Differential diagnosis of infectious laryngotracheitis from other avian respiratory diseases by a simplified PCR procedure

Elke Scholz [a], Robert E. Porter [a,c], Peixuan Guo [a,b,*]

[a] *Department of Veterinary Pathobiology, Purdue Cancer Center, B-36 Hansen Life Science Research Building, Purdue University, West Lafayette, IN 47907, USA*
[b] *Purdue Biochemistry and Molecular Biology Program, Purdue University, West Lafayette, IN 47907, USA*
[c] *Animal Disease Diagnostic Laboratory, Purdue University, West Lafayette, IN 47907, USA*

Abstract

A simple polymerase chain reaction (PCR)-based procedure was developed for the detection of avian infectious laryngotracheitis virus (ILTV) in chicken trachea, chorio-allantoic membrane (CAM), infected hepatoma cells and infectious cell culture supernatant. Samples were prepared by dilution in distilled water. After boiling and low speed centrifugation, samples were used for PCR analysis with two primers without special labeling. The PCR analysis for ILT virus could be completed in less than 8 h. Standard agarose gel electrophoretic analysis of the PCR products revealed a prominent band of 300 base-pairs in samples from ILTV-infected specimens, but not from specimens containing Newcastle disease virus, infectious bronchitis virus, avian adenovirus, fowlpox virus, Pachecoz or Marek's disease virus. One single ILTV infected cell or 10 plaque forming units of ILTV could be detected with this procedure. The procedure can be used for the identification of ILTV and the differentiation of ILTV from other avian respiratory tract infectants.

Keywords: Polymerase chain reaction; Avian respiratory disease; Laryngotracheitis

1. Introduction

Avian infectious laryngotracheitis (ILT) is a severe upper respiratory infection of chickens that has a morbidity rate approaching 100% and a mortality rate of 10–40% (Roizman, 1982; Hanson, 1984). Presently the disease occurs worldwide and is one of the major problems in the poultry industry (Bagust, 1986). The disease is caused by an

---

* Corresponding author; at address a. Fax: +1 (317) 4961795.

α-herpesvirus, ILTV. Soon after the recognition of ILTV in the USA, the detection of long-term carriers among convalescent birds was reported (Gibbs, 1933). There are approximately 2% of latently infected carriers among flocks recovered from acute ILTV infection; these carriers guarantee the long-term survival of the virus within a flock. At present, ILTV is controlled by the use of attenuated live vaccines. Current attenuated ILTV vaccines are not satisfactory due to the induction of latent infection and recurrence or even spread of the disease (Bagust, 1986; Hughes et al., 1991; Williams et al., 1992). Leib et al. (1986) have shown that the restriction endonuclease digests of nine European wild-type strains of ILTV were similar to that of the American vaccine strains, suggesting that the recurrence of the disease may in part result from a lack of attenuation of the vaccine strains. Hughes et al. (1991) reported that latent infection of ILTV can result in shedding of the virus, and is capable of causing disease in susceptible birds similar to that seen in the field. Immunization with the attenuated vaccine protects birds against clinical disease, but does not protect against infection with virulent virus or the development of a latent carrier status due to reactivation of infectious ILTV, either alone or in concert with other pathogens. In certain countries, evidence has been provided that suggests that with increased frequency of ILTV vaccination, there is an increase in the occurrence of ILT disease. Rapid disease diagnosis is essential for effective control and management of an ILT outbreak. Rapid diagnosis of ILT in chickens is difficult because similar clinical signs and gross lesions are induced by a number of avian respiratory pathogens such as avian infectious bronchitis virus, avian influenza virus, Newcastle disease virus, and avian mycoplasma. Additional microbiological and histopathological analyses are usually required to identify the causative agent as infectious laryngotracheitis virus (ILTV). Common procedures for the diagnosis of ILT are the demonstration of epithelial intranuclear inclusion bodies in histological sections of trachea or conjunctiva or the identification of pocks on chorio-allantoic membranes (CAM) of embryonic eggs inoculated with the virus. Unfortunately, the inclusion bodies are usually observed only during the early stages of infection (van der Heide et al., 1967), and other viruses such as fowlpox virus and avian influenza virus can also induce pocks on CAMs. Other approaches to ILT diagnosis are virus isolation (Hughes et al., 1988), cytopathic effect (cpe) and syncytial cell formation on epithelial cells (Scholz et al., 1993a; Guo et al., 1994), immunofluorescence (Hitchner et al., 1977), virus neutralization (Hitchner et al., 1958), enzyme-linked immunosorbent assay (Adair et al., 1985), double gel diffusion test (Guo, 1982), and electron microscopy (Watrach et al., 1958; Guo et al., 1993). These tests vary in difficulty and usually do not provide results in less than 24 h.

