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Comparison of Diagnostics Techniques in an Outbreak of Infectious Laryngotracheitis from Meat Chickens

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SUMMARY. Various diagnostics techniques were compared for their ability to detect infectious laryngotracheitis (ILT) during an outbreak in chickens aged between 4 and 21 wk. Gross lesions ranged from excess mucus to accumulation of fibrinonecrotic exudate in the larynx and trachea. Syncytial cells with intranuclear inclusion bodies were found in sinus, conjunctiva, larynx, trachea, lung, and air sac. Virus isolation in chicken embryos was attempted in every case. Negative-stain electron microscopy detected herpesvirus in only 6% of the cases. Yet, isolation of ILT virus in the chorioallantoic membrane was presumed by histology in >20% of the samples and confirmed by fluorescent antibody (FA) in 35% of the embryos inoculated with conjunctivas or tracheas from affected birds. Overall, results from histology and FA tests were highly correlated. FA test has the advantage over histology of being diagnostically specific for ILT virus. Polymerase chain reaction was the most sensitive test and detected the viral DNA even in cases where histology and FA were negative. ILT virus DNA was quantified by real-time polymerase chain reaction (Re-Ti ILTV). Histologic and FA results from larynx and trachea were negative if the concentration of the viral DNA was ≤4 of $\log_{10}$. A viral DNA concentration higher than $\log_{10}$ 4, as determined by Re-Ti ILTV, was required for clinical ILT to be manifested.

RESUMEN. Comparación de técnicas diagnósticas en un brote de laringotraqueítis infecciosa en líneas de engorde.

Se compararon varias técnicas diagnósticas para su capacidad de detectar el virus de laringotraqueítis infecciosa durante un brote en aves entre las 4 y 21 semanas edad. Las lesiones microscópicas variaron desde la presencia excesiva de moco hasta la acumulación de exudado fibrinonecrótico en la laringe y la tráquea. Se encontraron células sincitiales con inclusiones intranucleares en senos, conjuntiva, laringe, tráquea, pulmón y sacos aéreos. En cada caso se intentó el aislamiento del virus en embriones de pollo. Mediante la microscopía electrónica con preparaciones con tinción negativa, se detectó el virus Herpes solamente en el 6% de los casos, sin embargo, el aislamiento del virus en membranas corioalantoideas de embriones inoculados analizadas por histopatología indicó positividad para el 20% de las muestras, confirmándose por medio de la prueba de inmunofluorescencia una positividad del 35% de los embriones inoculados con conjuntivas o tráqueas de las aves afectadas. Los resultados de histología y las pruebas de inmunofluorescencia estuvieron altamente correlacionados. La prueba de inmunofluorescencia tiene como ventaja sobre la histología de que es específica para el diagnóstico del virus de laringotraqueítis. La reacción en cadena de la polimerasa (PCR por sus siglas en Inglés) fue la prueba que ofreció mayor sensibilidad y detectó el DNA viral aún en los casos cuando la histología y la inmunofluorescencia fueron negativas. El virus DNA de laringotraqueítis fue cuantificado usando PCR en tiempo real. Los resultados de histología y la inmunofluorescencia de la laringe y la tráquea fueron negativos cuando la concentración del DNA viral era menor o igual a 4 $\log_{10}$. Una concentración de DNA viral mayor de 4 $\log_{10}$, determinada por medio de la prueba de PCR en tiempo real para el virus de ILT, fue requerida para que la laringotraqueítis se manifieste clínicamente.

