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# Detection of infectious laryngotracheitis virus (Gallid herpesvirus-1) from clinically infected chickens in Egypt by different diagnostic methods

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## Summary

Infectious laryngotracheitis (ILT) disease is an acute highly contagious viral disease leading to massive economic losses to the national poultry industry. This study aimed to identify the most accurate and rapid diagnostic methods to rescue layer poultry farms from intense outbreaks in Egypt. Fifty pathological specimens were collected and subjected to virus isolation (VI), histopathology, direct fluorescent antibody technique (FAT) and polymerase chain reaction (PCR). Egg inoculation revealed stunted growth and white pock lesions on chorioallantoic membranes (CAM) in 23 samples. Isolation and propagation of infectious laryngotracheitis virus (ILTV) in cell culture showed syncytia formation 5 days post infection in 20 inoculated samples. PCR resulted in successful amplification of a 647 bp fragment of the thymidine kinase (TK) gene in 25 field samples. Histopathological examination of inoculated CAM showed intranuclear inclusion bodies with infiltration of inflammatory cells. Direct FAT showed intra-cytoplasmic apple green reactions in 18 examined tracheal tissues. PCR has been shown to be more sensitive, accurate and rapid than VI, FAT and histopathological examination.

**Key words:** FAT, Histopathology, ILTV, PCR, Virus isolation

## Introduction

Respiratory tract infections are significant in poultry industry and the leading causes of morbidity and mortality in poultry farms all over the world (Crespo *et al.*, 2007; Ahmed *et al.*, 2009). Infectious laryngotracheitis (ILT) is an acute, contagious, upper respiratory disease of chicken (Guy and Garcia, 2008) and has been considered as a serious disease with varying degrees of severity in Egypt (Shehata *et al.*, 2013) and worldwide, particularly in areas of intensive chicken production. The disease may result in heavy economic losses as a result of increased mortality, low weight gain and decreased egg production (Loncoman *et al.*, 2017).

The etiological agent is the Gallid herpesvirus-1 (GaHV-1) which is classified under the genus Iltovirus, subfamily Alphaherpes-Virinae and family Herpesviridae (Ou and Giambone, 2012). Virions are icosahedral in shape and about 100 nm-110 nm in diameter with a linear 155-kb double-stranded molecule of DNA genome (Lee *et al.*, 2011). Antigenically, infectious laryngotracheitis virus (ILTV) strains are considered to be homogenous, but some ILTV strains may naturally vary in virulence (Islam *et al.*, 2010). The ILTV has the capacity to establish a latent infection in the central trigeminal ganglia, and virus reactivation could be upgraded by stress factors such as beak trimming or transportation (Kaboudi *et al.*, 2016; Craig

*et al.*, 2017).

In ILTV infections, clinical signs are highly suspicious to the disease. Even so, rapid and accurate diagnostic techniques must be applied to confirm the diagnosis. A post-mortem examination of the diseased chickens will mainly reveal bloody exudate in the tracheal lumen. Virus isolation (VI) and identification can be performed in embryonated chicken eggs (ECE) or tissue culture (TC) from embryo liver or kidney origin (Hidalgo, 2003). The viral strains could be differentiated on the basis of the plaque size, their morphology in TC and the size of the pock lesions produced in the CAM of the inoculated eggs (Kirkpatrick *et al.*, 2006).

A rapid diagnosis can be attained by detection of the viral intranuclear inclusion bodies in the epithelial cells of the tracheal tissue in the acute stage of the disease (OIE, 2014). Results of the microscopic examination can be rapidly confirmed by direct electron microscopic examination of tracheal scrapings (Hidalgo, 2003) or by detection of viral genome using molecular assays (Zhao *et al.*, 2013). Serological tests such as virus neutralization, enzyme linked immunosorbent assay (ELISA), agar gel immunoprecipitation and indirect immunofluorescence assays are successfully performed for the detection of antibodies to ILTV (Koski, 2015).

