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Full Length Research Paper

Protein expression and phylogenetic analysis of *Thymidine kinase* gene of avian infectious laryngotracheitis virus in Iran

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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. In order to cloning of the coding region of *TK* gene of ILT virus , PCR product of the open reading frame of the gene from DNA extracted of infectious tissue of involved poultry farms in different parts of south west of Iran was amplified by PCR. An 1141bp PCR product of the *TK* gene with *BamH*I, *Xho*I restriction sites were subcloned of pTZ57R/T and digested by the mentioned endonucleases. Digested insert cloned in to pGEX-4T-3 and transfected in *E. coli* cells. For the expression of TK protein, the pGEX-4T-3 recombinant vector was transformed and then induced in BL21 (DE3) strain of *E. coli* competent cells using IPTG, the presence of TK expressed protein was shown in immunoblotting and SDS-PAGE system. Analysis of the partial ILT virus *TK* gene sequences obtained from insert and was carried out. The Iranian ILT *TK* sequences were compared to 11 other corresponding sequences of ILT isolated in different countries. Nucleotide analyzing of the sequences were shown a variation of 0 - 62.8% and constructing phylogenetic tree revealed two clusters in it. Most of agreement was related to the known sequences in the *TK* gene in USA (S83714.1), China (DQ522947.1, AF435453.1), Switzerland (EU360946.1) and most of differences were related to Australian strains of this virus (GQ180115.1).

Key words: Avian infectious laryngotracheitis, *Thymidine kinase*, pTZ57R/T, pGEX-4T-3, Phylogenetic analysis, protein expression.

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 herpesvirus 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 attenuated vaccines have been widely utilized to control the disease: the vaccines attenuated by serial passages 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 used as part of ILTV control programs, but they 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

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Abbreviations: ILT, Infectious laryngotracheitis; ***TK,*** *Thymidine kinase.*

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 to characterize and differentiate 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 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 differentiation 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; Vgtlin 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*), glycoproteinG (gG) and glycoprotein E (E) 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 2kp (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. In addition, sequence analysis of the *TK* gene can be used to differentiate between isolates of high and low virulence (Han and Kim, 2001). Considering that the lack of information about ILT virus in Iran, in the present study, we compared sequences of the ILT virus *Thymidine kinase* gene obtained from three ILT virus strains isolated in different regions of Iran with other *T. kinase* gene sequences of ILT virus strains isolated in other countries and registered in GenBank and was expressed in *E. coli* for design of recombinant protein.

MATERIALS AND METHODS

Sample, plasmids and bacterial strains

The extracted DNA from 3 ILT virus isolated in Iran were selected to be cloned .Plasmid pTZ57R/T (Ins T/A clone PCR Cloning kit, Fermentas) and *E. coli* strain JM107 (Fermentas) were used for initial cloning, sequencing and maintenance of DNA fragment. For recombinant protein production, a prokaryotic expression vector pGEX-4T-3 (Pharmacian) was used. The recombinant pGEX-4T-3 (pGEX-4T-3-*TK*) is transformed into *E. coli* BL21 (DE3) (Fermentas) as host strain. The required antibiotics were added to LB media according to the reference recommendation. The extracted DNA from ILT vaccine CEVAC® LT L (Budapest, Hugary) was used positive control in PCR assay.

Primers design

Primers were designed according to the published sequence for *TK* gene of ILT (accession number: D00565.1).

The forward primer, *TK* F:5-ATGGATCCGATTCCGGCATTTAGTCTA-3 contain *BamH*I site. Reverse primer, *TK* R:5-GGCTCGAGATTATGGAAGCGGAACATTA-3 contain recognition site for *Xho*I. The restriction enzyme sites (underlined) were added to the primers for subsequent cloning procedure.

Gene amplification of *TK* (encoding the *T. kinase* enzyme)

The amplification reaction was performed in 50 µL reaction mixtures containing 0.1 mM of each dexoynucteotide, 1 µM of each primer, 50 mM Kcl, 10 mM Tris-Hcl (pH = 9), 1.5 mM $MgCl_2$, 10% dimethyl sulfoxide (DMSO, Sigma), 1 U of Taq DNA polymerase (Roche applied science) and 40 ng of template DNA. The PCR reaction was carried out in a PCR programmed thermocycler (Eppendrof, Mastercycler 5330, Eppendorf-Nethel-Hinz GmbH, Hamburg, Germany) using the thermal profiles: initial cycle 95°C for 4 min, followed by a further 35 cycles: denaturation at 94°C for 60 s; annealing at 60°C for 60 s and extension by polymerase at 72°C for 60 s. The final cycle was run at 72°C for 7 min. The PCR product was analyzed by electrophoresis in 1% agarose gel in 1X TBE buffer and visualized by ethidium bromide staining on UV trans illuminator. The PCR product was purified by High pure PCR product purification kit (Roche applied science) according to the manufacturer recommendation.