Key words: chicken, fluorescent antibody, herpesvirus, infectious laryngotracheitis, syncytial cell

Abbreviations: CAHFS = California Animal Health and Food Safety Laboratory System; CAM = chorioallantoic membrane; CEO = chicken embryo origin; C_T = threshold cycle number; ELISA = enzyme-linked immunosorbent assay; EM = electron microscopy; FA = fluorescent antibody; ILT = infectious laryngotracheitis; ILTV = infectious laryngotracheitis virus; PCR = polymerase chain reaction; PCR-RFLP = polymerase chain reaction–restriction fragment polymorphism; Re-Ti ILTV = real-time PCR detection for ILT virus; SPF = specific pathogen-free; VTM = virus transport medium

Avian infectious laryngotracheitis (ILT) is a viral respiratory disease of chickens with worldwide distribution, caused by infectious laryngotracheitis virus (ILTV), of the family *Herpesviridae* subfamily *Alphaherpesvirus*. The disease is usually diagnosed in layers, but an increasing number of meat-type chickens are being affected. Vaccine-derived ILTV strains have been responsible for outbreaks in poultry (2,3,5). Clinical characteristics of the disease in chickens include respiratory signs, oral and ocular discharge, conjunctivitis, and increased mortality (4). Early and rapid detection of infected birds is essential to prevent and to avoid the spread of the disease.

Presumptive diagnosis of ILT can be made reliably in cases of severe acute disease based on high mortality with typical signs of the disease such as expectoration of blood. Otherwise, diagnosis should be based on one or more confirmatory laboratory diagnostic procedures. The most common, rapid laboratory diagnostic procedures are histology, which is confirmed by virus isolation, detection of ILTV antigen in tissues, and detection of ILTV-specific DNA (4). Comparison between diagnostic techniques has been performed on experimental studies (4,6) but not in field cases. This study compares histology, virus isolation, fluorescent antibody (FA) test, and polymerase chain reaction (PCR) techniques for their usefulness in the diagnosis of ILT during an outbreak in meat-type chickens in California.

MATERIALS AND METHODS

History. In October 2005, 10 birds from a commercial meat chicken flock were submitted to the California Animal Health and Food Safety

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Laboratory System (CAHFS), Fresno branch. The main complaint was sudden increased mortality, with 20% of the birds in the flock exhibiting respiratory distress and mouth breathing. Subsequently, between October 2005 and September 2006, 59 ILT suspect cases (a total of 517 birds, 423 live and 94 dead) from commercial chickens, between 4 and 21 wk of age (average age almost 8 wk, median age 6 wk), and raised for meat consumption, were submitted to the Fresno branch of the CAHFS. All 60 flocks were from two different companies, and the affected farms were located within a 48 km (30-mile) radius, in the Central Valley of California. The main complaints were excess tears, difficulty breathing, and increased mortality. None of flocks from the submitted cases had been vaccinated with chicken embryo origin (CEO) vaccine before presentation to the laboratory. Condemnation at slaughter was only increased by 0.5% from that normally expected.

After affected flocks were sent to market, cleaning and disinfection of affected houses were performed. Spray vaccination with CEO strain was performed only in flocks diagnosed with ILT and being kept on the farm for >5 wk (typically 16–25 wk of age) because those birds were to be sold at specialty markets. Because the virus continued spreading, affected companies commenced vaccination of 18-day-old embryos (*in ovo*) or 1-day-old chickens (wingweb), with a recombinant pox-ILT vaccine (Biomune, CEVA Animal Health, Lenexa, KS) in February 2006. After March 2006, only 14 cases were submitted from vaccinated flocks and 7 from nonvaccinated flocks.

Serology. A total of 67 sera samples from 13 cases were tested for ILT by enzyme-linked immunosorbent assay (ELISA; ProFLOCK® LT ELISA Kit, Synbiotics Corp., San Diego, CA).

Virus isolation. Conjunctiva and larynx–trachea were submitted for virus isolation. The tissues were mixed with virus transport medium (VTM) at a concentration of 1:10 (w/v), triturated with sterile silica and a pestle and mortar, and clarified by centrifugation at 1780 × *g* for 15 min at 4 C. An aliquant of the supernatant was passed through a 0.45-μm membrane filter. The filtrate was inoculated on to the chorioallantoic membrane (CAM) of 9-to-11-day-old developing specific pathogen-free (SPF) chicken embryos. These were incubated at 37 C and candled daily for 7 days. Eggs with dead embryos were chilled at 4 C. After 7 days of incubation, eggs with surviving embryos were transferred to 4 C. The CAM was examined for the presence of plaques and thickening. If no thickening was observed, CAMs were harvested, and a 10% w/v suspension in VTM was prepared for passage in SPF embryonating chicken eggs as before. All CAMs from second passage were processed for virus identification by electron microscopy, histology, and FA test.

