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Isolation and sequencing of field isolates of infectious laryngeotracheitis virus in Iraq

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Abstract
This study concerned with isolation and diagnosis of avian infectious laryngotracheitis (ILT) virus in Iraq by different diagnostic methods. Fifty laryngeal and tracheal samples were collected from Al-Taji, Diala, Al-Sowara, Al-Mahmodia, Al-Kut-Azizah, Al-Diwania, Holy-Karbala, Al-Hilla, and Al-Najaf from January-August/2016 for isolation of ILT virus. Infectious laryngotracheitis (ILT) virus was isolated from three samples out of fifty samples. The positive samples were collected from Al-kut, Diala and Diwania. Chicken embryo fibroblast (CEF) cell culture was used for isolation of ILT virus. Cytopathic effects of isolated virus on CEF were rounded of cells with foci of degenerated cells and formation of giant cells. The titer of isolated virus was $10^{3.16}$ TCID 50/50*ml* on third passage, the titer of virus was increased on fourth passage to $10^{4.7}$TCID 50/50*ml*. Pock lesions were detected on Chorio-allantoic membrane of chicken embryos after five days post inoculation with isolated virus at 12 days. Sequence analysis of one wild isolate revealed that 99% nucleic acid identity over 266 bp of glycoprotein H gene (265/266bp) to different data bases entries. Based on sequenced region identical relation to field strains GHV1 strain 81658 as well as to vaccine strains GHV-1 vaccine laryngeo Vac has been identified.

Keywords: PCR, Sequencing, Avian infectious laryngotracheitis

1. Introduction
Avian infectious laryngotracheitis (ILT) is an acute contagious disease of chickens that affect upper-respiratory tract, ILT was detected in the USA for the first time in 1925 [1]. The clinical manifestation of the disease are nasal discharge, conjunctivitis, gasping, coughing, reduced egg production, and expectoration of bloody mucus with marked dyspnea that may cause suffocation [2]. ILT is responsible for worldwide economic losses in poultry industries [2].
Infectious laryngotracheitis (ILT) is belong to the family of *Herpesviridae*, the genus of *Iltovirus* and species of *Gallid herpesvirus 1* [3]. ILT virus is icosahedral viral particles, the nucleocapsid of ILT virus is about 80-100 nm in diameter; the nucleocapsid has icosahedral symmetry and are composed of 162 elongated hollow capsomeres [4]. The complete virus particle has irregular envelope surrounding the nucleocapsid has a diameter of 195-250 nm; the envelope contains fine projections representing viral glycoprotein spikes on its surface, the genome of ILT virus is linear and approximated150 kb of double-stranded DNA [5]. It contain long and short unique regions (UL, US) and two inverted repeat sequences (internal repeat; terminal repeat); An assembled complete genome sequence of ILTV from different strains contains 148 665 base pairs, and a G + C content of 48.16% [6]. It was anticipated that the genome had 77 open reading frames (ORFs), and 63 of these were homologous to herpes simplex virus-1genes [7].
Gross lesions which were noticed in the larynx and trachea in the severe form, inflammation and necrosis of the mucosa of the respiratory tract with hemorrhage [4]. The main characteristic feature is intranuclear inclusion bodies in epithelial cells; the presence of inclusion bodies are for a few days at the early stage of infection before death of epithelial cells, epithelial cells also form multinucleated cells (syncytia). When the necrotic epithelial cells detached from the trachea, bloody mucus is observe [8]. In USA and Australia many vaccine strains [9-11] and virulent strains [12-13] were sequenced completely [14], also in China many vaccine strains and isolated virulent strains were sequenced completely [15].
The present study was aimed to investigate the presence of avian infectious laryngotracheitis virus in Iraq and the virus was isolated and detected by using several conventional methods and new molecular techniques and the field isolate was sequenced for the first time in Iraq in compare with vaccine strain of ILT virus.