Cloning of *TK* gene

The PCR product was digested with *BamH*I and *Xho*I and ligated to pTZ57R/T and pGEX-4T-3, which were digested by the same restriction enzymes, using T4 DNA ligase (Fermentas) at 4°C over night. *E. coli* JM107 and *E. coli* BL21 (DE3) competent cells were prepared by calcium chloride method and were used for transformation of pTZ57R/T-*TK* and pGEX-4T-3-*TK* vectors, respectively. The transformed bacteria were selected by screening the colonies on LB media containing antibiotic. The suspected colony was further analyzed by restriction enzyme digestion and PCR.

Expression and purification of recombinant *T. kinase* protein

E. coli strain BL21 (DE3) transformed with the recombinant pGEX-4T-3 plasmid was grown at 37°C in 2× YT medium to an optical density of 0.8 - 1.0 ($OD_{600\ nm}$) before induction with 1 mM IPTG for 4 h. Bacterial cells were harvested and lysed by sonication in phosphate-buffered saline (PBS, 10 mM sodium phosphate, pH 7.3; 150 mM NaCl). Triton X-100 was then added to a final concentration of 1%, and the lysates were incubated for 30 min at 0°C and subsequently clarified by centrifugation at 12,000 g for 30 min at 4°C. The clarified supernatants were passed over glutathione–Sepharose 4B column (Pharmacia) that was equalized with PBS. The GST fusion protein-bound column was washed by

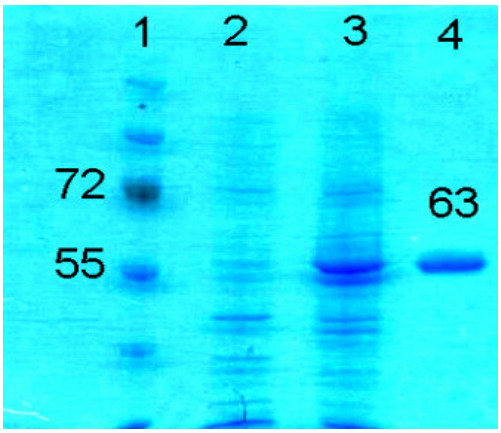

Figure 1. Expression of recombinant TK protein and its purification (Lane 1: Protein marker; Lane 2: pGEX-4T-3-*TK* before induction; Lane 3: pGEX-4T-3-*TK* after induction; Lane 4: Purified TK recombinant protein).

PBS over 10 column volumes and eluted with reduced glutathione (10 mM) for three column volumes. Quantity of the purified recombinant TK protein was analyzed by Bradford methods and subsequently its quality was assayed by SDS-PAGE 12% (2.5 µg/well). In order to analyze the cross-reaction between fused segments of TK protein with infected sera, an *E. coli* BL21 (DE3) containing pGEX-4T-3 vector was induced by IPTG.

Immunoblot analysis

For western blot analysis, 0.5 µg of purified recombinant TK protein was used per well. As a negative control, the bacterial lysate from induced *E. coli* BL21 (DE3) contain pGEX-4T-3 vector was analyzed by western blot. The gel was blotted on to Polyvinylidine difluoride (PVDF Membrane, Roche Diagnostics GmbH) membrane using transfer buffer containing 25 mM Tris (pH=8.3), 192 mM glycine and 20% methanol at 55v for one hour at 4°C. The blotted membrane was blocked with 3% (w/v) BSA in TBST buffer (0.5 M Nacl, 0.02 M Tris pH = 8.5, 0.05% Tween 20) for one hour at room temperature (RT). Membrane was incubated for 2 h at 37°C with ILT-infected bird serum, diluted 1:25, respectively. Negative serum from apparently health bird that had negative results in PCR and ELISA was used as control. After reaction the primery antibody, the blotted membranes were washed three times with TBST and incubated with peroxidase conjugated anti-chicken IgG (Rabbit anti-Chicken IgG F(ab\')2, HRP, antibodies-online GmbH, Germany) at a 1:7500 dilution in TBST. The blots were then washed three times with TBST and reaction was developed by diamino benzidine (DAB) solution (Sigma).

Sequence analysis

The nucleotide sequences were edited using Edit View v.1.0.1 (Applied Bioscience, Australia) and the 11 sequences registered in GenBank (accession numbers: FJ444832.1, FJ444847.1, S83714.1, EU360946.1, DQ522947.1, AF435453.1, DQ522949.1, D00565.1, EU423897.1, EU360950.1, GQ180115.1) were aligned separately using the Clustal X v1.81 in order to obtain a consensus sequence. Subsequently, the sequences were analyzed using the BioEdit package v.7.0.4.1 to compare the nucleotide sequences.