Electron microscopy (EM). A 10% w/v suspension in VTM of CAM tissue was clarified by centrifugation 1780 × *g* and then centrifuged at 140,000 × *g* for 75 min to pellet the virus. Pellets were resuspended in 0.3–1.0 ml deionized water, and 2–3 μl was mixed with 100–200 μl of 0.8% phosphotungstic acid; a drop was then placed onto a 200-mesh formvar-coated grid for ≤3.5 min. Unabsorbed material was wicked away with filter paper. Grids were examined on a Zeiss EM10A electron microscope.

Histology. Sections of nasal passages, larynx, trachea, lung, conjunctiva, air sac, heart, liver, spleen, bursa of Fabricius, kidney, brain, and sections throughout the entire digestive tract were collected from the birds submitted to the laboratory. The tissues were fixed in 10% buffered neutral formalin, embedded in paraffin, sectioned at 4 μm, stained with hematoxylin and eosin, and examined by light microscopy.

Additionally sections of CAMs harvested from chicken embryos inoculated with either conjunctiva or larynx–trachea homogenate were processed for standard light microscopy examination as described above.

FA. Fresh sections of tracheas, just below the larynx, were collected from the birds and prepared for FA testing. Sections of CAMs from chicken embryos inoculated with either conjunctiva or larynx–trachea homogenate were also processed for an FA test. The tissues were trimmed, placed in cryomolds, covered with Tissue-Tek O.T.C compound (Miles Scientific, Naperville, IL), frozen at −80 C until sectioned, and processed for direct immunofluorescence using a polyclonal serum reagent (National Veterinary Services Laboratories, Ames, IA), and examined at 495-nm wavelength using a fluorescence microscope.

PCR. Larynx–tracheas were used for total DNA extraction. DNA extraction was performed using the QIAamp Mini kit (Qiagen, Valencia, CA) with the following modifications from the manufacturer's suggestions. Briefly, 10 μl of proteinase K was used instead of 20 μl, and 400 μl of lysis buffer AL was used instead of 200 μl during the initial lysis step. After the 56 C incubation, only 100 μl of ethanol was added instead of 200 μl. All remaining steps were performed as described by the manufacturers. The final elution volume used was 100 μl. The DNA was stored at −20 C until needed.

Real-time PCR detection of ILTV (Re-Ti ILTV). The assay and quantification of viral DNA was performed as previously described by Callison *et al.* (1). Briefly, the primers and probe are located in the viral glycoprotein C gene and were synthesized by Integrated DNA Technologies (Coralville, IA) and BioSearch Technologies (Novato, CA). The final amplification reaction volume was 25 μl, including 12.5 μl of 2X Master Mix (Quantitect Probe PCR kit, Qiagen), primers to a final concentration of 0.5 μM, probe to a final concentration of 0.1 μM, 1 μl of HK™–UNG (Epicentre, Madison, WI), 2 μl of water, and 5 μl of DNA template. The tubes were closed and cycled in a Smart Cycler thermocycler (Cepheid, Sunnyvale, CA) using a thermocycle program of 50 C for 2 min, 95 C for 15 min, and 40 cycles of 94 C for 15 sec, 60 C for 60 sec with optics on. For each ReTi ILTV assay reaction, the threshold cycle number (C_T value) was determined to be the PCR cycle number at which the fluorescence of the reaction exceeded 30 units of fluorescence. To determine the detection limit of the ReTi ILTV assay, three independent runs using gC gene plasmid dilutions containing 100, 75, 50, 25, and 10 template copies were used. A standard curve and equation were generated using the gC plasmid dilutions mentioned above. The ILTV genome copy number per amplification reaction was estimated using the standard curve equation generated from the gC plasmid and expressed as $\log_{10}$.

