

Molecular Diagnosis of Explosive Outbreak of Infectious Laryngotracheitis(ILT) by Polymerase Chain Reaction in Palestine

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Summary

This study was conducted during an explosive outbreak of infectious laryngotracheitis (ILT) in layers flocks of Hyline breed with a total number 120.000 housed in cages system in Bal'a, East of Tulkarem and North Palestine. The clinical findings of the disease were gasping, coughing, gurgling, marked dyspnea and expectoration of vigorously in an blood stained mucous obstructing the trachea or larynx . Some layers showed existence of dried blood around the nostrils and lower beaks, closed eye or eyes, lacrimation and the egg production was decreased 30%. The morbidity rate was high, mortality rate reached 12%. The necropsy findings of dead birds showed mucoied tracheitis, laryngitis, severe hemorrhages in the trachea and the lumens were filled with mucus mixed with blood, exudates, caseous materials and existence of blood casts along the entire length of the larynx and trachea. The disease was diagnosed by isolation of the causative agent from the dead and sick birds trachea suspension onto the chorioallantoic membrane (CAM) of 10-12 day- old embryonated chicken eggs and identified by neutralization test using reference antiserum ,This study reports the application of PCR in detection of ILTV Thymidine kinase gene(TK gene) using Larynx and tracheal specimen from infected birds.

التشخيص الجزيئي لتفشي السريع التهاب الحنجرة و الرغامى المعدية (ILT) باستخدام تفاعل البلمرة المتسلسل في فلسطين

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الخلاصة

أجريت هذه الدراسة خلال التفشي الواسع لمرض التهاب الحنجرة والرغامى المعدية في قطعان من الدجاج البياض من سلالة الهاي لاين والذي يبلغ مجمل عدده 120,000 والمربي في نظام الأقفاس في منطقة بلعا شرق مدينة طولكرم, شمال فلسطين . كانت العلامات السريرية للمرض اللهثة والسعال والغرغرة وضيق التنفس وتتخ مخاط ملون بالدم بقوة والذي تسبب بانسداد القصبة الهوائية والحنجرة, أظهرت بعض الدجاجات البياضة وجود دم جاف حول فتحات الأنف والمنقار السفلي وانغلاق الأجنان و التدمع و هبوط في إنتاج البيض بنسبة تصل 30% . كانت معدلات الإصابة عالية ووصل معدل النفوق 12% , كما أظهرت نتائج التشريح المرضي للطيور النافقة وجود التهاب مخاطي داخل الحنجرة والقصبة الهوائية وحدوث نزف شديد من القصبة الهوائية بحيث امتلئ تجويف القصبة بالمخاط المدمم والقيح ومواد متجبنة على كامل طول الحنجرة والقصبة الهوائية. تم تشخيص المرض عن طريق عزل العامل المسبب للمرض من المستحلب المحضر من القصبات الهوائية للطيور المريضة والنافقة على الغشاء المشيمي اللقائقي لأجنة الدجاج بعمر 10-12 يوم وتم تحديده بإجراء اختبار التعادل المصلي بالاستعانة بمصل مضاد مرجعي . هذه الدراسة تسجل استخدام تقنية تفاعل البلمرة المتسلسل في الكشف عن جين التايميدين كاينيز(TK)لفيروس التهاب الحنجرة والرغامى المعدية لعينات من الحنجرة والقصبة الهوائية للطيور المصابة.

Introduction

laryngotracheitis is a viral respiratory tract infection of chickens that may result in severe production losses due to mortality and/or decrease egg production(1). Clinically,the disease may appear in three forms, namely peracute , subacute , and chronic or mild. In the preacute form, onset of disease is sudden with a rapid spread. The morbidity is high and mortality may exceed 50%. Some birds may die in good body condition before the appearance of signs

which are characteristic and comprise difficulty in breathing with extension of the neck and gasping in an attempt to inhale. There is gurgling, rattling and coughing when birds try to expel obstructions in the trachea. Clots of blood may be coughed up and can be found on the floor and walls of the house. In the subacute form, the onset of illness is slower and respiratory signs may extend over some days before death are seen. The morbidity is high but the mortality is lower than in preacute form between 10% and 30%. Chronic or mild ILT may be seen among survivors of either of the above forms of the disease(2). laryngotracheitis virus is classified as a member of Genus Iltovirus within the family herpesviridae, subfamily alphaherpesvirinae, 195nm to 250nm in diameter, with a DNA buoyant density of 1.704g/ml.