The nucleotide sequence of the Iranian ILT *TK* gene was compared with the corresponding sequences from other regions of the world. An unrooted dendrogramme was constructed using the Njplot software and statistical support for the dendrogramme was obtained by bootstrapping using 1000 replicates.

RESULTS

The recombinant plasmid (pGEX-4T-3-*TK*) was sequenced by specific primers and Sanger sequencing method (Macrogen, Korea). The sequencing result was confirmed by comparing with databases and using basic local alignment search tool (BLAST) software.

Expression of pGEX-4T-3-*TK* in *E. coli* BL21 (DE3) induced and the expressed protein was purified by glutathione-Sepharose 4B column (Figure 1). The result showed that the best conditions for recombinant TK protein expression can be achieved when 1 mM of IPTG and OD_{600} = 1 for three hours were used.

To determine the reactivity of recombinant protein TK, the purified recombinant protein was assayed by western blotting method. The infected bird serum (which had previously shown positive result based on PCR) was used. A negative serum from disinfected bird used as a control. Figure 2 illustrated the specific interaction between positive serum and purified recombinant TK protein. There was no reaction between the expressed pGEX-4T-3 in *E. coli* BL21 (DE3) and ILT infected serum (Lane 5 in Figure 2).

The recombinant plasmid (pTZ57R/T-*TK*) and 3 positive samples were sequenced by specific primers and Sanger sequencing method (Macrogen, Korea). The sequencing result was confirmed by comparing with databases and using basic local alignment search tool (BLAST) software.

The result of sequencing of the *TK* fragment using Clustal X software was aligned with some of the registered sequences in Genbank such as the sequences of this gene in Brazil, Australia, USA, China, Taiwan, and Switzerland. After comparing differences and similarities applying Njplot software, the phylogenetic tree was drawn which is shown in Figure 3 and the diagram is demonstrated in Table 1.

DISCUSSION

As ILT is a disease involves chickens, the economic impact due to this infection is more than expected. So that design of a procedure for prevention of this infectious disease is an initial step in hygiene program of poultry forms. Determining virulence of the routine vaccines is a decisive factor for biosafety evaluation of them.

Chang et al. (2007) compared four different ILT vaccine strains by digestion of *Tk* gene with the enzymes *Hae*III and *Msp*I as this gene is conserved within all ILT species (Griffin and Boursnell, 1990). After that Han and Kim (2001) discriminated the virulent strains of ILT based on RFLP patterns of the *Tk* gene. Also in another study that

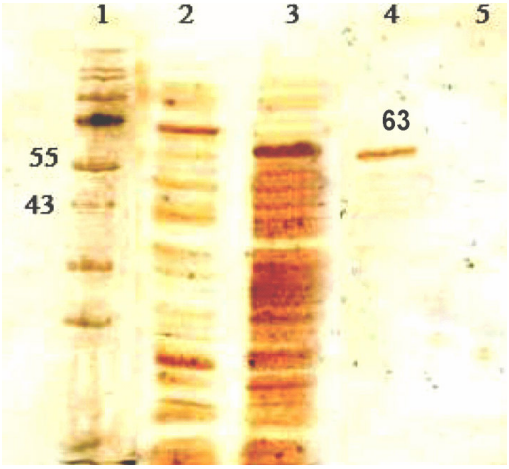

Figure 2. Western blot analyses against recombinant TK protein by ILT-infected serum (Lane 1: Protein marker; Lane 2: Western blotting pGEX-4T-3-*TK* before induction; Lane 3: Western blotting pGEX-4T-3-*TK* after induction; Lane 4: Western blotting Purified TK recombinant protein by infected sera; Lane 5: Western blotting reaction between the expressed pGEX-4T-3 and positive serum).

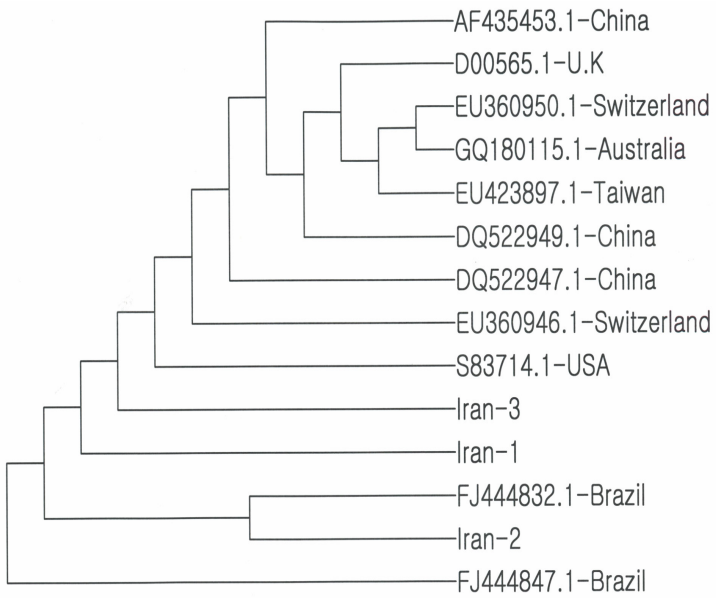

Figure 3. Phylogenetic tree of the sequence of the ILT virus *TK* gene in Iran and in other six countries.