Molecular techniques, including conventional and real-time polymerase chain reaction (PCR) assays, have been used successfully in the diagnosis of ILTV (Chacón and Ferreira, 2008). Due to the antigenic homogeneity

among ILTV strains, diagnostic assays based on antigenic differences are usually useless and the identification of the ILTV strain that is responsible for the outbreaks is very difficult (Guy and Garcia, 2008). Hence, instead of serological methods, molecular assays such as sequencing of PCR products and restriction fragment length polymorphism (RFLP) are adopted. The RFLP analysis classifies ILTV strains based on genetic markers including glycoprotein G and M, ICP4 and thymidine kinase (TK) genes (Koski, 2015). The differentiation of ILTV strains has been achieved by targeting genomic differences between isolates from natural outbreaks and vaccine strains. Lately, full genome sequences have been applied to identify the origin of ILTV isolates related to the disease outbreaks (Oldoni *et al.*, 2008).

In general, molecular techniques have identified live attenuated vaccine-related isolates as the main cause of the disease outbreaks worldwide (Kimberly *et al.*, 2014). Even in the absence of multiple ILTV serotypes, molecular characterization is still epidemiologically useful to rate the virus circulation in certain regions (Craig *et al.*, 2017). On the other hand, ELISA using monoclonal antibodies to specific glycoproteins could also be valuable in differentiating birds vaccinated with recombinant vaccines from those vaccinated with chicken embryo origin (CEO) or tissue culture origin (TCO) vaccines or infected with field strain viruses (Coppo *et al.*, 2013).

Infectious laryngotracheitis virus receives less attention from veterinarians and researchers in Egypt as the diagnosis of a severe, acute ILTV outbreak can be made based on the high mortality rates along with expectoration of bloody exudate usually in layer chicken flocks, but the milder forms of the disease may simulate respiratory disease caused by other viruses such as avian influenza (AI), Newcastle disease (ND) and infectious bronchitis (IB). Thus, for confirmation of the disease diagnosis, fast and accurate laboratory methods are required (Bagust *et al.*, 2000). Most of the previous studies compared the different ILTV diagnostic tools only on experimentally infected chickens. Herein, we report the diagnosis of field cases of suspected ILTV in chicken flocks by PCR compared with other diagnostic tools such as VI in ECE and chicken embryo fibroblast (CEF) cell culture, FAT and histopathological examination.

## Materials and Methods

### Collection of samples

Pooled specimens were collected from fifty clinically diseased commercial layer chickens flocks located in different provinces in Egypt including Kafrelsheikh, Elbeheira, Elgharbia, Elsharqia, and Elmenofia provinces. The examined chicken flocks were aged between 22-28 weeks and all flocks were not vaccinated against ILTV. Tissue samples included larynges, tracheas and lung tissues. All birds suffered from severe respiratory manifestations including sneezing, dyspnea,

gasping, rattling, conjunctivitis, in addition to severe drop in egg production with high mortality rates. Post mortem examination revealed hemorrhage within the tracheal lumen of the infected birds (Figs. 1a-b).



**Fig. 1:** Postmortem lesion of examined birds. **a:** Trachea of a normal non infected bird, and **b:** Trachea of a bird infected with ILTV shows haemorrhage in the tracheal lumen

### Isolation and propagation of ILTV in ECE

Specimens were pooled and minced to prepare 10% suspensions which were centrifuged at 13,000 rpm for 20 min at 4°C. The collected supernatants were inoculated into the CAM of 13-day-old SPF eggs for up to three blind passages. The eggs were incubated at 37°C for 5-7 days and daily examined for abnormalities.

### DNA extraction

DNA extraction from tissue samples was performed using Gene Jet Genomic DNA Extraction Kit (Fermentas) as per manufacturer's protocol. The purified DNA was stored at -20°C till used.

### PCR amplification of TK gene

The PCR was conducted in 25 µL volumes, in which the reaction mixture consisted of 5 µL of 5X PCR Master Mix (Jena Bioscience, Germany), 1 µL of forward and reverse primers, 5 µL of DNA and 13 µL of PCR grade water. PCR was conducted in an applied Biosystem 2720 thermal cycler using 2 sets of primers which specifically amplify a 647 bp fragment of the TK gene according to (Pang *et al.*, 2002) (Table 1). The PCR cycle condition consisted of initial denaturation at 95°C for 5 min, then 40 cycles of denaturation at 95°C/1 min, annealing at 53°C/1 min and extension at 70°C/1 min with a final extension at 72°C/10 min. For positive control, DNA

extracted from a lyophilized ILTV vaccine was used. The negative control contained only PCR master mix and primers.

