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Infectious Laryngotracheitis

Cytology, Virology and Serology

J. J. York

CSIRO Division of Animal Health, Animal Health Research Laboratory, Private Bag No. 1, Parkville, Vic. 3052, Australia.

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1. Introduction

The first Australian outbreak of infectious laryngotracheitis (ILT) was recorded in the State of New South Wales in 1935, and the disease has since been reported in all other States of Australia. In New South Wales, Victoria and Western Australia the acute form of the disease was frequently observed, while a mild form was generally seen in South Australia and Queensland.

In Australia, which has been free of velogenic Newcastle disease, the acute form of ILT has been easily recognised, and many field diagnoses have been made on clinical manifestations only. However, mild outbreaks, which have become more frequent, can easily be confused with some other respiratory diseases of chickens. A diagnosis of the disease should, therefore, be confirmed by the demonstration of infection with ILT virus and, depending upon the urgency of the diagnosis and laboratory facilities available, one or more of the following should be carried out:

(a) cytological examination;
(b) electron microscopy;
(c) virus isolation and identification;
(d) demonstration of viral antigen;
(e) antibody examination.

2. Diagnosis by Cytological Examination

Cytological examination, either in histological sections or in smears, is directed primarily towards the demonstration of syncytial cells and intranuclear inclusion bodies in the epithelium of the upper respiratory tract and in the bronchial epithelium of the lungs. The inclusion bodies were shown to be diagnostic by Beveridge and Burnet (1946), and a presumptive diagnosis of ILT may be made once their presence has been established. The probability of demonstrating the above changes is increased by taking sections or smears from chickens as soon as they begin to show clinical signs of disease.

Histological sections may be prepared by conventional methods (Cover and Benton, 1958), by freezing (Pirozok *et al.*, 1957), or by a rapid tissue fixation method (Sevoian, 1960). Smears may be prepared either by a method described by Armstrong (1959) or by the method of Odagiri and Yoshimura (1965).

Of the diagnostic techniques based on cytological examination, the smear technique by Odagiri and Yoshimura (1965) and the rapid tissue fixation method by Sevoian (1960) are recommended because of the speed and simplicity these methods offer (Jackson and Sinkovic, 1969).

2.1. Smear Method

(a) Scrape off an intact sheet of epithelium, including as little blood and exudate as possible, from affected trachea and/or conjunctiva with a round blade scalpel. Deposit the scraping near the mid-point of a clean, oil-free slide. With a second clean slide press the tissue onto the first slide with a gentle rotary pressure until it is flattened out in a thin layer. Draw the two slides apart parallel to one another.
(b) While the smears are still wet hold them face downwards for 10 s over the fumes of modified Schaudinn's fixative [32 mL absolute alcohol (C_2H_5OH), 63 mL of 7.5% saturated mercuric chloride ($HgCl_2$) and 5 mL acetic acid (CH_3CO_2H)] kept at 45°C. Touch the surface of fixative for a further 10 s and then immerse the slides for 15 min in the fixative.
(c) Transfer the slides into 70% iodised alcohol (a few drops of Lugol's iodine to 100 mL of alcohol) for 15 min.
(d) Wash in 70% ethanol.
(e) Immerse in 50% ethanol for two to three minutes.
(f) Wash in tap water then stain with haematoxylin and eosin, dry and mount (Lillie and Fullmer, 1976).

2.2. Rapid Tissue Fixation Method

(a) Incubate small pieces of affected trachea and/or conjunctiva for 30 min in the following solution which has been heated to 56°C:

95% Ethanol saturated with picric acid $(O_2N)_3(C_6H_2OH)$	75 mL
Formalin, HCHO	15 mL
Glacial acetic acid, CH_3CO_2H	5 mL

(b) Dehydrate in two changes of absolute ethanol heated to 56°C for a total of 20 min.
(c) Clear in two changes of toluene ($C_6H_5CH_3$) heated to 56°C for a total of 30 min.
(e) Place tissues in melted paraffin wax (56°C) for 20 min and then imbed.
(f) Cut sections 6 µm thick and place them on slides which have been smeared with egg albumin. Dry with mild heat for 20 min.
(g) Bring sections through xylol and alcohol, stain with haematoxylin and eosin, dehydrate, clear and mount (Lillie and Fullmer, 1976).

The smear method is speedier and can be performed in a most modestly equipped laboratory, but the inclusion bodies can frequently be demonstrated more easily in histological sections which also allow examination for other cellular changes commonly seen in ILT.