was done by Han et al. (2002), the role of *Tk* gene was investigated as the major gene related to virulence of ILT. Since the aim of this study was determining the capacity of ILT vaccines as a source of ILT infectious, the diversity of *Tk*, form different strains of Iran, was evaluated by sequencing. Sequence and blast analysis of *Tk* gene indicate that all three ILT isolates produce and use in Iran, are in correlation with virulent strains isolated either in Iran or other counties. In conclusion however the request of a new vaccine is seemed indeed, the use of previous vaccines must be limited.

For sequencing of the *TK* gene and comparing its genetic diversity in the Iranian isolates with other available viruses in the world, we compared the known

Table 1. The results of comparison the sequence of the ILT virus *TK* gene in Iran with other countries (Sequence identity matrix).

Seq->	Iran-2	FJ444847 Brazil	Iran-1	Iran-3	S83714 USA	EU360946 Switzerland	DQ522947 China	AF435453 China	DQ522949 China	DQ522949 China	D00565 U.K	EU423897 Taiwan	EU360950 Switzerland	GQ180115 Australia
FJ444832 Brazil	0.77	0.983	0.762	0.677	0.677	0.677	0.677	0.677	0.673	0.673	0.673	0.675	0.675	0.142
Iran-2	ID	0.783	0.987	0.878	0.878	0.878	0.878	0.878	0.874	0.874	0.874	0.877	0.877	0.272
FJ444847 Brazil	0.783	ID	0.775	0.688	0.688	0.688	0.688	0.688	0.684	0.684	0.684	0.687	0.687	0.154
Iran-1	0.987	0.775	ID	0.885	0.885	0.885	0.885	0.885	0.881	0.881	0.881	0.884	0.884	0.28
Iran-3	0.878	0.688	0.885	ID	1	1	1	1	0.995	0.995	0.995	0.998	0.998	0.394
S83714 USA	0.878	0.688	0.885	1	ID	1	1	1	0.995	0.995	0.995	0.998	0.998	0.394
EU360946 Switzerland	0.878	0.688	0.885	1	1	ID	1	1	0.995	0.995	0.995	0.998	0.998	0.394
DQ522947 China	0.878	0.688	0.885	1	1	1	ID	1	0.995	0.995	0.995	0.998	0.998	0.394
AF435453 China	0.878	0.688	0.885	1	1	1	1	ID	0.995	0.995	0.995	0.998	0.998	0.394
DQ522949 China	0.874	0.684	0.881	0.995	0.995	0.995	0.995	0.995	ID	ID	1	0.994	0.994	0.392
D00565 U.K	0.874	0.684	0.881	0.995	0.995	0.995	0.995	0.995	1	1	ID	0.994	0.994	0.392
EU423897 Taiwan	0.877	0.687	0.884	0.998	0.998	0.998	0.998	0.998	0.994	0.994	0.994	ID	0.997	0.394
EU360950 Switzerland	0.877	0.687	0.884	0.998	0.998	0.998	0.998	0.998	0.994	0.994	0.994	0.997	ID	0.394
GQ180115 Australia	0.272	0.154	0.28	0.394	0.394	0.394	0.394	0.394	0.392	0.392	0.392	0.394	0.394	ID

sequences of this gene in Genbank of NCBI. The results indicated that there was 0-62.8% genetic diversity in the fragment. Most of agreement was related to the known sequences in the *TK* gene in USA (S83714.1), China (DQ522947.1, AF435453.1), Switzerland (EU360946.1) and most of differences were related to strain of this virus in Australia (GQ180115.1) (Table 1).

The phylogenetic tree of compared sequences was drawn using Clustal X and Njplot software's. As it shown in Figure 3 the Iranian isolate is set in the branch of American, China and Switzerland sequences.

We observed no much diversity in the sequences of ILT in the other studies. Despite of little diversity in the sequences which are being studied, we can justify the genetic diversity of the virus based on its geographical distribution. As the origins of many Iranian noble birds refer to the European, American and Far-East countries, so the perceived genetic similarities in this research can justify this claim. In the other hand, transportation of fowls and biologic materials between Australia and Iran basically does not have historical background. Thus, placing of Australian strains in other branches of phylogenetic tree are indicating more differences in the sequence of this virus between Iran and the mentioned countries.

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