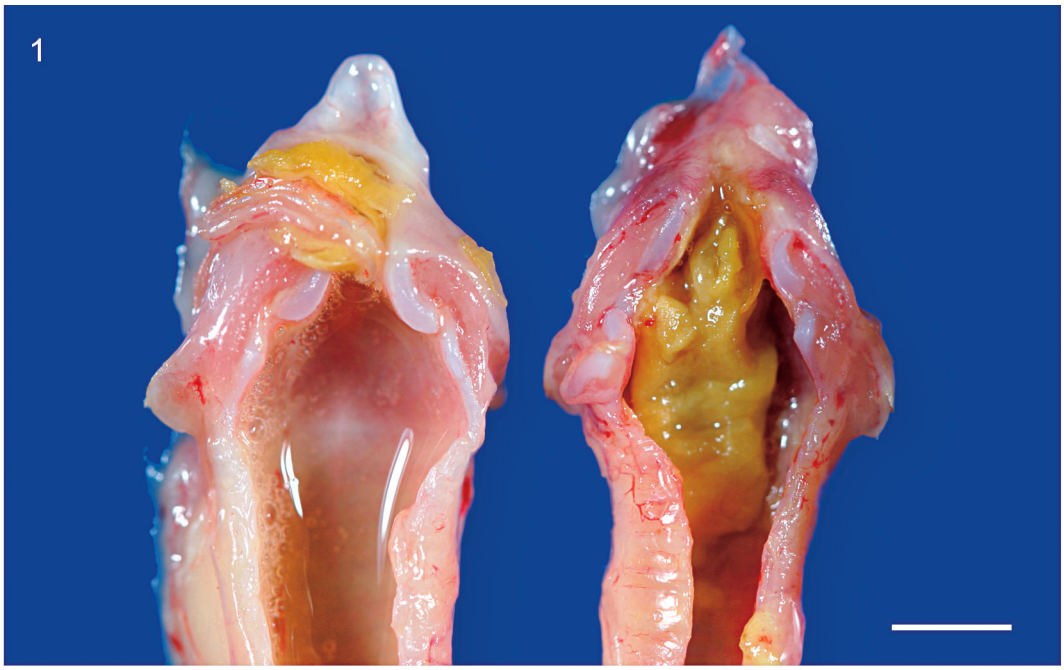
RESULTS

At necropsy, the most striking gross finding was excess mucus, with or without fibrinous exudate, in the lumen of the larynx and upper trachea (Fig. 1). Mild laryngeal and tracheal hemorrhages were observed in 24 birds out of 94 submitted dead. Other changes included conjunctivitis and occasionally pneumonia and airsacculitis.

Histologically, the lesions were typical of ILT. Epithelial necrosis and sloughing, heterophilic exudation, and syncytial cells with intranuclear inclusion bodies were found in 83% (49/59) of the cases (Table 1). Syncytia were found in the sinus, conjunctiva, larynx, trachea, lung, and air sac. There was also extensive deciliation, and flattening, of the epithelium in larynx and trachea. Numerous lymphocytes and heterophils infiltrated the lamina propria of affected tissues.

Apple-green fluorescence, indicating positivity for ILTV, associated with the epithelial cells of the upper trachea (Fig. 2) was found in 65% (26/40) of the cases (Table 1). Direct FA results on frozen sections of upper trachea showed an 85% correlation with the histology findings.

