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Detection of Infectious Laryngotrachities Virus PCR and Nested PCR

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Abstract

In this study a polymerase chain reaction (PCR) was optimized for rapid and sensitive detection of infectious Laryngotrachietis (ILTV). A 443bp DNA fragment of ILTV was amplified. After DNA extraction, PCR was carried out using specific primers and then specificity of the results was confirmed by sequencing of PCR products. Sensitivity of the test was found to be 2×10^{3} *EID_{50} by this method. After using Nested-PCR a 201bp fragment amplified which increased the sensitivity of the test* 2×10^{1} *EID_{50}.. using this technique has many advantages compared with other methods that are currently used. Therefore, it can be used as a reliable diagnostic test for rapid detection of ILTV infections*

Key words *:* infectious laryngotrachitis, polymerase chain reaction, Nested-PCR

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Introduction

Avian Infectious laryngotracheitis virus (ILTV) is an alphaherpes virus of herpesviride family (jordan, F.T.W. 1993). The virus is an important pathogen of chicken, causing an acute respiratory tract disease of laying flock and chicken that characterized by tracheal rales, coughing, mucosal layer in trachea, exuda of conjunctivitis in nasal and oral (Hughes, C.S, jones, R.C 1998). Declines in egg

production and quality in laying flocks (Hanson, LY, Bagust, T.J. 1991). In Iran, ILTV is one of the most important respiratory diseases in laying flocks.

There are some serological and pathological tests that containing Eliza, agar gel diffusion, searching in tracheal exuda cell to find Inclusion bodies and injecting in embryo chorio allantoic membrane for isolating virus. (Abbas. F., And reasen J.R et al 1996). The purpose of this study was using PCR to detect ILTV and in order to understand better their epidemiology in Iran for controlling this disease.

Materials and Methods

Virus. In this research for setup and regulation used vaccine virus strain Gallid herpes virus that produced from Razi Institute. The vial of vaccine mixed with 2ml DEPC-water and then divided in 10 vials. Each of them had 2×10^5 EID_{50} rate of viruses.

Total DNA extraction. Viral DNA was extracted from vaccine virus by a procedure, which was developed in National Research Center for Genetic Engineering and Biotechnology (NRCGEB). Briefly in a 1.5 ml tube, $200\mu l$ of solution vaccine was added to $900\mu l$ of extracted solution (DNA fast). The sample was vortexed and $400\mu l$ chloroform was added.

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After centrifugation at 12000g for 15 min in $4^{\circ}C$ temperature. the aqueous phase was transferred to other tube and equal volume of cold isopropanol was added. The sample was incubated for 15 min on ice and DNA was precipitated by centrifugation at 12000g for 10 min. The pellet was washed with 70% ethanol, semi dried in air and resuspended in $20\mu l$ dH_2O.

PCR primers. For setup PCR Test was using primers $ILTV_1$ "$ILTV_2$". The sequences of primers are as below.

5′- AGA AGG AGA CAA TCC TCC $ILTV_1$

5′- GGT CGG TTC AGT CAG TAA $ILTV_2$

Condition of PCR. PCR was performed in total volume of $50\mu l$ containing $4\mu l$ of DNA template $(5\,{}^{u}/_{\mu l})$, $1/5\mu l$ Mg cl_2 50 mM, $1\,\mu l$ of each primers $(250\,{}^{ng}/_{\mu l})$, $5\mu l$ of PCR buffer 10X , $1\,\mu l$ of d NTP_s (mix) 10 mM, $36\mu l$ DEPC-water and $0/5\mu l$ of Taq DNA polymerase. The following cycling program was performed in thermocycler : $94^{\circ}C$ for 3 min (on cycle), $94\,{}^{\circ}C/_{30}$ seconds for denaturation, $52\,{}^{\circ}C/_{30}$ seconds for annealing and $72\,{}^{\circ}C/_{30}$ second for amplicon (30 cycles) and $72^{\circ}C$, 10 min for final extension. Optimization of PCR was carried out using Negative controls. Then $7\mu l$ of amplified product was analyzed by electro phoresis using 1% agars gel (120v for 40 min). Gels were stained with ethidium bromide and amplified DNA was visualized by a UV transluminator.