2. Materials and Methods

2.1. Materials

2.1.1 Collection of Samples

Fifty (50) tracheas and larynxes were collected from chickens with respiratory tract infection after post mortem examination; these samples were collected from January-August/2016 from different areas in Iraq as following:

- Al-Taji broiler chicken farm, at Al-Taji province (10 Tracheas and larynxes specimens).
- Diala broiler breeder chicken farm, at Diala (6 Tracheas and larynxes specimens).
- Sowara brown table egg layer chicken farm, at Al-Sowara (7 Tracheas and larynxes specimens).
- Al-Mahmodia broiler chicken farm, at Al-Mahmodia (5 Tracheas and larynxes specimens).
- Azizah broiler breeder chicken farm, at Al-Kut-Azizah (2 Tracheas and larynxes specimens).
- Al-Diwania brown table egg layer farm, at Al-Diwania (3 Tracheas and larynxes specimens).
- Karbala brown table egg layer farm, at Holy-Karbala (10 Tracheas and larynxes specimens).
- Al-Najaf broiler chicken farm, at Al-Najaf (9 Tracheas and larynxes specimens, 5 serum samples).

2.1.2 Chicken embryos (10 days old)

Chicken embryos were supplied by local Iraqi farm from Abu Ghraib, used for tissue culture preparation and chicken embryos inoculation.

2.1.3 Stains

Methylene blue stain was used, 1% solution prepared in 10% formalin used for staining and fixation of cell culture [16].

2.1.4 DNA Extraction kit (QIAamp®):

The QIAamp extraction kit was used for DNA extraction of ILT virus (isolated virus) as instruction of manufacturer Company [17].

2.1.5 DNA amplification kit (Genekam®):

ILT Real time PCR kit (Genekam®): This ready to use amplification kit has been manufactured by Genekam biotechnology AG, Germany, to detect ILT virus in real time PCR. The kit need DNA which can be isolated from nasal swab, respiratory swab, cell culture, vaccine, blood, lung tissue and other tissue and any body fluid [18].

2.2 Methods

2.2.1 Preparation of samples

One gram of tissue was ground into a mortar with adding phosphate buffer saline (1:10), then a 10 mg of streptomycin and 10000 I.U of crystal penicillin were added to the suspension.

2.2.2 Chicken embryo fibroblast cell culture

These cells were prepared according to [19-20]; this cell culture was used for isolation of ILT virus.

2.2.3 Titration of isolated viruses

The virus was titrated on CEF cell culture according to [21]; the titer of virus was calculated according to [22].

2.2.4 Staining of infected cell culture

After fixation of infected cell culture with buffered 10% formalin for 24 hrs. Infected cell culture was stained with 1% Methylene blue for 4 hrs. Then washed with tap water and examined by inverted microscope [16].

2.2.5 Inoculation of Chorio-Allantoic membranes with isolated virus

The isolated virus was inoculated on Chorio-allantoic membranes (CAM) of chicken embryos at 12 days old according to [23] and control chicken were inoculated with PBS by artificial air-sac method.

2.2.6 Extraction of DNA from isolated virus

DNA was extracted according to instructions of kit [17].

2.2.7 Amplification of isolated virus

Amplification of extracted viral DNA was done by Real Time PCR the primers and probe sequence of ILT can be explained as the following [18]:

Forward primer: 5'-CAGATCTGGCATCGCCTCAT-3'
Reverse primer: 5'-CCTGGGAACAGAACCTGAACT-3 '
Probe: 5' FAM-CTAACCCGTTCGCCGCACTCG-BHQ-3'

2.2.8 Sequencing of isolated virus

The field isolate, (isolated virus) at the virology laboratory at college of veterinary medicine/University of Baghdad; was sent by URUK Center to University of Veterinary medicine / Vienna, Austria, for sequencing by amplification of H gene for the first time in Iraq. The sample was taken from Al-Kut farms for layers aged 30 weeks old.

Statistical analysis section is still missing.

3. Results

3.1 Results of virus isolation on chicken embryo fibroblast cell culture

Three samples out of fifty tracheal and laryngeal samples were positive on cell culture, the cytopathic effect (CPE) was not clear on the first passage after inoculation with prepared ILT samples while on the second passage, CPE was noticed after 48hr., Completed CPE was noticed after five days post infection(P.I.). CPE of isolated virus on CEF cell culture was rounded of cells with degeneration of cells as seen in Fig. 3.1, with not CPE was seen in control CEF cell culture as seen in Fig. 3.2. The CPE of isolated virus on the third and fourth passages was noticed after 24hr P.I. with isolated ILT virus and completed after 4 days. CPE of isolated virus on CEF cell culture was rounded of cells, foci of degenerated cells with giant cells formation as seen in Fig.3.3, 3.5, with not CPE was seen in control CEF cell culture as seen in Fig. 3.4, and 3.6.