Attempts to isolate ILTV by inoculation in the CAM of embryonating chicken eggs were notably unsuccessful (Table 1). ILTV was detected from the CAM of embryonating chicken eggs in 9 cases. In 45 cases, other viruses were isolated. The most common virus isolated was adenovirus (18 cases), followed by infectious bronchitis virus (8 cases), and avian paramyxovirus type 1 (3 cases). Rarely were any macroscopic changes, such as thickening, edema or pale foci, detected in the CAMs. Detection of herpesvirus in CAMs by negative stain EM was successful in only 3 cases (5.1%). Syncytia and inclusion bodies were detected by histology in the CAMs in



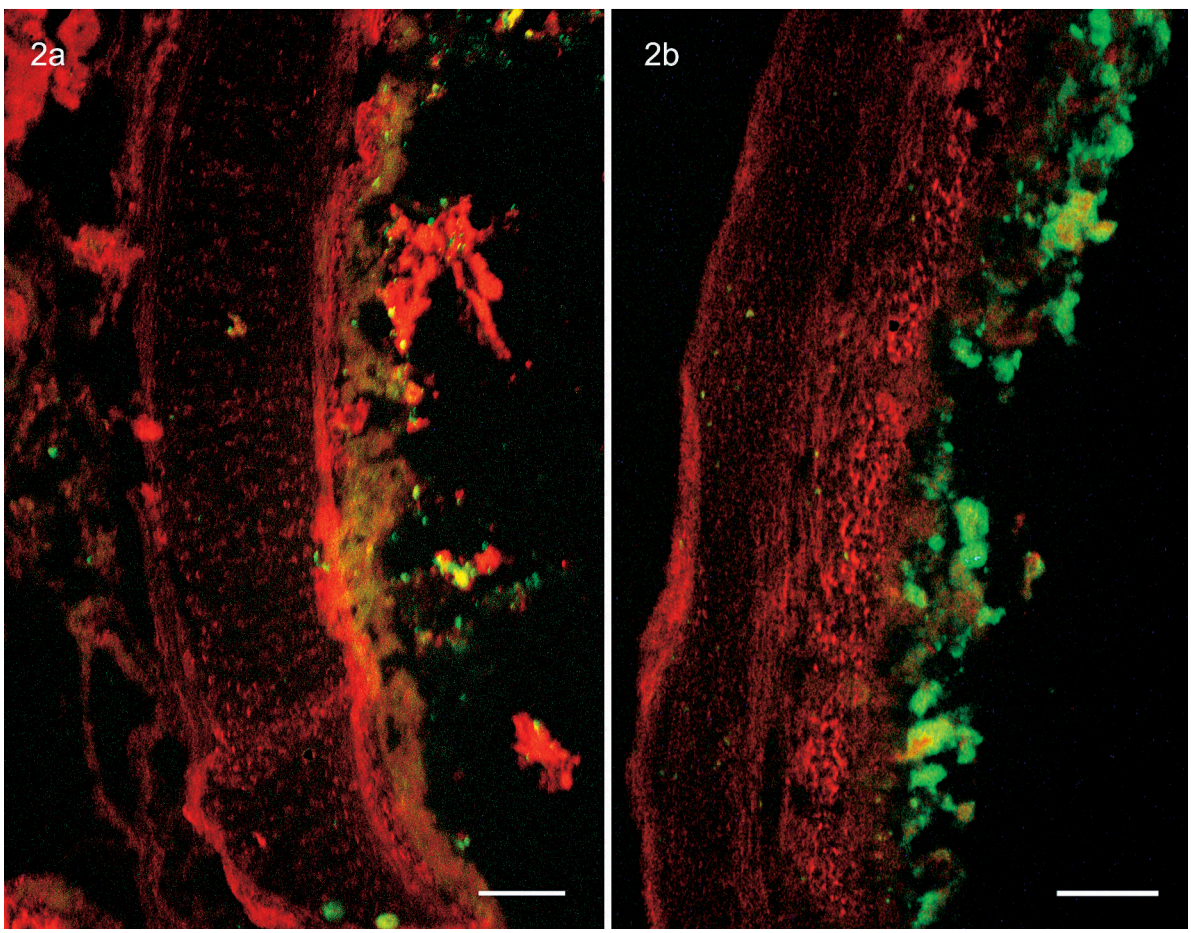


Fig 1. Typical gross finding in larynx and upper trachea from live birds. There is excess mucus with or without fibrinous exudate. Note the lack of hemorrhage in larynx and trachea. Bar = 0.5 mm.