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Sequencing. The PCR products were purified using High pure PCR product purification Kit (Roche-Germany) and sequenced. The nucleotide sequences were aligned dustral method of DNA STAR soft ware (DNA STAR Inc., madison, wis.)

Nasted-PCR. Optimization of Nested-PCR was planning primers and primers planed with aligo software. Nested-PCR was performed in total volume of $50\mu l$ contained $5\mu l$ PCR buffer 10x, $1/5\mu l$ $Mgcl_2$ 50mM, $1\mu l$ of each primer $(250ng/\mu l)$, $1\mu l$ of $dNTP_s$ (mix) 10mM, $39\mu l$ Depc-H_2o, $1\mu l$ PCR and $0/5\mu l$ Taq DNA

polymerase $5^{U}\!/_{\mu l}$. The thermalcycler profle was the same as described above for PCR, Denaturing Temperature must be changed from $55^{\circ}C$ to $60^{\circ}C$.

The obtained products were electrophoresed on 1/5% agaros gel to determine the size of the products.

Results

Amplification of DNA sequences. A correct size of DNA fragment (443bp) was observed in vaccine sample and negative control didn't show nonospecific DNA (figure 1)

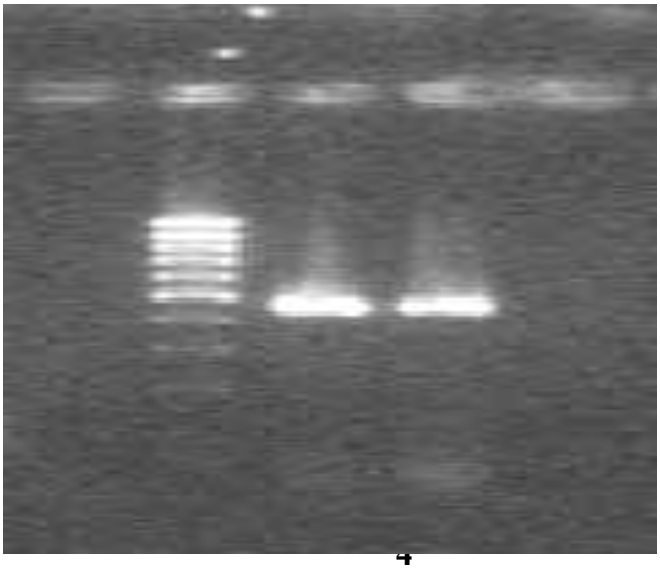

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Figure1.optimization of PCR condition using negative control.

A 443bp fragment was observing in a 1% agarose gel .Lane 1:DNA size Marker(ladder 100), lane 2,3: Amplification of a443 bp fragment from ILTV,lane 4:negative control

Sequencing PCR product. after purification of PCR product for detected the sequencing of PCR product and certificate for result of specific test comparison of sequenced data with Gen Bank revealed the specificity of the results. sequence alignments of the PCR product showed a close relationship of all the studied isolates. (figure 2-3)

PRODUCT COMPLET SEQUENCE

ILT-1

AME0003D37 28 410 ILTV1 >ILT-1-ILTV1 28..410 of trace file

GTTTGTCACGTGGGCTCAGCTTGGGGCCGCCATACCCATACGAAACGCGCGACA

GGGGCTGGACCATGAACTCCTCCACAAGGCGCTCTAACTGTT

CCACTGGCATCCCGTACTCTTCGAGATACACGGAAGCTGATTTCGGA

TTGGCATGGAATTCGGCCGCGGCCTCCCGAACCTGCGGGTCATCGACC

AAAGACTGTCTATAGCCACTTCCATGATATACTTGTCCTCTACTGGGTG

GGGACGGGATAATAGCTTCTGGAACAAAAACGCGAGCCTCGGAACTATGGAGG

TCGACGGCCAACGCTCGTGAGAGTACATTCAGGGTAATGCCGCGGCCTAGGT

CTGGGAGCTCCGTAG GCGCGGTTACTGACTGAAACCG

Figure 2 : A part of the ILTV sequenced and aligned with each other

LOCUS HSMICP4MIE 8364 bp DNA linear VRL 27-FEB-1996

DEFINITION Gallid herpesvirus 1 major immediate early protein (ICP4) gene, complete cds.