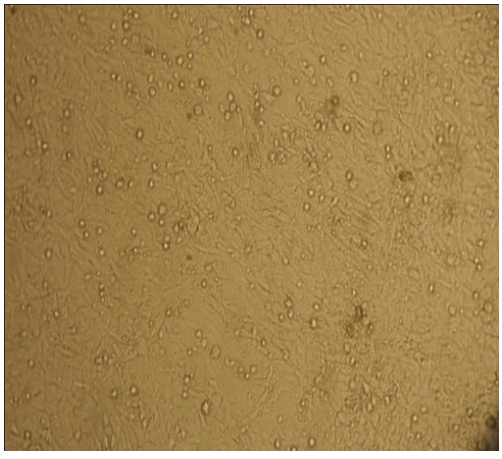

Fig 3.1: CPE of isolated virus on chicken embryo fibroblast cell culture after 5 days post infection with isolated ILT virus (2nd passage). (100X)

Fig 3.2: None infected chicken embryo fibroblast cell culture (control cell culture). (100X)

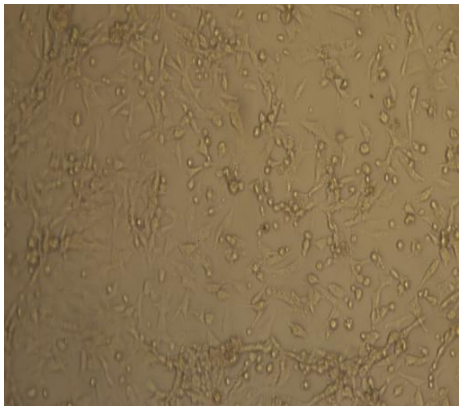

Fig 3.3: CPE of Isolated virus on chicken embryo fibroblast cell culture after 5 days post infection with isolated ILT virus (3rd passage). (100X)

Fig 3.4: none infected chicken embryo fibroblast cell culture (control cell culture). (100X)

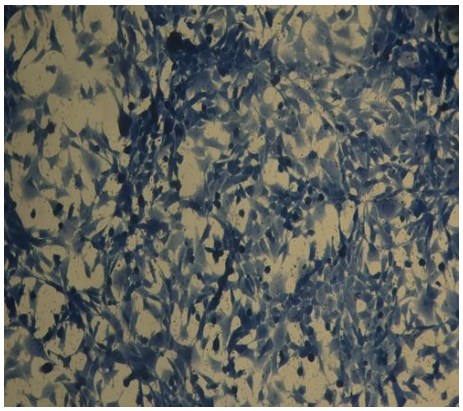

Fig 3.5: CPE of isolated virus on chicken embryo fibroblast cell culture after 5 days post infection with isolated ILT virus (2nd passage).(100X)

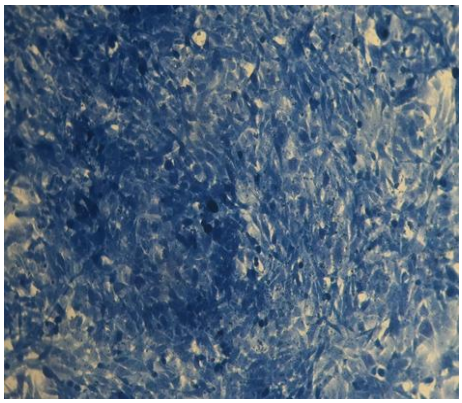

Fig 3.6: None infected chicken embryo fibroblast cell culture (control cell culture). (100X)

3.2 Titer of isolated virus

The titer of isolated virus was $10^{3.16}$ TCID 50/50*ml*, the titer of virus was increased on fourth passage to $10^{4.7}$ TCID 50/50*ml*.

