILTV isolates, including CEO and TCO strains (Chang et al., 1997; Clavijo and Nagy, 1997; Graham et al., 2000; Han and Kim, 2001; Kirkpatrick et al., 2006; Ojkic et al., 2006; Vogtlin et al., 1999). PCR-RFLP methods have been utilized successfully in several countries and include the amplification of the infected cell protein 4 (ICP4), Thymidine kinase (TK), glycoprotein G (gG) and glycoprotein E (gE) genes (Creelan et al., 2006; Garcia and Riblet, 2001; Kirkpatrick et al., 2006). Sequencing analysis of the gG, ICP4 and UL47 genes has also been used to characterize ILTV isolates (Creelan et al., 2006; Han and Kim, 2001; Ojkic et al., 2006). Although the differentiation by PCR-RFLP was shown to be efficient in discriminating ILTV isolates in many countries, these approaches included simultaneous amplification of several genes and the need of viral propagation for amplification of fragments bigger than 2 kp (Neff et al., 2008; Oldoni and Garcia, 2007). On the other hand, DNA sequencing has more discrimination potential and may reveal possible evolutionary and recombination events, as well as other avian pathogens. Sequence analysis of the TK gene can be used to differentiate between isolates of high and low virulence (Han and Kim, 2001). In addition, amplification and sequencing of the ICP4 gene for characterization and differentiation of TCO and CEO vaccine strains and field isolates of ILTV (Chacon et al., 2009).

MATERIALS AND METHODS

Iranian vaccine

One chicken embryo origin (CEO) vaccine strains Razi laboratory, Hesarak Karaj, Iran.

Clinical samples

Two ILTV isolates obtained from a severe outbreak of disease in two states (Isfahan and Tehran) of Iran follow the lack of foreign vaccine in Iran (2008-2009).

All samples were isolated from commercial birds with severe signs of disease which included conjunctivitis, gasping, coughing, nasal discharge and expectoration of bloody mucus, with 20-30% of mortality.

Samples of trachea, lungs and conjunctive tissues from five birds per flock were collected before the use of new attenuated vaccine in these flocks.

Positive control

The extracted DNA from ILT vaccine cevac LTL (Budapest, hungry) was used as a positive control in PCR assay.

Extraction of DNA

Total DNA from the field isolates and CEO vaccine strains were extracted according to the method of chomczynski. Briefly, 200 µl of homogenized tissue suspension was incubated for 5 min at 37° Cwith 1000 µl of phenol/guanidine thiocyanate solution. Chloroform (200 µl) was then added to the solution, the mixture was centrifuged and propanol (750 µl) was added and the whole cooled at -20° Cfor at least 2h. Precipitated DNA was collected by centrifugation. Any remaining DNA adhering to the wall of the tube was rinsed off with 70% ethanol and the solvent was allowed to evaporate. DNA was previously incubated in 200 µl of tris HCL-EDTA, PH 8.0 at room temperature for 15 min the field isolates were extracted directly from tissue from clinically infected chickens.

Characterization by DNA sequencing

Design of primers of ICP4 gene

On the basis of sequences previously published in Genbank, two sets of primers were designed to amplify two 688, and 635 bp fragments of the ICP4 gene. The primers ICP4-1F(5' ACTGATAGCTTTTCGTACAGCACG-3') and ICP4-1R, (5' AGTCATGCGCATCGGGACATTCTCCAGGTAGCA-3'): ICP4-2F(5' -CTTCAGACTCCAGCTCATCTG-3') and ICP4-2R(5'- TCTATGGCGTTGAC-3'), located at positions 181-204, 846-869, 3804-3824 and 4418-4440 of the ILTV ICP4 gene sequence (Genbank accession number NC-006623). Respectively, were designed using the FAST PCR software version 3.3.64.

PCR of ICP4 gene

DNA extracted directly from tissue of infected chickens was used for amplification of two fragments of the ICP4 gene. The PCR was performed with the addition of 5 µl of extracted DNA to PCR mix containing 1X PCR buffer (Invitrogen TM, Carlsbad, CA), 0.2 mM of each dNTP, 0.5 µM of each primer, 1.5 mM $MgCl_2$, 25.25 µl of ultra-pure water and 1.25 U of platinum Taq DNA polymerase (Invitrogen TM, carlsad CA) to afinal reaction of 50 µl. The conditions for DNA synthesis were: 94 C/3 min for initial denaturation, followed by 35 cycles of 94°C/1min and 62°C /1 min, 72°C /1.5 min, and a final extension at 72°C /10 min. finally, 10 µl aliquot of the PCR product was separated by agarose gel (1.5%) electrophoresis and stained with ethidium bromide solution (0.5 µg/ml). the PCR product was purified by high pure PCR product purification kit (Roche applied science) according to the manufacturer recommendation.