### Histopathology

Infected CAM of ECE were fixed in 10% formalin and then dehydrated in an ascending series of ethanol, cleared in xylene and embedded in paraffin wax. 4  $\mu$ m sections were stained with hematoxylin and eosin (HE) stain and microscopically examined.

### Isolation of ILTV in CEF cell culture

After removal of growth medium, 0.2 ml of virus suspensions were inoculated onto CEF monolayers, incubated for one hour at 37°C to allow virus adsorption to cells then fresh maintenance media were added to the cell cultures. The cells were examined daily until pathognomonic cytopathic effects (CPE) were observed.

### Antiserum production

Antiserum to ILTV (local isolate ILTV\_Egypt 2015) (GenBank: KX021881.1) was produced in rabbits. Briefly, 3-month-old female rabbits were inoculated with 0.5 ml of virus suspension subcutaneously at multiple sites. The rabbits were re-immunized weekly for 6 weeks. After 2 weeks from the last immunization, blood was collected by heart puncture under general anesthesia and sera were stored at -20°C till used (Leenaars and Hendriksen, 2005).

### Fluorescein labeling

Fluorescein isothiocyanate (FITC) isomer I was purchased from Sigma-Aldrich and labeled to ILTV antibodies according to protocol devised by (Kanto *et al.*, 2016). Briefly, 10 mg of FITC were dissolved in 1 ml anhydrous Dimethylsulfoxide (DMSO). FITC was mixed with the antibody at a ratio of 40-80  $\mu$ g/mg and

incubated with stirring at room temperature for at least 1 h in dark place. The free FITC was cleared out by dialysis to separate the conjugates. The F/P ratio and protein concentration was recorded by evaluating the absorbance at 280 and 495 nm.

### Direct fluorescent antibody technique (FAT)

Tracheal specimens were fixed to the slide with cold acetone at 20°C overnight. The conjugated antiserum was added and incubated for 1 h, rinsed by PBS and examined under fluorescent microscope.

### Statistics

Sensitivity and specificity were estimated as described by Villarreal (2015).

## Results

### Virus isolation on ECE

Congestion and stunting in growth were evidenced in the inoculated embryos. CAMs of eggs inoculated with the viral suspensions of 15 samples showed white pock lesions on the first egg passage except for 8 samples which showed pock lesion only after three passages (Figs. 2a-c). The remaining inoculated samples were negative even after three passages.

### PCR amplification of TK gene

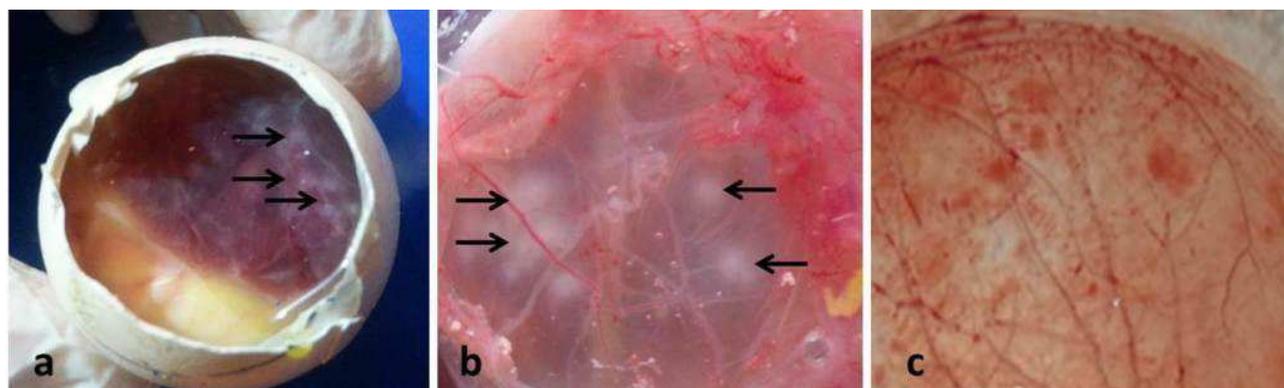
Twenty-five samples were positive by PCR which was represented by clear bands at the expected band size (647 fragment of the TK gene) (Fig. 3).