ACCESSION L32139

VERSION L32139.1 GI:493597

KEYWORDS major immediate early gene.

SOURCE Gallid herpesvirus 1

Gallid herpesvirus 1ORGANISM

Viruses; dsDNA viruses, no RNA stage; Herpesviridae;

Alphaherpesvirinae; Infectious laryngotracheitis-

like viruses.

Figure 3 : comparison of sequenced data with Gen Bank revealed the specificity of the results

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determination of sensitive PCR. It must be determine the sensitively test after diluted

The ILTV virus was set PCR and observed dilution in 2×10^3 EID_{50} that in this rage of product 443bp amplified fragment other diluted and negative control didn't has any fragment (figure 4)

1 2 3 4 5 6 7 8 9 10 11

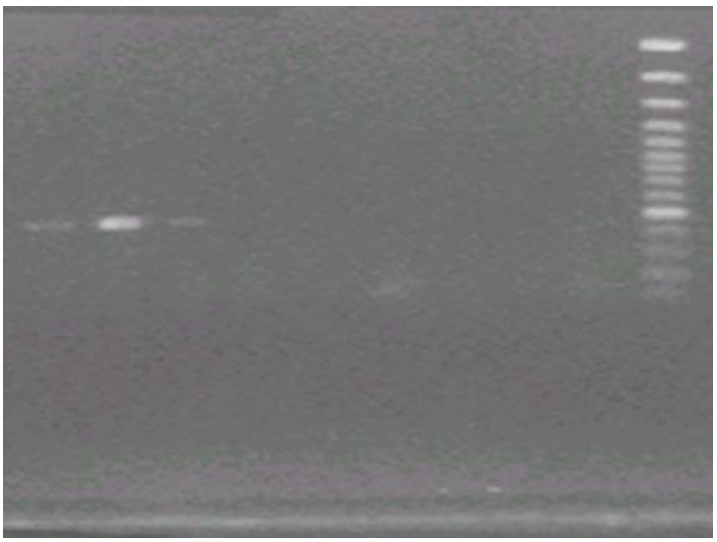
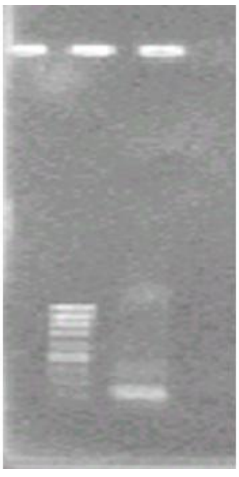


figure 4 : Sensitivity of PCR for ILTV

1- 2 X 10^5 EID_{50} viral titer
2- 2 X 10^4 EID_{50} viral titer
3- 2 X 10^3 EID_{50} viral titer
4- 2 X 10^2 EID_{50} viral titer
5- 2 X 10^1 EID_{50} viral titer
6- 2 EID_{50} viral titer
7- 2 X 10^{-1} EID_{50} viral titer
8- 2 X 10^{-2} EID_{50} viral titer
9- 2 X 10^{-3} EID_{50} viral titer
10- PCR Negative Control
11-DNA Marker(Ladder 100)

Nested. PCR : Nested-PCR a 201bp fragment amplified (figure 5)

1 2 3



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Fiqure5 :Lane 1 : :negative control, Lane 2 : DNA size Marker (ladder 100), Lane 3 : Nested-PCR a201bp fragment amplified

Determine of sensitive Nested-PCR : It observed in 2×10^{1} EID_{50} virus (figure 6)