3.3 Results of inoculation of isolated virus on Chorio-allantoic membrane

The isolated ILT virus produced pock lesions on CAM after 5 days of inoculation with isolated virus as seen in Fig. 3.7 in compare with control CAM which inoculated with phosphate buffer saline as seen in Fig. 3.8:

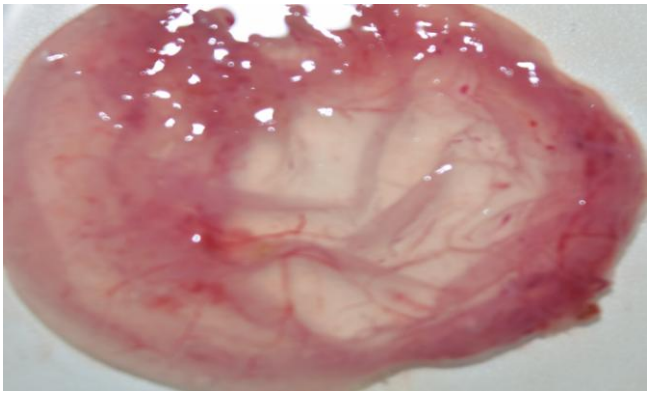

Fig 3.7: Infected CAM membrane with isolated ILT virus revealed congestion and hemorrhage with pock lesions on infected membrane.

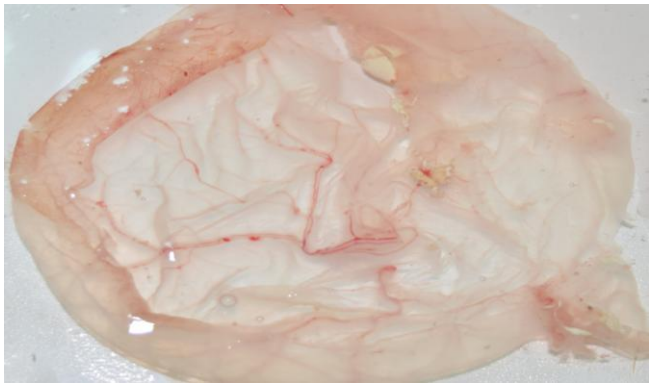

Fig 3.8: None infected CAM inoculated with PBS revealed normal membrane without any lesions.

3.4 Results of Real-time PCR technique

Real-time PCR technique showed three samples positive out of fifty samples (larynx and trachea) before isolation into cell culture Fig.3.9, 3.10, and then these samples were positive after isolation into CEF cell culture by using Real-time PCR technique as seen in Fig.3.11:

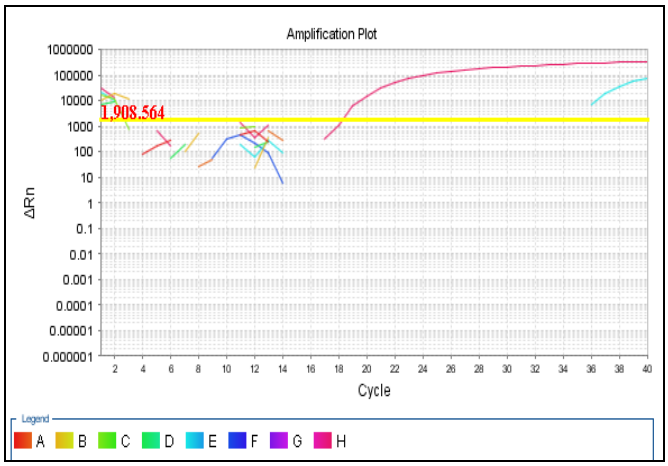

Fig 3.9: Real - Time PCR amplification plot of M gene of ILT virus of one laryngeal and tracheal sample indicate positive result with FAM labeled probe. ( ) Positive control curves and ( ) Positive sample curves.