DNA sequencing and phylogenetic analysis Sequencing of the 688 and 635 bp DNA fragments of ICP4 gene deriving from the two field isolates and the two CEO vaccine strains was performed. Using the sequences from the field isolates and one Iranian CEO Vaccine aligned by clustal-w method available in the Bioedit software package, with homologous sequences retrived from the genbank database.

In phyologenetic analysis, the follow sequences were used:

EU 104915 20/F/04/BR, EU 104913 305/K/05/BR, FJ 794469 ILT/ Peru/2008/ USP-81, EU 104 907 13/E/03/BBR, EU 104905 401/A/06/BBR, EU 104909 strain USDA, EU 104908 TCO vaccine, EU 104906 14/E/03/BBR, FJ 477357 ILT/Brazil/2003/USP-9, FJ477356 ILT/Brazil/2003-USP-6, FJ477352 ILT/Brazil/2002/USP-1, FJ477353 ILT/Brazil/2002/usp-2, FJ794467 ILT/Brazil/2008/ USP-74, FJ 794468 ILT/Brazil/2008/usp-80, EU 104910

24/H/91/BCK, DQ 995291 strain Wang Gang, EU 104899 25/H/88/BCK EU 104911 12/D/02/BCK, EU 104900 CEO vaccine, Nobilis-ILT CEO Vaccine, EU 104904 10/C/97/BR, EU 104901 21/G/05/BR, EU 104916 205/J/06/BR, EU 104917 402/A/06/BR, EU 104903 301/K/06/BR, EU 104920 501/C/ 06/BR, CEO Laryngo-Vac vaccine and EU 104918 102/B/05/BR.

RESULTS

Characterization by DNA sequencing

PCR of ICP4 gene

All PCR products of the ILTV field isolates and vaccine strain analyzed in this study resulted in one DNA band of approximately 688 bp using the set of primers ICP4-1F and ICP4-1R, whereas that one unique band of 635 bp observed in all samples with the primers ICP4-2F and ICP4-2R.

DNA sequencing and phylogenetic analysis

All analyzed samples had 688 and 635 bp in length using the primers ICP4-1F-ICP4-1R and ICP4-2F-ICP4-2R, respectively. Identical results occurred when the sequences obtained from the two fragments the ICP4 genes were analyzed separately.

Although both fragments of ICP4 gene could differentiate among field isolates and vaccine strains, in the 688-bp fragment the CEo Strains present absence of the three amino acids which permit easily discriminate CEO vaccine from TCO strain and field isolates. However 635 bp fragment could be used when more discrimination is required.

Thus two field isolates could not distinguish between CEO vaccines. It was high relationship among two Iranian field isolates in phylogenetic tree with nobilis-ILT CEO vaccine, EU 104900 CEO vaccine, EU 104915 20/F/04/BR, EU 104913 305/K/05/BR, ILT/Iran/2009 Razi CEO vaccine, EU 104904 10/C/97/BR, EU 104901 21/G/05/BR, EU 104916 205/J/06/BR, EU 104917 402/A/06/BR, EU 104903 301/K/06/BR and EU 104920 501/C/06/BR.

All of this isolates and CEO vaccines and Iranian field isolates was in the same branch when phylogenetic tree was drown (Figure 1).

The nucleotide and amino acid sequences analysis couldn't differentiate between the CEO vaccine strains with Iranian isolates in this study. The CEO strains and Iranian field strains both showed the absence of four amino acids: alanine-alanine-glycine-aspartate in the analyzed fragment of 688-bp when they were compared with field isolates and TCO strains (Table 1).

The one Iranian CEO strain analyzed in this study formed some branch with other viruses characterized as CEO-origin and many of strains published in the genebank (Figure 1).

DISCUSSION

The molecular characterization and differentiation between ILTV strains and isolates are required in epidemiological studies whose intent is to know the origin of the virus involve in outbreaks or typing the virus circulating in the farms. We employed eight PCR-RFLP assays described in the literature and developed a novel approach based on sequencing of two regions of the ICP4 gene for differentiation among field isolates and vaccine strans of ILTV.