Fig 2. Frozen sections of trachea stained by direct immunofluorescence. (A) Negative control. There is no fluorescence in the epithelium of trachea negative for ILT. Bar = 0.2 mm. (B) Typical apple-green fluorescence, indicating positivity for ILT, associated with the epithelial cells of the trachea. Bar = 0.2 mm.

Table 1. Summary of results for all the tested cases performed for detection of ILTV.

	Tissue examination		Virus isolation and identification			
Results	Histology[A]	FA[B]	EM[C]	HistoCAM[D]	FA CAM[E]	PCR[F]
Positive	49	26	3	9	7	40
Negative	10	14	56	35	12	4
Total	59	40	59	44	19	44

[A]Histology = interpretation of the examination of larynx, trachea, and conjunctiva. A positive result indicates syncytia or intranuclear inclusion bodies in at least one of these tissues.
[B]FA = examination performed on sections of upper trachea by fluorescence antibody.
[C]EM = negative stained electron microscopy of chorioallantoic membranes (CAM) harvested from chicken embryos inoculated either with conjunctiva or larynx–trachea homogenate.
[D]HistoCAM = interpretation of the histologic examination of CAMs harvested from chicken embryos inoculated either with conjunctiva or larynx–trachea homogenate.
[E]FA CAM = examination performed on sections CAMs harvested from chicken embryos inoculated either with conjunctiva or larynx-trachea homogenate by fluorescence antibody.
[F]PCR = polymerase chain reaction performed on scrapings of larynx and upper trachea.

approximately 20% (9/44) of the embryos inoculated with conjunctival or tracheal-tissue homogenate from suspected birds. Detection of ILTV on frozen sections of CAM by FA was successful in 35% (7/19) of the attempted cases. There was a 90% correlation between the findings in the CAM by histology and FA.

DISCUSSION

Based on these results, the diagnosis of ILT was more reliable when based on histology and FA findings than by virus isolation. The strain of virus circulating was not easily propagated in embryonating chicken eggs. Also, negative-stain EM on CAMs was not sensitive enough to detect the virus, even when the CAM was thickened. It is recognized that EM is less sensitive than other tests and, therefore, may not detect viruses if they are present in low numbers. A limited number of samples were inoculated in kidney cells, but results were similar to isolation in CAM (data not shown). Guy *et al.* reported (6) virus isolation in chicken kidney cells to be the most-sensitive test. Examination of different tissues or differences in virus strains may account for the differences between this and previous studies.

Additionally, different ILTV strains have different tissue tropism (8). Therefore, it is important to examine not only trachea but also conjunctiva and even other portions of the respiratory tract. In several cases, syncytial cells and intranuclear inclusion bodies were detected in lung and air sac. In two cases, inclusion bodies were found by histology only in the conjunctiva. The lower detection rate by FA might have been, in part, because of testing of tracheas only. On the other hand, larynges, tracheas, and conjunctivas were examined microscopically in all cases. Histology and FA examination of CAMs from ILT suspect cases were more successful tests for detecting ILTV than EM. As compared to histology examination, the FA test had the advantage of being diagnostically specific for ILTV.

Table 2. Comparison between the average concentration $\log_{10}$ of ILTV obtained by Re-Ti ILTV and identification by histology and virus isolation.

		PCR[A] (average $\log_{10}$)		
Test	Results	0 (negative)	$1 < x \leq 4$	>4
Histology[B]	Positive	0	0	35
	Negative	4	4	1[C]
VI[D]	Positive	0	0	8
	Negative	4	4	28

[A]PCR = polymerase chain reaction performed on larynx–trachea scrapings.
[B]Histology = histologic results from tissues by light microscopy and fluorescent antibody (FA).
[C]Log_{10} value of this case = 6.545.
[D]VI = virus isolation is positive when virus demonstrated by negative-staining electron microscopy, histology, or FA of the chorioallantoic membrane harvested from chicken embryos inoculated either with conjunctiva or larynx–trachea homogenate.