### Histopathology

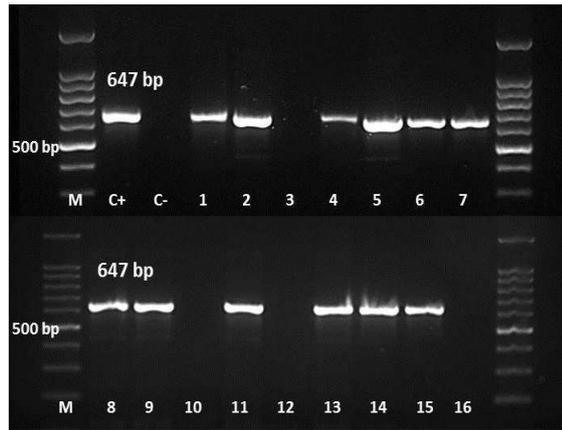
Microscopic examination showed degenerative changes with infiltration of lymphocytes and some macrophages, with intranuclear inclusion bodies in the infected cells of all CAM (Figs. 4a-b).

**Table 1:** Oligonucleotide primers used for amplification of TK gene of suspected ILTV infected field cases according to (Pang *et al.*, 2002)

Primer	Oligonucleotide sequence	Gene	Length of amplified fragment
Forward	5' - ACGATGACTCCGACTTTC -3'	TK gene	647 bp
Reverse	5' - CGTTGGAGGTAGGTGGTA -3'		



**Fig. 2:** Pathological lesions of ILTV infected ECE. **a** and **b**: CAM of ILTV infected embryo represented white pock lesion after 3rd egg passage, and **c**: CAM of non-infected embryo



**Fig. 3:** PCR amplification of 647 bp of the TK gene from ILTV infected field samples. Lane M: 100 bp DNA size marker, Lane C+: Control positive, Lane C-: Negative control. Lanes 1, 2, 4, 5, 6, 7, 8, 9, 11, 13, 14 and 15 showed positive amplification of TK gene

**Isolation and propagation of ILTV in cell culture**

Cytopathic effect of ILTV on CEF cell culture was detected by formation of syncytia (multinucleated cells) at 5th day post infection and detachment of the cells in 9 samples after the 1st cell passage and 11 samples after the 3rd passage (Figs. 5a-b).

**Direct fluorescent antibody technique**

Intracytoplasmic apple green reactions (positive

FAT) could be detected in eighteen of the examined tracheal tissue samples (Figs. 6a-b).

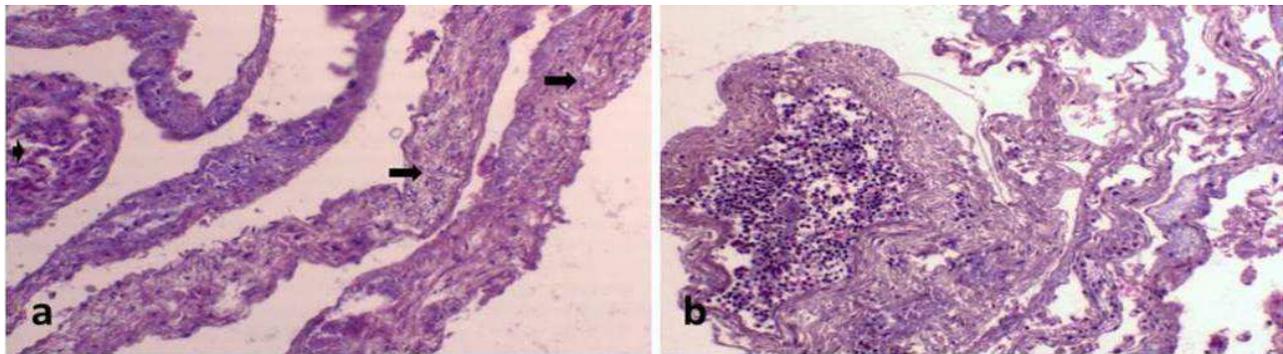
**Statistics**

Sensitivity and specificity were summarized in Table 2.