The ICP4 gene plays a role in regulation of gene expression early in infection and commonly used in epidemiological studies in order to know the origin of the strain involved in outbreaks (Chacon et al., 2009) had suggested that this gene could serve as a good target for RFLP analysis (Chang et al., 1997). The amplification and sequencing of two different fragments of the ICP4 gene were selected because the approaches using the PCR-RFLP with amplification of 4.9 kb of this gene were efficient to differentiate between field isolates and attenuated vaccine strains.

This study included the unique CEO vaccine as positive control and two field isolates from two state of Iran with high morbidity and nearly 30% mortality in autumn of 2008 when the attenuated vaccine were not access (specially abroad vaccines).

First the field isolates and vaccine strain were amplified and sequenced using primers designed in this study. The same samples were characterized using PCR-RFLP assays.

Although both fragments of ICP4 gene could differentiate among field isolates and vaccine strains, in the 688-bp fragment the CEO strains present absence of three amino acids which permit easily discriminate CEO vaccines from TCO strains and field isolates (Chacon et al., 2009).

However 635 bp fragment could be used when more discrimination is required in this study phylogenetic analysis of the two region not showed differences in nucleotide and amino acid sequences between field isolates and attenuated vaccines.

Many of studies show that the attenuated vaccines or vaccine like ILTV can Induced high pathogenese. For example Guy et al. (1991) compared virulence of six modified-live (Ml) infections ILT vaccine viruses with that of 11 field isolates by intratracheal exposure of 4-week old SPF Chickens. intracheal pathogenicity indices for ML vaccine viruses was ranged from 0-0.14 while those for field isolates were 0.20-0.82 and one of six ML vaccine viruses produced mortality.

Too Guy et al. (1989) in a study passage modified live (ML) ILT vaccine (both CEO and TCO vaccine) 20 time in SPF chickens. After serial bird-to-bird passage increased virulence was observed for CEO virus but not TCO virus. Increased mortality and increased severity and duration of respiratory disease were observed in chickens inoculated with chicken-passaged CEO viruses.