The overall value of cytological examination may be questioned in the light of the work by Kawamura *et al.* (1966) and Lim *et al.* (1973) who have demonstrated intranuclear inclusion bodies in cases of a respiratory disease in chickens due to an adenovirus infection. However, clinical manifestations combined with the demonstration of intranuclear inclusion bodies should be sufficient in most instances for a presumptive diagnosis of ILT. In cases where conclusive evidence of infection with ILT virus is required, virus isolation and/or serology must be undertaken.

3. Diagnosis by Demonstration of Virus

3.1. Isolation of Virus

ILT virus can be isolated on the chorioallantoic membrane (CAM) of fertile eggs or in chicken embryo kidney (CEK) or chicken kidney (CK) cell cultures.

In most diagnostic laboratories primary isolation is undertaken in developing chicken embryos. Any ILT virus isolated can be identified by inoculation into ILT immune and susceptible chickens, by neutralising or reacting the virus with specific antisera, or by electron microscopy.

3.1.1. Samples for Virus Isolation

Tissues must be sampled from birds in the early stages of disease as virus is only readily isolated for two to three days following the development of clinical signs.

Virus is most likely to be isolated from the trachea and larynx. Tracheal scrapings or homogenised tissues are preferable to swabs because some bacteriological swabs which are available commercially may contain alginate which inactivates herpesvirus. However, buffered cotton swabs could be used if desired.

Tissues or scrapings are homogenised in a cold balanced salt solution such as Hanks or phosphate buffered saline (PBS) with 0.5% (w/v) bovine albumin or gelatin, and antibiotics (1000 units penicillin, 1000 µg streptomycin, 2.5 µg amphotericin B per mL) to an approximate 10% suspension. The homogenates should be kept at 4°C and they should be inoculated into cultures or eggs as soon as possible. If they are not inoculated within eight hours, they must be stored in airtight containers at -60°C or lower.

Material to be inoculated is centrifuged at 2000 *g* for five minutes, preferably in a refrigerated centrifuge as heat will rapidly inactivate ILT virus. The pellet is discarded. The supemate, which contains 90% of any ILT virus in the sample, may be passed through a 0.45 µm membrane filter, but this will reduce infectivity by about 90% and thus reduce the likelihood of detecting virus if the sample has a low initial titre. The supernate should be serially diluted through five 10-fold steps and each dilution should be inoculated to at least five eggs or cultures. Each culture unit is incubated and examined as described in 3.1.2. This will enable defined foci of cytopathology or pocks to be observed in those specimens with high titres of virus.

3.1.2. Growth of Virus

3.1.2.1. Embryonated eggs

When attempts are made to isolate virus on the CAM, 9–12-day-old embryonated chicken eggs are used and 0.1–0.2 mL of the tissue supernate is inoculated per egg. If inoculation is performed after dropping the CAM, eggs should be tilted gently to distribute the inoculum over the surface.

Eggs are incubated at 37°C for four to five days after inoculation before CAMs are harvested and examined for pocks. The presence of typical pocks is generally considered to confirm a field diagnosis of ILT, but virus identity should be confirmed as described in 3.2.1–3.2.4.

3.1.2.2. Tissue cultures

Washed and drained CEK or CK cultures containing glass coverslips are inoculated with a volume of tissue homogenate which is equal to 1/10th the volume of maintenance medium used. Adsorption is allowed to continue for one hour at room temperature after which serum-free maintenance medium (such as 199 medium with 0.1% sodium bicarbonate and antibiotics) is added to the cultures which are then incubated at 37–40°C. Cultures are examined daily for cytopathological changes characterised by syncytium formation. Coverslip cultures are fixed in Bouin's fluid (Lillie and Fullmer, 1976), stained with haematoxylin and eosin and examined for characteristic intranuclear inclusion bodies. Coverslips may also be fixed in acetone and examined by immunofluorescence for ILT viral antigen (see 3.2.4). The remainder of the tissue cultures are used to identify any virus isolated as described in 3.2.1.–3.2.4.

3.2. Identification of Virus Isolate

3.2.1. Identification Using Chickens

(a) A presumptive identification can be achieved by the intratracheal inoculation of suspensions containing the suspected ILT virus to susceptible and ILT-vaccinated chickens. Some of these are killed two to four days post inoculation and samples of trachea and larynx are examined as described in Section 2. If the isolate is ILT virus, inclusion bodies will be observed in the epithelial cells of susceptible chickens and there will be associated inflammation. In the sections from vaccinated chickens no inflammatory response or inclusion bodies will be observed.