Real-time PCR detected viral DNA in cases where ILT was suspected, but other tests (histology, FA, virus isolation) failed to detect the virus (4 cases). Detection of syncytia or inclusion bodies by histology or viral antigen by FA on frozen sections of trachea and conjunctiva sections was successful when the average $\log_{10}$ viral DNA concentration was higher than 4. In general, when the concentration of the viral DNA was ≤ 4 of $\log_{10}$, the case was negative by histologic examination and FA (Table 2). Only 1 case found negative by histology and FA had a $\log_{10}$ value of 6.545. This might have been an early infection where lesions were not yet developed. Unfortunately, no follow-up was submitted to prove this hypothesis. Low levels of viral DNA in the absence of other indication of infection (e.g., without histologic lesions or viral antigens detected by FA) may be interpreted as a false-positive result, where the flock might have been exposed to the virus, but the viral load might have not been sufficient to cause disease, causing some birds to have low viral DNA titers.

In recent years, the use of PCR for the detection of ILT DNA in clinical cases has become widespread (4). Real-time PCR has the ability to quantify viral DNA copies present per sample tested (11). From our data, the number of viral DNA copies was correlated with the presence of syncytial cell or inclusion bodies by histology and viral antigen by FA on frozen sections of conjunctiva or larynx–trachea. Positive histology and FA results for ILT occurred when the average $\log_{10}$ viral DNA concentration was >4, whereas negative histology and FA results were associated with viral DNA concentration ≤ 4 of $\log_{10}$. This suggests clinical ILT will not be apparent if the number of viral DNA template copies obtained by Re-Ti ILTV is <4. On the other hand, the concentration of viral DNA found in the trachea by Re-Ti ILTV did not necessarily correlate with the ability to isolate the virus in the CAM. The isolation of ILTV was unsuccessful in 9 samples with concentrations of viral DNA >7 of $\log_{10}$, whereas adenovirus virus was isolated in 6 of these cases. Commonly, samples from clinical cases of ILTV can be overgrown by other viruses (1,7). In this study, adenovirus was isolated in 31% (18/59) of the cases analyzed. Consequently, the

correlation of ILTV isolation with either histology or Re-Ti ILTV was 20% (12/59). Also, it maybe a characteristic of the strain of ILTV circulating in this outbreak to have limited growth in embryonating chicken eggs and may require more than two passages for the virus to adapt to the system.

Of the 13 cases selected for serologic testing, four were negative for ILT by histology, FA from trachea, and virus isolation, and the amount of ILTV DNA detected by PCR was <4 of $\log_{10}$; nine cases were positive for ILTV by at least two tests (histology, FA from trachea, virus isolation, or PCR). Of the nine positive cases, two of them had been vaccinated *in ovo* with the recombinant vaccine. No significant differences were observed between positive, positive-vaccinated, and negative cases. Ninety-three percent of the birds tested were seronegative to ILT because the recommended threshold for seropositive titer is >1070. The titers varied between 0 and 2600, with an average of 352. Based on these results, serology was considered of no diagnostic use. Previous researches have recommended the use of pair samples to obtain meaningful results (12).

Even though ILT diagnosis was made in flocks vaccinated with the recombinant pox-ILT vaccine, the morbidity and mortality in these flocks were lower than reported in nonvaccinated flocks. The exact origin of the outbreak virus was not determined; however, epidemiologic evidence suggests that the source of the virus might have been either 1) a vaccinated breeder flock on a ranch 16 km (10 miles) from the index case, or 2) a contaminated truck moving live birds. Polymerase chain reaction–restriction fragment polymorphism (PCR-RFLP) of four genome regions was used to characterize 25 isolates from commercial poultry and backyard flocks from the United States. Combinations of PCR-RFLP patterns classified the ILTV isolates into nine (I to IX) different genotypes (10). Using this technique, early isolates from this outbreak were categorized as either PCR-RFLP group IV or group V; these two genotypes were identified as closely related to the CEO-viral strains. The use of multiple PCR-RFLP analyses has made it possible to differentiate vaccine strains from wild type viruses from Australia (9).