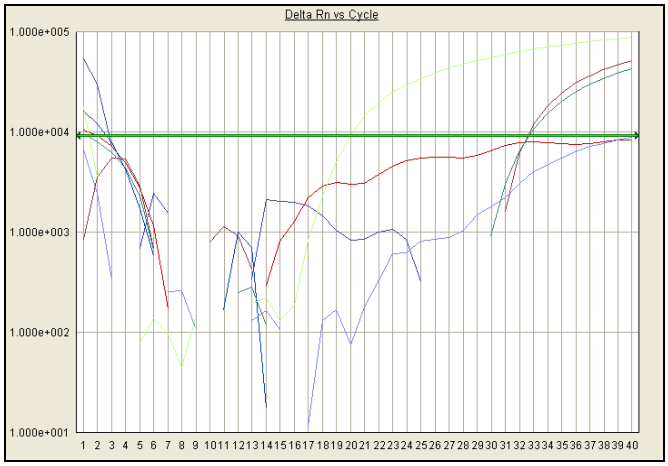

Fig 3.10: Real - Time PCR amplification plot of M gene of ILT virus of two laryngeals and tracheal sample indicate positive result with FAM labeled probe. ( ) Positive control curve, ( ) Positive sample curve and ( ) Positive sample curve.

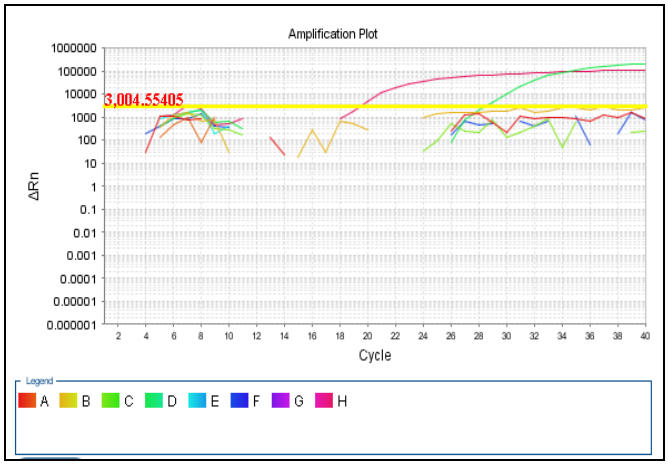

Fig 3.11: Real - Time PCR amplification plot of M gene of ILT virus of isolated ILT virus on CEF cell culture indicates positive result with FAM labeled probe. ( ) Positive control curve and ( ) Positive sample curve.

3.5 Results of sequencing

Sequence analysis of one wild isolate revealed that 99% nucleic acid identity over 266 bp of glycoprotein H gene (265/266bp) to different data bases entries. Based on the sequenced region showed identical relation to field strains GHV1 strain 81658, Accession number JN542535 as well as to vaccine strains, Accession number: JQ083494 GHV-1 vaccine laryngeo Vac has been identified.

4. Discussion

Avian Infectious laryngotracheitis virus is a Herpesvirus which causes acute respiratory infection in poultry [24]. This study concerned with isolation of field isolates of ILT virus and sequencing of isolated virus in compare with vaccinal ILT strain for the first time in Iraq by using one gene.

Chicken embryo fibroblast cell culture was support growth of ILT virus therefore it is very suitable for primary isolation ILT virus in agreed with [25]. The main cytopathic effect of virus growth on CEF cell culture was rounding of cells, foci of degenerated cells with giant cell formation in agreement with [25], other researches explained that primary chicken embryo liver cell culture [26] and primary chicken embryo kidney cell culture [27] were very suitable for isolation of ILT virus but primary chicken embryo liver cell culture was the most sensitive in compare with other cell culture [28].

The isolated ILT virus produced pock lesions on chicken embryos when inoculated by chorioallantoic method after 5 days of inoculation in agreement with [29]. Real-time PCR technique showed high sensitivity to detect ILT virus before isolation of the virus into cell culture and after isolation into CEF cell culture by amplification of matrix gene in agreement with [30-31] which described this technique and showed that Real-time PCR technique have the advantage that they can be performed in less than two hours and consequently provide a fast method for detection of ILT in compare with conventional virus isolation, or conventional Polymerase chain reaction followed by gel electrophoresis.

Results of virus sequencing indicate that trading of infected birds or introducing of live attenuated vaccine that capable to cause latency in vaccinated birds with shedding of reactivated virus periodically [32], and also bird to bird passage vaccine strain can result in reversion of attenuated strains to virulent strains [33. 34], all these factors responsible for evolution of virulent field isolates in Iraq.

5. Conclusion The result of sequencing of isolated ILT virus, the trading of infected birds and using of live attenuated vaccine result in evolution of ILT infections in Iraq. Chicken embryo fibroblast tissue culture is very suitable for ILT virus Isolation.

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7. References

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