(b) Two to four days after inoculation there should be signs of respiratory distress in the remaining susceptible chickens, while the immune chickens remain free of the disease. The severity of clinical signs will depend on the virulence of virus involved in the outbreak.

3.2.2. Identification of Virus Using Standard Serum

ILT virus which grows from any sample can be identified by one of the two methods described below (see 3.2.2.1. and 3.2.2.2.)

Initially a stock suspension of the virus is prepared either by infecting CAMs with sufficient virus to produce confluent pocks after four to six days at 37°C, or by infecting CEK or CK tissue cultures with sufficient virus to produce a confluent CPE within three days at 40°C.

Homogenates of either preparation are prepared in PBS (pH 6.6) containing 0.5% gelatin and antibiotics and are clarified by low-speed centrifugation (2000 *g*). The preparation is then dispensed into small volumes and stored at -60°C or less.

Representative samples of the preparation are assayed for virus titre in the system to be used for the neutralisation test.

3.2.2.1. Identity test using inhibition of pock formation on the chorioallantoic membrane

The stock suspension of virus is diluted in PBS (pH 6.6) containing 0.5% gelatin and antibiotics to a concentration of 600 pock forming units/mL. The reference antiserum is diluted four-fold in the same diluent and equal volumes of diluted virus and serum are mixed. A control mixture consisting of equal volumes of normal fowl serum diluted four-fold and of the diluted virus suspension diluted two-fold further is prepared at the same time. All mixtures are incubated at 37°C for two hours. A volume of 0.1 mL of each mixture is inoculated onto the dropped CAM of each of 10 chick embryos, 9–12 days old. The hole in the shell is sealed and eggs are incubated for four days at 37°C, after which each CAM is harvested and the number of pocks produced on it is counted. The test is invalid if more than two embryos die in either group or if the mean number of pocks in the control group is <10 or >50.

The mean number of pocks per membrane in the test group is calculated and if it is less than 10% of that of the control group, the virus which has been isolated is ILT virus.

3.2.2.2. Identity test using inhibition of plaque formation in tissue culture

The stock suspension of virus is diluted in PBS (pH 6.6) containing 0.5% gelatin and antibiotics to a concentration of 600 plaque forming units/mL. The reference antiserum is diluted four-fold in the same diluent and equal volumes of diluted virus and serum are mixed. A control mixture, consisting of equal volumes of normal fowl serum diluted four-fold and of the diluted virus suspension diluted two-fold further, is prepared at the same time. All mixtures are incubated at 37°C for two hours. Eight confluent monolayer cultures are rinsed with PBS and drained. A volume of 0.5 mL of test mixture is inoculated per 20 cm^2 of tissue culture onto each of four cultures and the control mixture is inoculated similarly onto the other four cultures. After 30 min at room temperature (20–28°C), cultures are overlaid with agar overlay medium (see 3.2.2.2.1.) and incubated at 40°C in a humidified incubator containing an atmosphere of about 5% CO_2 in air. A second staining overlay (see 3.2.2.2.1.) is added to each culture after three days and the cultures are then incubated for a further day under the same conditions. Cultures may be incubated for a further two days if plaques are not clearly visible. Plaques may also be visualised using MTT tetrazolium dye [3-(4,5-dimethylthiazol-2-y')-2,5-diphenyltetrazolium bromide; thiazolyl blue] (Klebe and Harris, 1984; see 3.2.2.2.2.). All plaques which form on the monolayers are counted and the total plaque count for each group is obtained. The test is invalid if the total number of plaques in the control group is fewer than 80 or greater than 400.

If the total plaque count of the test is less than 10% of that of the control group, the virus which has been isolated is ILT virus.

3.2.2.2.1. Agar overlay medium. A two-fold concentrate is prepared aseptically as follows:

Medium 199 (without phenol red and sodium bicarbonate, $NaHCO_3$, 10-fold concentrate)	160 mL
$NaHCO_3$, 2.8% (w/v)	30 mL
Tryptose phosphate broth (Difco)	200 mL
Glutamine, 2.92% (w/v)	16 mL
Calf serum (heat-inactivated at 56°C for 30 min)	200 mL
Penicillin and streptomycin (1×10^5 IU/mL and 100 µg/mL, respectively)	2 mL
Sterile glass distilled water to	1 L

The medium is dispensed in convenient amounts and stored frozen until required.

