Detection of Infectious Laryngotracheitis by real-time PCR in Baghdad

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Abstract

The present study is concerned with developing detection method for avian infectious laryngotracheitis (ILT) virus in Iraq using real-time PCR assay. Total of 64 clinically suspected birds believed to be infected with ILTV , were chosen from 6 diverse commercial poultry farms in several areas of North Baghdad. The infected birds were examined and the tissue samples collected from the lungs, spleens, tracheas of each farm. The positive collected samples were used for DNA extraction using High Pure Viral Nucleic Acid Kit (Patho Gene-spin DNA/RNA Extraction Kit) according to the manufacturer's instructions. The extracted DNA was immediately assayed via RT-PCR.

Tissues were presented to a real-time PCR assay for detection of ILTV. The test is based on a fast and sensitive real-time PCR method with the use of TaqMan probes. The primers were designed to detect the conserved region of G pathogenic proteins of ILTV. Infectious laryngotracheitis virus is detected in forty-two of forty-six clinically suspected birds within less than 2hours.

Keyword: Laryngotracheitis, real-time PCR.

Introduction

Viral infection in chicken by Infectious Laryngotracheitis (ILT) is a significant respiratory tract disease. It was first depicted in 1925 and it has been portrayed in numerous nations in which stays as a genuine ailment predominantly in zones of severe creation and vast centralizations of chicken, for example, North America, South America, Europe, China, Southeast Asia and Australia. ILT infection can be transmitted through (a) chickens with intense upper respiratory tract ailment inactively tainted "transporter" fowls' fomites and debased people.

These chances of ILT diseases are expansive populaces of gullible, unvaccinated flying creatures i.e. in concentrated territories of the grill. Chicken rushes which are endemically tainted with ILT infection happen just in a few locales of nations or even specifically numerous age generation farms. It produces extreme creation of misfortunes because of mortality of tainted grills, pullets and grown-up winged creatures or potentially diminished weight gain and egg production.[11] Sporadic instances of ILT happen in all classes of winged creatures including pastime/appear/amusement chickens, ovens, overwhelming reproducers and business laying hens. Rapid detection methods such as Realtime PCR assays are required in the field for fast pathogen detections.[2] So, we aimed to create this assay to help in accurate and rapid detection for the viral diseases.

Material and Methods

Collection of samples: A total number of 64 clinically suspected birds believed to be infected with ILTV, were chosen from 6 different commercial poultry farms in various areas of North Baghdad, brought to "Uruk Lab for the molecular and serological test" by the owner, in Middle of Baghdad. The infected birds were examined and the tissue samples collected from the lungs, spleens, tracheas of each farm were removed and transferred into sterile tubes separately. The samples were kept at -20°C for later analysis. Clinically, the signs showed difficulty in breathing with the extension of the neck and gasping in an attempt to inhale, reduced egg production.

There are more symptoms like coughing, gurgling and rattling. Conjunctivitis was also reported. Clots of blood may be coughed up and can be found on the floor and walls of the house. Most of the flocks showed respiratory signs of ILTV, Post-mortem changes are confined to the upper respiratory tract (URT) and are also characteristic consisting of hemorrhagic tracheitis associated by blood clots, mucoid rhinitis, as well as bloody mucus covering all the length of the trachea (fig. 1).

Trachea samples from infected birds taken from each farm were separately homogenized using a sterilized pestle and mortared in 5 ml tryptose phosphate broth solution containing penicillin (2000 IU/ml), gentamicin (50 µl/ml) and mycostatin (1000 units/ml). The suspension was incubated at 25°C for 30 minutes and centrifuged at 13000 rpm for 1 minute.

Viral DNA preparation and one step RT-PCR assay: The ILTV DNA was extracted directly from infected tissue using a viral DNA purification kit. The primers for RT-PCR were designed by aligning multiple sequences of ILTV G pathogenic genes using the ApE software (http://biologylabs.utah.edu/jorgensen/ wayned/ape/).

The conserved regions in the G genes among the ILTV isolates were selected for primer design. The primers were synthesized by Biocorp company, Canada, five µl of DNA were added in 25 µl of the final volume, the primers and hydrolysis probe used were:

(G g+ve- FAM Prob 5-TGAGCGTTCAGTAACATAGGAT CGA-3BHQ-1).
g G+ Forward 5'-CAGCTCGAAGTCTGAAGAGACA-3'
gG+ Reverse 5'AGCGAGCATACTAGGGAAACGGT-3'.

Thermal cycling reaction was performed using Smartcycler thermal cycler. Optimization of PCR reaction was accomplished for adopting which temperature yielded the desired band product. The PCR reaction was carried out as ILTV wild (FAM)/common(cy5) protocol shown in the table 2.

Results and Discussion

Results of the samples collected from 6 poultry flocks (64 sample) showed positive results in 42 samples and the other 22 samples failed to show any positive results as in fig. 2.

In this study, we presented a real-time PCR assay for detection of ILTV. The test is based on a fast and sensitive real-time PCR method with the use of TaqMan probes. The primers were designed to detect the conserved region of G pathogenic proteins of ILTV. Infectious laryngotracheitis virus is detected and the primers and the probe also were designed based on the conserved fragment coding pathogenic protein (G gene). These genes of the virus show the highest level of conservation and therefore the test is adaptable for detection of ILTV as in fig. 3.