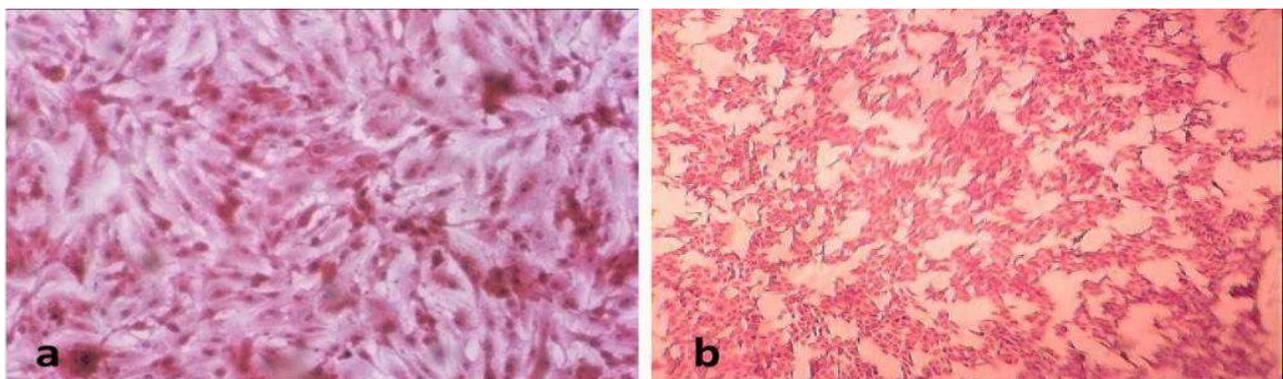
**Table 2:** Sensitivity and specificity of VI, FAT, and histopathology in relation to PCR for the detection of ILTV in field samples

	PCR		Sensitivity	Specificity
	P	N		
<b>VI (ECE)</b>	P 23	25	92%	100%
	N 2	0		
<b>VI (CEF)</b>	P 20	25	80%	100%
	N 5	0		
<b>FAT</b>	P 18	25	72%	100%
	N 7	0		
<b>Histopathology</b>	P 23	25	92%	100%
	N 2	0		

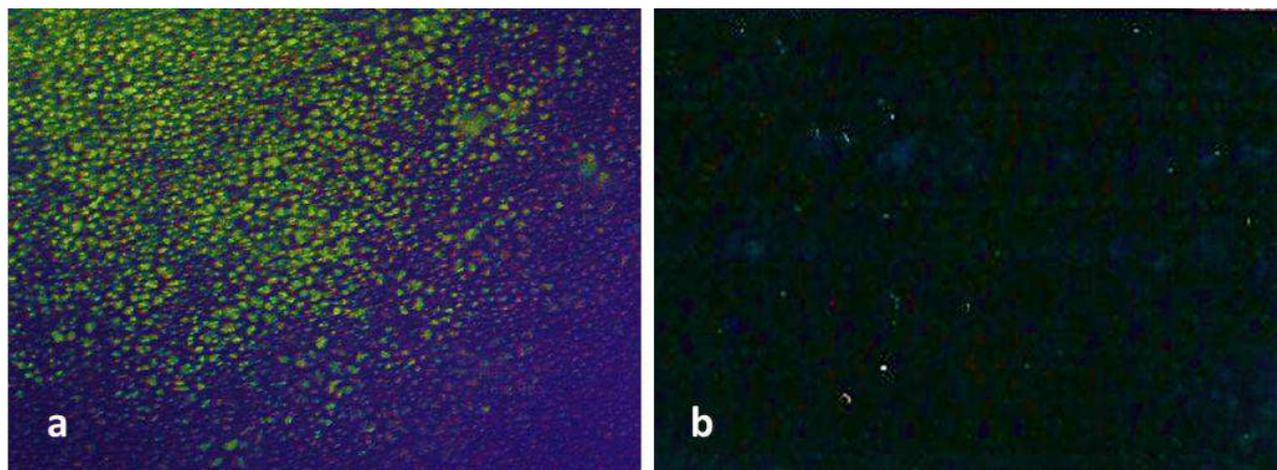
P: Positive, N: Negative, VI (ECE): Virus isolation in embryonated chicken eggs, VI (CEF): Virus isolation in chicken embryo fibroblast cells, PCR: Polymerase chain reaction, and FAT: Fluorescent antibody test