For use, the double strength overlay medium is thawed, warmed at 45°C and toluidine blue added to give a concentration of 2.4 µg/mL. This is then saturated with carbon dioxide and mixed with an equal volume of 2% agar (Difco Bacto Agar) which has been melted and cooled to 45°C.

3.2.2.2.2. Staining overlay media. Stained agar overlay consisting of 70 mL of 3% agar (Difco Bacto Agar), 100 mL of double strength overlay medium and 30 mL of neutral red (0.1% w/v) is prepared for staining monolayers for plaque counting.

Plaques may also be stained by adding 0.4 mg/mL MTT tetrazolium dye in 0.15 mol/L sodium chloride to the cultures for at least two hours.

3.2.3. Identification of Virus by Electron Microscopy

This method has been shown to be the fastest for the direct diagnosis of ILT (Mayr *et al.*, 1964), although the method appears to be less sensitive than histopathology. The cost involved in the installation of the equipment is high and experienced staff are required to use it. However, where such a facility is available, examination of negatively stained tracheal scrapings may provide a rapid diagnosis of ILT.

Demonstration by electron microscopy of herpesvirus particles in CAM or cell culture preparations will enable a rapid differentiation to be made between ILT virus and adenoviruses

and reoviruses, which also produce pocks on the CAM and confusing cytopathology in CEK and CK cell cultures.

3.2.4. Identification of Virus by Other Methods

Immunofluorescence or gel precipitin techniques may also be used for identification, provided the relevant monospecific sera and control antigens are included in the tests.

The fluorescent antibody test has been used successfully for the detection of ILT virus in tracheal smears (Braune and Gentry, 1965; van der Heide *et al.*, 1967). The technique has subsequently been refined by Ide (1978), Meulemans and Halen (1978) and Wilkes and Kogan (1979) and it may prove to be a more specific diagnostic tool than the detection of intranuclear inclusion bodies. However, experience with the technique is needed to consistently produce accurate results.

Immunofluorescence may also be used to confirm the identity of an isolate grown in CEK or CK cultures. Fixed virus-infected coverslips are stained with a reference antiserum for 30 min in a humid atmosphere. They are rinsed twice in PBS and stained with antichicken immunoglobulin conjugated to fluorescein. After incubation for 30 min the coverslips are rinsed twice in PBS, counterstained briefly with 0.01% (w/v) Evan's blue and rinsed in distilled water. They are mounted in 50% glycerol in PBS, pH 8.6 and examined using a fluorescence microscope.

3.3. Detection of Viral Antigen by Enzyme-linked Immunosorbent Assay

An accurate and rapid method of diagnosis of ILT is by the demonstration of viral antigen in tracheal scrapings using ELISA (York and Fahey, 1988).

(a) Purified rabbit antibody to ILT virus is adsorbed to the wells of a polyvinylchloride microtitre tray overnight.
(b) Tracheal scrapings or exudate are diluted two-fold in PBS containing 1% (v/v) Nonidet P40 and vortexed for 30 s. The sample is centrifuged and the supernate is added to duplicate wells and incubated for one hour.
(c) The tray is washed and monoclonal antibody to ILT virus is added for one hour.
(d) The tray is washed and antimouse immunoglobulin conjugated to horseradish peroxidase is added for one hour.
(e) The tray is washed and an appropriate substrate is added. After incubation, the mean optical density is determined for each sample. The corrected mean optical density is obtained by subtracting the mean optical density of control wells from the mean optical density of each sample. A corrected mean optical density of greater than 0.15 is considered positive.

4. Diagnosis by Antibody Examination

A rise in serum ILT virus neutralising antibody of at least four-fold occurs following recovery from ILT, and the demonstration of such a rise in specific virus neutralising antibody is definitive evidence of ILT virus infection. However, serology is rarely used for diagnosis of flocks suspected of being infected with ILT virus unless they are associated with research projects. This is due to the ease and speed of the techniques described above. It is usually about four days after infection that antibody is detectable in serum. Serum samples must be collected immediately after an ILT outbreak for this test to be useful.

Howes *et al.* (1969) have increased the sensitivity of the neutralisation test. The test is similar to that described under 3.2.2 except that a standardised ILT virus preparation is used instead of a standard antiserum. The tissue culture system is used in preference to embryonated eggs. A microneutralisation test has also been described (Robertson and Egerton, 1977).

An alternative method to demonstrate serum ILT virus-specific antibodies is to use ELISA (York *et al.*, 1983).