In summary, results from histology of tissues, FA from trachea, and Re-Ti ILTV tests were comparable. FA from CAM was more useful than electron microscopy of the CAM for identifying ILTV in chicken embryos inoculated by the CAM route. Lastly, Re-Ti ILTV was a sensitive test; and the quantification of viral DNA template copies per sample was important in the interpretation of the PCR results and helped to minimize possible false-positives when comparing PCR results with other rapid diagnostic assays, such as histology and FA.

REFERENCES

1. Callison, S. A., S. M. Riblet, S. Sun, G. Zavala, S. Williams, R. Resurreccion, E. Spackman, and M. Garcia. Development and validation of a real-time TaqMan–PCR assay for the detection and quantification of infectious laryngotracheitis virus in poultry. J. Virol. Methods 139:31–38. 2007.

2. Garcia, M., and S. M. Riblet. Characterization of infectious laryngotracheitis virus isolates: demonstration of viral subpopulations within vaccine preparations. Avian Dis. 45:558–566. 2001.

3. Graham, D. A., I. E. McLaren, V. M. Calvert, D. Torrens, and B. M. Meehan. RFLP analysis of recent Northern Ireland isolates of infectious laryngotracheitis virus comparison with vaccine virus and field isolates from England, Scotland, and the Republic of Ireland. Avian Pathol. 29:57–62. 2000.

4. Guy, J. S., and T. J. Bagust. Laryngotracheitis. In: Diseases of poultry, 11th ed. Y. M. Saif, H. J. Barnes, J. R. Glisson, A. M. Fadly, L. R. McDougald, and D. E. Swayne, eds. Iowa State University Press, Ames, IA. pp. 121–134. 2003.

5. Guy, J. S., H. J. Barnes, L. L. Munger, and L. Rose. Restriction endonuclease analysis of infectious laryngotracheitis viruses; comparison of modified live-vaccine viruses and North Carolina field isolates. Avian Dis. 33:316–323. 1989.

6. Guy, J. S., H. J. Barnes, and L. G. Smith. Rapid diagnosis of infectious laryngotracheitis using a monoclonal antibody-based immunoperoxidase procedure. Avian Pathol. 21:77–86. 1992.

7. Hughes, C. S., and R. C. Jones. Comparison of cultural methods for primary isolation of infectious laryngotracheitis virus from field material. Avian Pathol. 17:295–303. 1988.

8. Kirkpatrick, N. C., A. Mahmoudian, C. A. Colson, J. M. Devlin, and A. H. Noormohammadi. Relationship between mortality, clinical signs and tracheal pathology in infectious laryngotracheitis. Avian Pathol. 35:449–453. 2006.

9. Kirkpatrick, N. C., A. Mahmoudian, D. O'Rouke, and A. H. Noormohammadi. Differentiation of infectious laryngotracheitis virus isolates by restriction fragment length polymorphic analysis of polymerase chain reaction products amplified from multiple genes. Avian Dis. 50:28–34. 2006.

10. Oldoni, I., and M. García. Characterization of infectious laryngotracheitis virus (ILTV) isolates from United States by polymerase chain reaction and restriction fragment length polymorphism (PCR-RFLP) of multiple genome regions. Avian Pathol. 36:167–176. 2007.

11. Oldoni, I., S. Riblet, S. Sun, K. Jones, S. Callison, G. Zavala, and M. García. Application of real-time PCR in the diagnosis of infectious laryngotracheitis during outbreaks of the disease. In: Proc. 142nd Annual Meeting American Veterinary Medical Association Minneapolis, MN. 2005.

12. Sander, J. E., and S. G. Thayer. Evaluation of ELISA titers to infectious laryngotracheitis. Avian Dis. 41:429–432. 1997.