**Fig. 4:** Histopathological examination of CAM of ILTV infected ECE. **a:** CAM of ILTV infected embryo shows presence of intranuclear inclusions (arrows), together with infiltration of lymphocytes (arrow head) (H&E, ×20), and **b:** Massive lymphocytic infiltration in the mesoderm of CAM



**Fig. 5:** chicken embryo fibroblast cell culture inoculated with ILT virus suspected samples. **a:** Normal “CEF” (H&E, ×100), and **b:** Infected CEF with ILT virus showing syncytia formation 5 days post infection and cell detachment (H&E, ×100)



**Fig. 6:** Fluorescent antibody technique on ILTV suspected tracheal samples. **a:** Tracheal cells infected with ILTV showing positive fluorescence reaction ( $\times 100$ ) **b:** Normal non-infected tracheal cells showing negative fluorescence reaction

## Discussion

Respiratory viral disease outbreaks with variable mortality rates have been increasing in the Egyptian chicken population during the past few years (Hassan *et al.*, 2016). Although ILT disease causes less mortality than AI and ND, nevertheless it greatly reduces the productivity of the surviving birds (Al Shekaili, 2015).

The key components for the prevention and control of the disease are accurate, fast and highly sensitive diagnostic tools which are critical to eliminate the economic losses and for facilitating the laborious implementation of control measures (Kim *et al.*, 2013). Sensitive, specific, and rapid tests are necessary to detect the infected flocks, prevent the disease transmission to susceptible flocks, prevent unnecessary treatment costs and to encourage vaccination in wider populations.

In the current study, diagnostic procedures for the detection of ILTV in tracheas of naturally infected field cases including VI, histopathology, PCR and FAT were applied and compared.

Virus isolation in CEF cell culture resulted in pathognomonic lesions in twenty samples which proved that the chicken embryo fibroblast TC is very suitable for ILT virus isolation (Taha *et al.*, 2017). In contrast, Bagust *et al.* (2000) mentioned that chick embryo fibroblasts and Vero cells have been shown to be relatively insensitive for ILTV growth from field samples. While Abbas *et al.* (2010) and Parra *et al.* (2016) reported that chicken embryo liver (CEL) cells were found to be the most sensitive for ILTV isolation.

Chicken embryo eggs inoculated with the ILTV suspected tissues homogenates showed characteristic pock lesions on the CAM in fifteen samples on the first egg passage while eight samples needed a further two blind passages in ECEs. This high rate of detection was consistent with previous studies (Crespo *et al.*, 2007; El Zowalaty *et al.*, 2011; Ali *et al.*, 2014). Although VI is considered one of the best choices between different methods for virus detection, it is a slow and laborious method (Spackman *et al.*, 2002). In addition, VI in TC

has the disadvantage of possible bacterial contamination even if it had been treated with high concentrations of antibiotics. Virus isolation on ECE similarly has the risk of contamination of the environment and workers during VI and propagation (Rashid *et al.*, 2009).

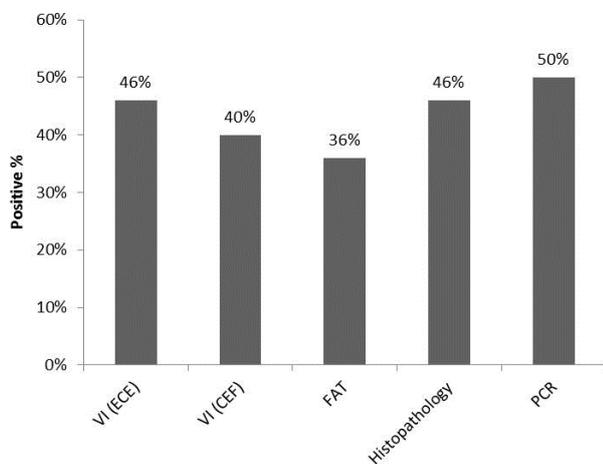
Histopathological analysis showed degenerative changes with infiltration of lymphocytes and macrophages with intranuclear inclusion bodies in the infected cells of all infected CAM (Madbouly *et al.*, 2005; Ojkic *et al.*, 2007; Ali *et al.*, 2014; Sary *et al.*, 2017). Conversely, other studies could not detect intranuclear inclusion bodies of ILTV in the infected cells which show positive VI and FAT results. This could be explained by the fact that the inclusion bodies are usually present in the early stages of infection, and disappear later due to necrosis and sloughing of the epithelial cells (Guy and Bagust, 2003; Hidalgo, 2003; Abdo *et al.*, 2017). Although histopathological diagnosis of ILTV by detection of inclusion bodies is highly specific, negative results will be suspicious because the appearance of these inclusions is temporary and inability to detect them does not exclude an ILTV infection (Bagust *et al.*, 2000; Preis *et al.*, 2014).