4.1. Serum Neutralisation Test to Show Rise in Anti-Infectious Laryngotracheitis Antibody

Representative birds are bled as soon as possible after the disease is noticed and again two weeks later. Serum samples are prepared and dialysed against PBS (pH 6.6) for 15–20 hours at 4°C.

Sera are diluted in a series of 10-fold steps using PBS (pH 6.6) containing 0.5% gelatin and antibiotics and equal volumes of each serum dilution and standardised ILT virus suspension containing 600 plaque forming units/mL are mixed and the mixtures are incubated at 40°C for two hours.

A control mixture consisting of equal parts of normal fowl serum diluted 1/10 and the ILT virus suspension diluted to 300 plaque forming units/mL is also incubated under the same conditions.

A volume of 0.5 mL of each mixture is inoculated to one washed and drained 5 cm CEK or CK monolayer culture. After one hour at room temperature all cultures are overlaid with agar overlay and are incubated at 40°C for three days. At that time 3 mL of staining overlay is added, and plaques are counted after a further incubation period of 24 hours at 40°C.

Serum titres are calculated as $\log_{10}$ (50% virus neutralising units) and a rise in titre of 1 log indicates infection of the bird with ILT virus. Titres are calculated by using the formula:

$$\log_{10} \text{VN 50\% Ab titre} = [\log_{10} + (\log_{10} d)\,(e - a)] \,/\, (b - a)$$

where;

e = the expected plaque count at 50% virus survival;

a = the plaque count at the serum dilution giving a count between 0 (zero) and 'e';

b = the plaque count at the next more dilute level of serum (such that b>e);

c = the reciprocal of the serum dilution giving count 'a'; and

d = the reciprocal of the dilution factor for each dilution step in the test serum dilution series (=10 for the test sera, and two for the standard serum).

It is preferable that paired sera from each bird are assayed together. However, if this is not possible, allowance can be made for differences in sensitivity of the assay system by including two-fold dilutions of the same standardised positive serum in each assay and adjusting the titres obtained in each assay on the basis of the two observed titres of the known serum as follows.

After the titres of the standard serum have been calculated, each value is compared to the designated titre of the standard. All other titres in that assay are adjusted for any inter-test variation in sensitivity by adding or subtracting the difference between the observed and designated titres of the standard.

4.2. Enzyme-linked immunosorbent assay

The ELISA test can be performed by either the antigen-capture method or the antibody-capture method.

(a) If the antigen-capture method is used, it is carried out by adsorbing purified antibody to ILT onto a microtitre tray and washing off unadsorbed antibody. Then a suspension of ILT virus antigen is adsorbed to the antibody, and after incubation for one hour at room temperature, unadsorbed antigen is washed off.

(b) If the antibody-capture method is used it is carried out by adsorbing a purified suspension of ILT virus antigen onto a microtitre tray and then, after incubation for a suitable time, washing off unadsorbed antigen.

(c) In either case the test for antibody is performed as follows. Add negative control serum, diluted 1/100 (to reduce non-specific binding) to at least two wells on each tray. Also add ILT positive antiserum diluted by a suitable factor to at least two wells on each tray. Then add each test serum, diluted 1/100 to at least two wells. Include at least two wells of controls of non-specific binding of reagents.
Incubate for one hour at room temperature and wash to remove unadsorbed serum then add antichicken immunoglobulin conjugated to an enzyme (such as horseradish peroxidase). Incubate for one hour at room temperature and wash the tray to remove unadsorbed conjugate. Finally, add a specific substrate for the enzyme used and incubate under conditions which allow the reaction between the enzyme and substrate to be detectable.
Determine the optical density of the solution in each well of the plate at a wavelength which is compatible with the product formed by the reaction of the enzyme and substrate used. Calculate the mean optical density for each serum and for the control wells and subtract the mean optical density of the control wells from all other mean optical density values to obtain the corrected mean optical density for each serum.

(d) The test is considered to be valid if the ILT positive antiserum has a value which is not less than half nor more than double the designated value, and the negative control serum has a corrected mean optical density of less than 0.10.

(e) A serum under test is considered positive for antibody to ILT virus if its corrected mean optical density is more than five times that of the negative control.

5. Conclusions

The diagnostic techniques most commonly practised in Australia include cytological examination and virus isolation or detection of viral antigen in the trachea. Techniques based on cytological examination have found wide use in the clinical diagnosis of ILT, while those based on serum antibody detection have been used in screening selected flocks for freedom from the disease. Electron microscopy is used in some laboratories for both diagnosis and screening flocks.

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