According to the fact that few reports are available on the existence of the ILTV in Iraqi poultry population. It was obvious in this study that the virus was detected in all strains/breeds revealing that all breeds have an equal susceptibility to ILTV infection. Furthermore, adults and young chicks showed severe respiratory disorders. This not agreed with the general assumption that most clinical signs are related only of adult birds.[11] The virulence of Iraqi strain circulating in poultry might have virulence for all ages. This study has confirmed remarkable variation among ILTV strains regarding their capacity, tropism to induce clinical signs, mortality and lesions in various tissues. Also, ILTV can keep its infectivity out of the host for many days under breeding conditions of farms and the presence of organic substances like faces helps in that.

PCR detection of ILTV gives the link of the clinical signs and pathology of ILT found in infected birds. Therefore, the PCR technique would be a beneficial tool to get evidence of the presence of ILTV. For the clinical samples it has been found that PCR is more sensitive than virus isolation, especially in the presence of contaminant viruses like adenoviruses.[1]

Evidence was mentioned that most field outbreaks are associated with chicken-embryo-origin vaccine strains. For this reason, broiler outbreaks called "vaccinal laryngotracheitis" (VLT).[7] Different previous studies explained that various ILTV strains circulating in the fields are genetically related to the vaccine strains In USA and Australia. Some field isolates were closely related to vaccine strains.

Real-Time PCR method is a very fast method of analysis (less than 2 hours) with very high sensitivity. The versatility and sensitivity of the test make it suitable for wide use in veterinary laboratories and fast detection of viruses in wild and domestic birds.

These result are in agreement with Oldoni and Garcia who showed that the genes commonly examined by glycoprotein G (gG) and contract with Mahmoudian et al showed Real-time PCR assays more sensitive methods described for ILTV and have the benefit that they can be finshed in less than 2 hours and therefore offer a very fast method of ILT diagnosis.

In conclusion, this study successfully developed an RT-PCR for ILTV pathogenic strain detection and differentiation. The study proved that ILTV could be detected directly from clinical samples without the need for virus propagation in the lab. Also, the pathogenic strain of ILTV is commonly examined by glycoprotein G (gG) gene. Furthermore, developing a new technique will contribute significantly to the control and prevention of the spread of the disease.

Figure 1: Post-mortem changes in URT consisting of hemorrhagic tracheitis with blood clots.

Table 1
Master mix amplification reaction Component, Concentration and volume

S.N.	Components	Volume
1	Gotaq RT- qPCR Master Mix (2X) (Promega USA)	10 µl
2	G g+veForward Primer (10 pmol)	1 µl
3	G g+veReverse Primer (10 pmol)	1 µl
4	G g+ve- FAM Prob (10 pmol)	0.5 µl
6	Water	7.5 µl
7	Template DNA	5 µl
Total volume reaction		25 µl

Table 2
PCR program and cycling protocol

S.N.	Step	Temperature	Duration	Cycle
1	Early denaturation	95 ºC	600 sec	1
2	Denaturation	95ºC	30 sec	40
3	Annealing/emission detection	60ºC	30sec	
4	Extension	72ºC	20sec	

Assay Information

Assay Name	Assay Version	Lot Number	Expiration Date	Assay Status	Assay Type
ILTV RT-PCR wild type	NA			Valid	Research

* indicates that a particular field is entered using a barcode scanner

Sample Results

Site ID	Sample ID	Assay Result	Warning/ Error Code	Sample Type	Notes
A14	T1	Positive		SPEC	
A15	T2	Negative		SPEC	
A16	T3	Positive		SPEC	

Number of Sites: 3

Wild

Site ID	Sample ID	Channel Result	Ct	EndPt
A14	T1	POS	39.1	20
A15	T2	NEG	0	-1
A16	T3	POS	33.5	33

Figure 2: Positive results (A=sample position, T=Trachea, POS=Positive, NEG=negative). FAM= (6-carboxy fluorescent)- probe pigments.

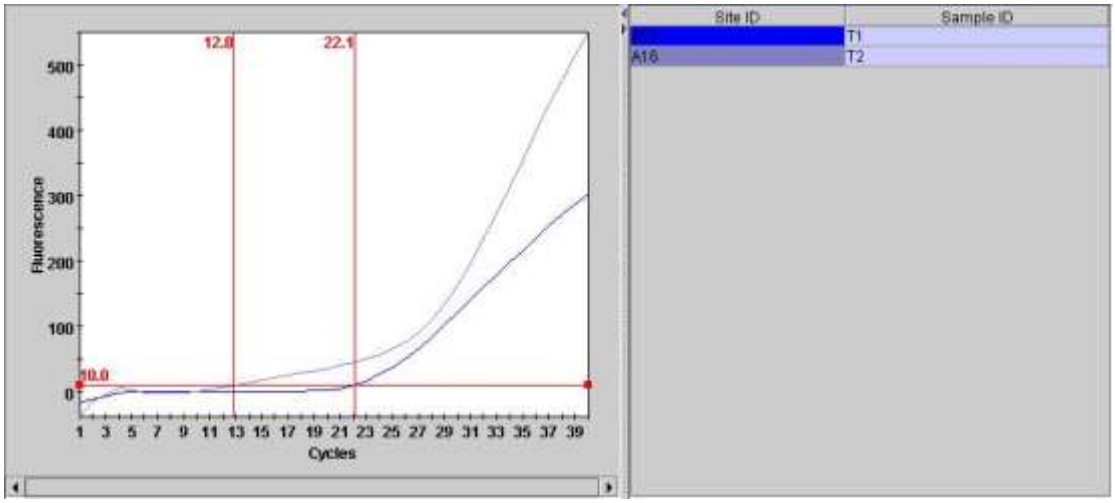

Figure 3: G pathogenic gene fragment containing the site amplified by G-gene primer which shows positive results of ILTV pathogenic strain.

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