Direct FAT showed intracytoplasmic fluorescence in 18 tracheal tissue cells. Similar cytoplasmic fluorescence has also been recorded by (Fuchs *et al.*, 2007). In contrast, (Helferich *et al.*, 2007) observed only intranuclear fluorescence in ILT-infected TC cells suggesting that different locations of the fluorescence reaction in ILT-infected cells are indications of the enveloped and non-enveloped virions which was used for immunization. These criteria of fluorescence were also explained by (Abbas *et al.*, 2010) who suggests that cytoplasmic fluorescence is due to the presence of viral antigens expressed on the surface of virus-infected cells, while nuclear fluorescence indicates the reaction of MCAs against viral structural proteins.

Molecular identification of ILTV using PCR technique for amplification of specific fragment of TK gene revealed the presence of ILTV in 25 samples. Parallel to previous studies, PCR has been found to be

more sensitive for the detection of ILTV in clinical samples compared with other diagnostic methods, as well as its rapidity and possibility of automation. In addition, PCR allows detection of the virus in samples contaminated with high numbers of other pathogens which may prevent VI in cell culture. Furthermore, results are obtained within few hours as compared with VI technique which needs a few days for conclusive diagnosis (Williams *et al.*, 1994; Humberd *et al.*, 2002; Crespo *et al.*, 2007; Noroozian and Vasfi-Marandi, 2007; Tao *et al.*, 2009; Cobo, 2016; Kaboudi *et al.*, 2016; Parra *et al.*, 2016). Conversely, (Abbas and Andreasen, 1996) reported that PCR was less sensitive than VI and FAT proposing that DNA may have been destructed and lost in the DNA extraction process. In addition, positive results with PCR assays are not always associated with the presence of replicating viruses and the viral DNA detected by this technique may be due to a latent virus and was not the primary cause of the ongoing disease (Crespo *et al.*, 2007; OIE, 2014).

The percentages of positive results detected by each test are shown in Fig. 7. PCR was able to detect the greatest number of positive samples and the PCR-negative samples were negative by the other tests. If PCR was considered as the standard, the sensitivity of the other tests were 92%, 92%, 80%, and 72% for VI (ECE), histopathology, VI (CEF) and FAT, respectively. These tests had 100% specificity when compared to PCR (Table 2).



**Fig. 7:** Comparison of ILTV detection methods from field cases of naturally infected chicken flocks (n=50)

Although FAT has the advantage over VI and histopathology of being virus specific, it is more effective for the diagnosis if the samples are collected during the acute stage as there is a low level of virus shedding in the early or late stage of the disease. This was inconsistent with previous studies which reported that direct FAT was less sensitive than VI for the detection of ILTV (Crespo *et al.*, 2007). On the other hand, histopathological examination and FAT resemble PCR in that they also do not detect viable viruses.

General comparison of PCR, VI on ECE and CEF cell cultures, histopathology and direct FAT as

diagnostic tools, demonstrated that they all were effectual systems for detection of ILTV which was in accordance with (Zaher and Syame, 2009). Polymerase chain reaction was the most sensitive diagnostic method as it was able to detect the viral genome even in cases where other tools tested negative, but overall results concluded in this study show that the detection rate of ILTV by the other tests was adequate to be used as diagnostic tool. However, during an epidemic, VI may not be the best choice as it is a laborious and time consuming task. But it should never be neglected because it is the only method to obtain viable viruses, which is critical for further studies on ILTV (Cattoli *et al.*, 2004). Positive results with an antigen detection system are good indications of ILTV infection, but it is recommended that the suspect cases that test negative should be retested by molecular techniques to make a presumptive diagnosis.

### Conflict of interest

The authors disclose any financial and personal relationships with other people or organizations that might inappropriately influence or bias this work.

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