The virus is taxonomically Identified as Gallid herpesvirus-1. AH strains have been demonstrated to be antigenically homogenous (3). Laboratory diagnosis depends on isolation of the virus, demonstration of the presence of the virus or viral antigens, and detection of specific antibodies in the serum. Histopathological examination of trachea for characteristic intranuclear inclusions may be of value (2). In Palestine infectious laryngotracheitis (ILT) was reported in three flocks Hyline breed of layers with a total number 120.000 housed in cages system in Bal'a , the disease was diagnosed by isolation of the causative agent from the dead and sick birds trachea suspension onto the chorioallantoic membrane (CAM) of 10-12 day old embryonated chicken eggs and identified by neutralization test using reference anti-ILT serum (4). This study deals with outbreak of ILTV in layer flocks where clinical, virological and molecular studies were conducted.

Materials and Methods

An outbreak of laryngotracheitis in layers flock was investigated. The disease appeared in April 2010 in commercial Hyline breed layers flock with a total number 120.000 housed in cages system in Bal'a, Tulkarem Governorate, and North Palestine. Complete clinical examination was performed on the layers flock, Dead birds and some sick were subjected to thorough Post-mortem examination. Larynx and parts of tracheal tissues and blood stained tracheal exudates were used for virus isolation and parts of larynx and trachea for PCR application.

Larynx, tracheal tissues and exudates were collected and processed for isolation of the virus in accordance (5), 100 I.U / ml penicillin and 100mg/ml streptomycin were added to the suspension of suspected specimens and inoculated onto CAMs of 10-12 day-old embryonated chicken eggs. The inoculated eggs were re-incubated at 37.8c and candled daily, dead embryos within first 24 hours after inoculation were discarded while other eggs CAMs examined for the presence of pocks lesion until the six days post incubation.

The isolated virus was titrated to determine EID₅₀ according to (6) where tenfold dilutions of the daily harvested virus was done for each dilution, five eggs were inoculated on the dropped CAM with 0.1ml of each dilution .

Specific ILT hyper immune serum supplied by Jordan – Bio industries center (JOVAC) was used in the neutralization test onto dropped CAMs of 10-12 day_ old embryonated chicken eggs, Doubling dilution of serum was added to equal volume of a constant concentration of the virus which equal 100 embryo infective doses (EID₅₀), the mixture was incubated at 37c for one hour to allow neutralization to occur before inoculation (7).

Total DNA was extracted from larynx and parts of tracheal tissues by using Intron biotechnology viral gene-spine DNA/RNA extraction Kit. Nested PCR was performed by using the Intron biotechnology PCR master mix kit according to the protocol described by the manufacturer. First round amplification PCR for ILTV detection parameters were 94°C for 5min, follow by 40 cycles of denaturation at 94°C for 30sec, annealing at 52°C for 30sec, and extension at 72°C for 40sec followed by an extension cycle at 72°C for 5min. The pair of primers in Table.1 were designed according to(8-9). These primers targeted the TK gene of ILTV and produced a 1024-bp fragment.

Table 1. The PCR primers sequence used in the present study.

Primers	Sequence
ILT-TKOP gene External primer	F = 5-CGGGATCCATCGTATAGGCCAGCCTT-3 R = 5-GCTCTAGACCACGCTCTCTCGAGTAA
ILT-TKPD2 gene Internal primer	F= 5-GTTCGAGAACGATGACTCC-3 R= 5- GCATTGTAGCGCTCTACTG-3

Results

Clinical signs: The disease occurred suddenly in a form of explosive outbreak in layers flock with marked decrease in egg production up to 30% and increase in number of dead birds. Sick birds showed respiratory distress, gasping, coughing, some birds extend their necks and prolonged inspiration through a wide open beak gurgling and hens shook their heads vigorously in an attempt to dislodge blood stained exudates obstructing the trachea. Some birds have a bloody beak or existence of blood on the face, head and feather, as seen in (Fig.1) .Some birds characterized emaciation closed eye or eyes. Lacrimation conjunctivitis and conjunctival edema as seen in (Fig.2). Morbidity rate was 90% and mortality 12%.



Fig.1 Infected layer shows a bloody beak associated with ILTV.



Fig.2 Infected layer with conjunctivitis and conjunctival edema.

Necropsy findings

Mucoied tracheitis , laryngitis , severe hemorrhages in the trachea and the lumens were filled with mucus mixed with blood , exudates , caseous materials and existence of blood casts along the entire length of the larynx and trachea .