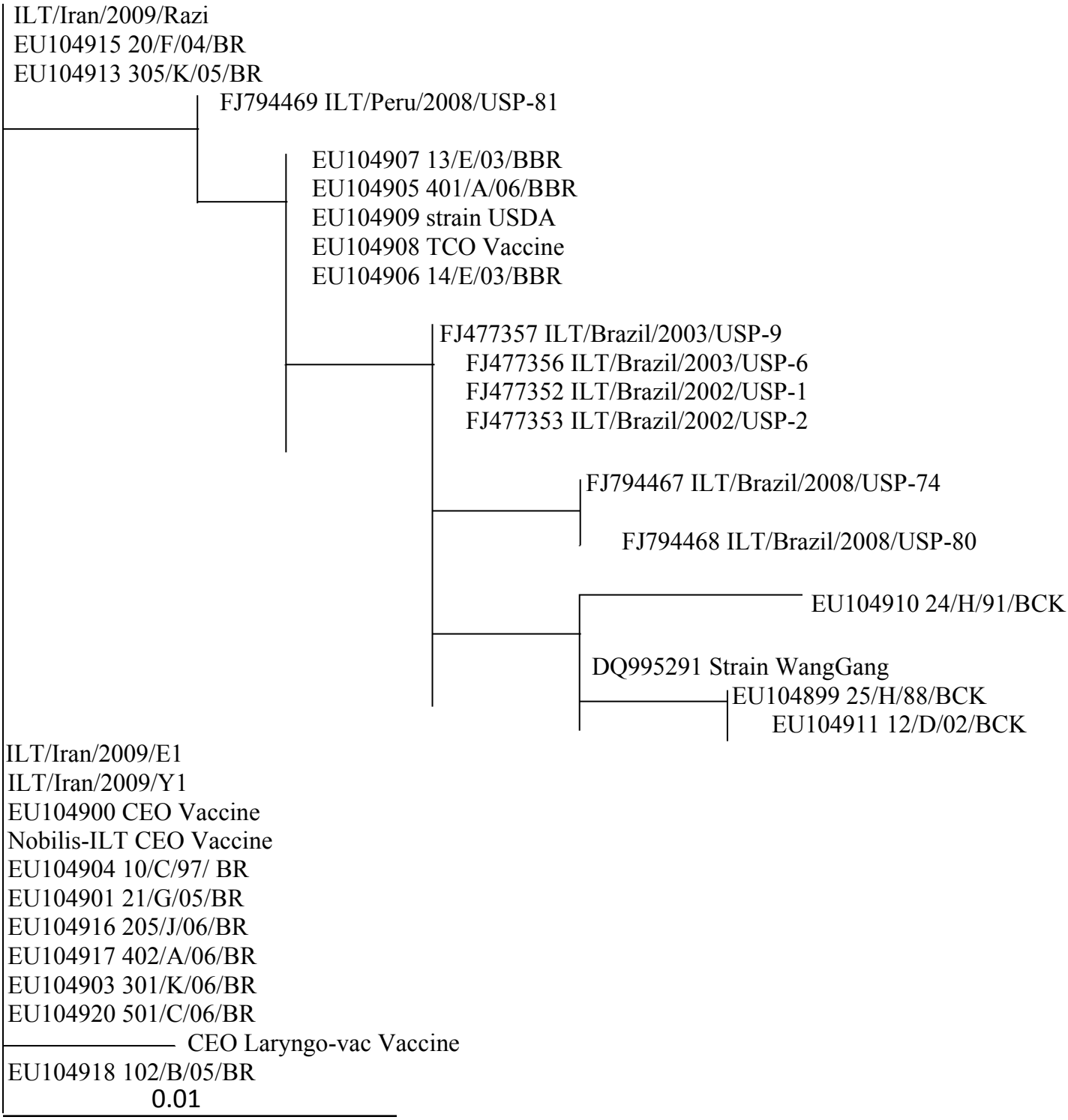

Figure 1. Phylogenetic relationships among ILTV. following of sequences of 688 bp from the ICP4 gene from nt 181 nt 869 of the ICP4 gene.

These findings suggest a possibility that field isolates originated from ML vaccine viruses through reversion to parental type virulence.

These examples show if the vaccination program was cut the vaccine viruses that used before with passaged bird-to-bird in farms can induce pathogenese and make mortality.

After the beginning production of CEO vaccines in Iran

Table 1. Comparison of nucleotide sequences of vaccine strains, Iranian isolates, Iranian vaccine and published field isolates. The analyzed sequences corresponding to 688 and 635 bp fragments of the ICP4 gene (from nt 181 to nt 869 and from nt 3804 to nt 4440 of the gene, respectively). The sequence NC-006623 (Genbank accession number was taken an reference. The dashes (-) indicate regions where the sequences are identical to those of ILtV Brazil/2002/ usp-1. Deletions within the sequences are shown with asterisks (*).

POSITION IN THE GENE	27 2	27 3	27 4	27 5	27 6	27 7	27 8	27 9	28 0	28 1	28 2	28 3	43 0	43 8	45 6	53 9	59 4	59 8	62 2	68 4	79 4	81 1	38 35	38 37	38 38	39 06	39 58	39 82	39 93	40 13	40 14	40 48	41 02	43 78
USP- 1	C	G	G	C	C	C	A	A	G	A	C	G	G	G	G	G	C	G	C	A	G	A	G	A	C	C	C	C	C	A	G	G	T	C
TCO LTI VAX	-	-	-	-	-	-	-	-	-	-	-	-	-	-	A	-	-	-	-	-	-	-	-	-	-	-	T	T	-	G	C	-	-	A
Iran-razi CEO VAC	*	*	*	*	*	*	*	*	*	*	*	*	-	A	A	-	-	A	----				-	-	-	T	-	-	-	-	-	A	-	A
Nc 006623	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	A	A	-	-	-	-	T	-	-	-	-	-	-	-
CEO nobilis ILT	*	*	*	*	*	*	*	*	*	*	*	*	*	A	A	-	-	A-------								T----				-	A	-	A
Iran E1	*	*	*	*	*	*	*	*	*	*	*	*	*	A	A	-	-	A	-	-	-	-	-	-	-	T	-	-	-	-	-	A	-	A
Iran Y1	*	*	*	*	*	*	*	*	*	*	*	*	*	A	A	-	-	A	-	-	-	-	-	-	-	T	-	-	-	-	-	A	-	A
Wang Gang	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	A	-	-	-	-	T	-	-	-	-	-	-	-
10/C/97/BR	*	*	*	*	*	*	*	*	*	*	*	*	*	A	A	-	-	A	-	-	-	-	-	-	-	T	-	-	-	-	-	A	-	A

this finding were not repeated.

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