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15

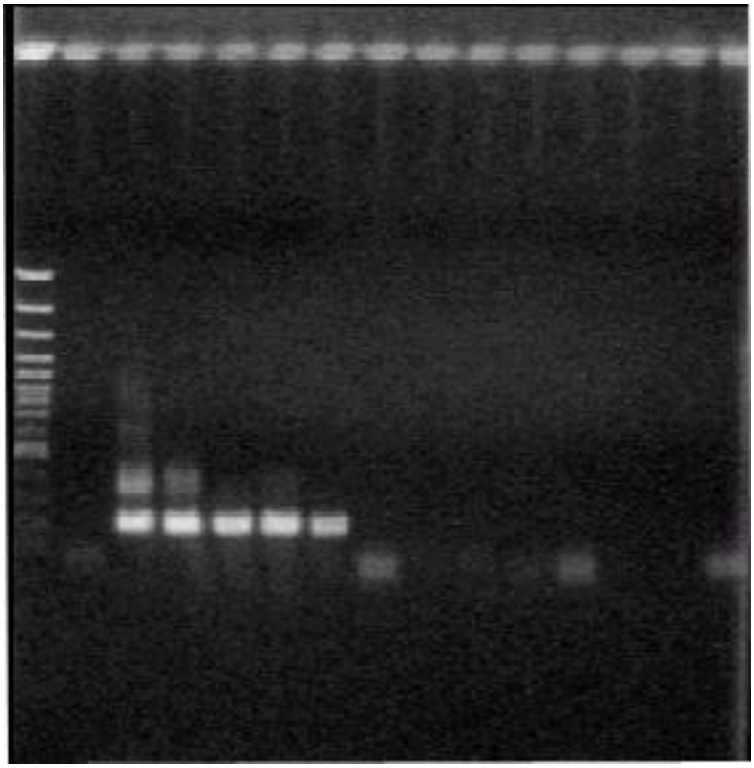

figure 6 : Sensitivity of Nested-PCR for ILTV
1- DNA Marker (Ladder 100)
2- Nested-PCR Negative Control (No. 1)
3- 2 X 10^{5} EID_{50} viral titer
4- 2 X 10^{4} EID_{50} viral titer
5- 2 X 10^{3} EID_{50} viral titer
6- 2 X 10^{2} EID_{50} viral titer
7- 2 X 10^{1} EID_{50} viral titer
8- Nested-PCR Negative Control (No. 2)
9- 2 EID_{50} viral titer
10- 2 X 10^{-1} EID_{50} viral titer
11- 2 X 10^{-2} EID_{50} viral titer
12- 2 X 10^{-3} EID_{50} viral titer
13- 2 X 10^{-4} EID_{50} viral titer
14- PCR Negative Control
15- Nested-PCR Negative Control (No. 3)

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Discussion

laryngotracheitis is one of the contagious diseases in poultry. It can create mortality between 10%-40% which causes an economical damage that decrease eggs products in layin flocks or reduces the weight in Bailler (Hayashi, s, y. odagiri et.al 1985) .

Rapid detection of ILTV is very important to control diseases (Alexander, H. S, Nagy, 1997).

the methods for recognizing ILTV are on the basis of observing the clinical signification, serological Isolation virus from embryo egg, or cell culture with observing inclusion body in Epithelium nuclear of palpebra eye or observing tracheal histopatological. therefore, for recognizing of ILTV usually with helping injected virus in Embryo egg or observing treacheal histopatological. virus isolation from embryo Egg or observation damaging tissue in histopatological lam needed specifics skill and very feasibility laboratory. Therefore, a lot of laboratories hadn't this feasibility, and so theses tests had lost a lot of time (Abbas. F.,Andreasen J.R et al 1996) .

in this manner to determine PCR that is one of very sensitive method to recognize virus in clinical samples (key, D.W., Gough, C.B and et al 1994).

This technique that contain proper clinical sample, DNA extraction, testing PCR, electrophoreses of PCR product, coloration of gel and observation, all of them do less than 8 hours. Detection virus with PCR was done in dilution 2×10^{3} EID_{50} virus and for Nested-PCR in dilution 2×10^{1} EID_{50} virus. rate of virus shows high sensitive test. Vogthin in Switzerland for detection of infection laryngotreachitis virus used PCR Technique. their researched on ILTV vaccine which was made in their country found sensitivity of PCR test in 2×10^{5} EID_{50} rate of virus (vogtlin. A, Bruckner.L et al 1999).

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in this study by (chun chang. P, ling lee. Y et al 1997) in Taiwan. He tested vaccine that showed 2×10^3 EID_{50} rate of virus. Humnerd. J, Garcia. M (et al 2002) researched clinical tissue. they recognized rate of 50 fementogram from DNA virus in PCR testes to appear comparison in these causes, showed that our studies and results had more sensitive and recognized results than other studies.

Sequence of PCR product with sequences of target fragment Genes of ILTV with Gene bank showed 98% similarity; this similar Gene was resulting specific tests.

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