Virus isolation

The virus was isolated from the larynx and tracheal suspension collected from the layers flock 10-12 day old chick embryonated eggs after incubation period 3-6 days ,The CAMS

showed generalized edema and the development lesion which varied from scattered pocks to large one with diameter 4-5mm (Fig.3).

Neutralization test

The results of this test showed absence of pocks from the reacted isolated virus with hyperimmune serum while untreated virus with hyperimmune serum developed pocks and edema onto the CAMs.



Fig.3 The CAMS showed generalized edema and pock lesions.

Virus titration

The titer of isolated ILT virus from the trachea after two passages on CAMs was 105.6 EID50 per/ml tracheal or CAMS suspensions.

Polymerase Chain Reaction (PCR)

As shown in Figure 4, the expected fragment of 1024bp of the TK gene of ILTV was obtained in two samples, as well as in the reference vaccine strain (Biovac) used as positive control; no bands were detected in the negative control (DNA\ RNA Nuclease-free water)

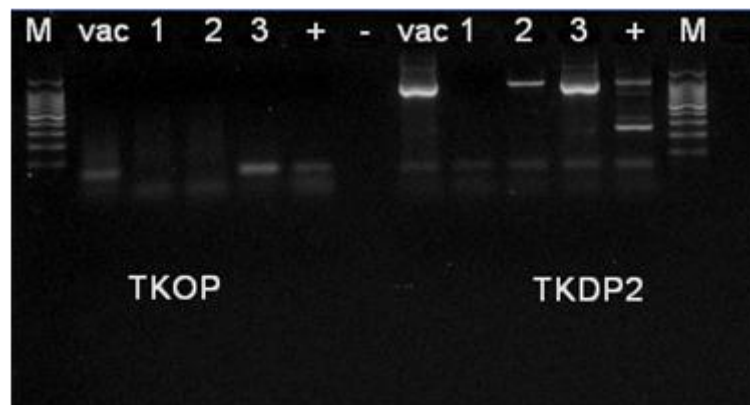


Fig .4. Visualization of the 1024-bp PCR product from the TK gene of ILTV by agarose gel electrophoresis (1.5%) . M=100-bp DNA ladder; 1-3 = Virus strains isolated in CAM , + = Positive control (ILTV reference vaccine strain-Biovac); - = Negative control (DNA\ RNA Nucleasefree water).

Discussion

Infectious Laryngotracheitis ILT is a respiratory disease of chickens , pheasant and peafowl caused by gallid herpesvirus-1 , result in severe production losses due to mortality and/or decreased egg production, severe epizootic forms of infection are characterized by signs of respiratory depression , gasping , expectoration of bloody mucus and high mortality (1). Mild enzootic form of infection are encountered increasingly in developed poultry industries and manifest variously as muroid tracheitis , sinusitis , conjunctivitis , general unthriftiness and low mortality (9,10).This study documents the explosive outbreak of infectious laryngotracheitis in Palestine in layer flocks in Bal'a East of Tulkarem Governorate

which was confirmed by PCR . The encountered clinical and postmortem findings are characteristic and similar to those previously reported by others (11,12,13,14,15)and characterized by respiratory distress, dyspnea , gurgling and decrease in the egg production. Some recovered layers and vaccinated ones became carrier and shed virus for long period of time or much later can shed virus following stress induced reactivation of latent infection, thus exposing other susceptible birds. In areas where ILT is enzootic , vaccination of layers is frequently practice and is quite effective in this outbreak ILT virus is introduced in the area via carrier birds and spread to neighboring flocks rapidly due to stress and absence of biosecurity measures(16).Three samples of larynx andtracheal specimen collected from layers were analyzed by PCR and the three showed positive results to ILTV. The positive results in the PCR directed to the TK gene of ILTV,the result were agreed with the characteristic symptoms of laryngotracheitis observed in the surveyed birds, as tracheitis, watery eyes and dyspnea. The PCR described in the present study is a usefulrapid tool to confirm the diagnosis of birds suspected of infectious laryngotracheitis and differentiate it from other respiratory disease which can be confused within less than 24 hours, which is an essential point in outbreaks, when fast decisions are required, considering that PCR applied to viral diagnosis is a highly sensitive technique (17)that allows the detection of infection in a very early phase when compare to serological reactions or viral isolation (18). The final diagnosis of ILT was based on characteristic clinical signs, Isolation of the virus and detection of thymidine kinase gene in larynx and tracheal suspension of sick birds which is indicative for virulent strain.

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