    This report introduces a new procedure based on the polymerase chain reaction (PCR) for the detection of ILTV in chicken trachea. This PCR technique is specific for ILTV, has high sensitivity, and can be completed within 8 h.

2. Materials and methods

2.1. Test samples

    ILTV positive trachea was collected from 7-week-old white leghorn chickens at 6 days after infection with a U.S.D.A. challenge strain NVSL (National Veterinary

Services Laboratory, Ames, IA) kindly provided by Dr. Robert Nordgren from Solvay Animal Health, Inc. Trachea from a healthy 10-week-old chicken was used as negative control. Brain and gonads from chickens infected with Marek's disease virus (MDV, strain RBIB) were kindly provided by Dr. Robert Silva, Avian Disease and Oncology Laboratory, USDA, East Lansing, MI. MDV was chosen as an additional control because both MDV and ILTV are herpesviruses. Allantoic fluid containing infectious bronchitis virus (Ark 99) or Newcastle disease virus (LaSota strain) was acquired by embryonated egg inoculation. Analyses were also carried out on avian viruses that were field isolates collected from trachea and propagated in embryonated eggs. These viruses included quail bronchitis virus (type 1 adenovirus, egg yolk), Indiana C virus (type 1 adenovirus, allantoic fluid, Winterfield et al., 1973), two fowlpox viruses (CAM), and four ILTV (CAM). Lyophilized live fowlpox virus vaccine (FP-Blen, CEVA Laboratories), fowlpox virus from a skin lesion, and Pachecoz virus, prepared from parrot liver tissue, were also tested. All samples were stored at $-70°C$ prior to analysis.

Tracheas from chickens experimentally infected with ILTV were thawed at 25°C. Tracheal mucosa and luminal exudate were scraped into 2 ml of sterile double-distilled H_2O. Brain and gonad were finely minced with scissors and then processed similarly. After homogenization by 12 strokes in a Tenbroek tissue grinder the samples were boiled for 10 min. One hundred μl of allantoic fluid specimens, yolk, or reconstituted fowlpox virus vaccine were added to 100 μl sterile distilled water, and all samples were then boiled directly. About 100–120 mg each of CAM specimens were finely minced, mixed with 800 μl of sterile distilled water, and homogenized by 24 strokes in a Tenbroek tissue grinder. After boiling, all samples were centrifuged at $2010 \times g$ for 5 min to pellet cell debris and coagulated protein. The supernatants were analyzed by PCR.

2.2. Isolation and purification of ILTV DNA

Control experiments were performed with purified ILTV DNA prepared from NVSL infected chicken hepatoma cells. ILTV DNA was purified using a NaI gradient procedure (Scholz et al., 1993a).

2.3. PCR conditions

PCR reactions were performed in a DNA thermal cycler (Perkins Elmer Cetus). Primer #1 (5'-CGTGGCTTCACCAGCAA-3') was derived from the 3' end of the ILTV thymidine kinase (TK) gene as previously described (Griffin and Boursnell, 1990; Keeler et al., 1991). Primer #2 (5'-CGAGTAAGTAATAGGCT-3') was selected from sequence data obtained at 300 base pair (bp) downstream from the ILTV-TK gene (Elke Scholz, Choong-Sik Lee, and Peixuan Guo, unpublished data). Each PCR reaction mixture (100 μl) contained 10 μl clarified test sample, 2.25 mM $MgCl_2$ (final concentration), 9 μl of $10 \times$ PCR buffer (Promega), 5 μl of sample and 2.5 units of *Taq*-polymerase (Promega). Reagents were taken from the Gene Amp PCR core reagents kit (Perkin Elmer Cetus) unless specified otherwise, and were used according to the manufacturer's instructions. The condition for PCR reaction was: annealing at 50°C,

extension for 2 min at 72°C. After 40 cycles, 10 μl reaction mixture was assayed in a 2% agarose slab gel stained with ethidium bromide.

2.4. Quantification of the diagnostic PCR procedure

To test the sensitivity of the PCR for ILTV, chicken hepatoma cells (Scholz, 1993a,b) were infected with ILTV at a multiplicity of infection > 1, to ensure that all cells were infected. 27 h after infection the hepatoma cells were scraped from the surface of the culture flask using a plastic scraper and then transferred to a 1.5 ml microcentrifuge tube. The cells were pelleted at $325 \times g$ for 3 min, and the supernatant was removed. Supernatants were tested in a separate experiment (see below). The cell pellet was resuspended in 100 μl of sterile distilled H_2O to a final concentration of 1×10^7 cells/ml and then homogenized by 12 strokes in a Tenbroek tissue grinder. After boiling for 10 min, serial log dilutions of the homogenate in sterile distilled H_2O were analyzed by PCR as described above.

To evaluate the sensitivity of the procedure for ILTV detection in cell culture supernatants, 500 μl of supernatant from ILTV-infected hepatoma cells were boiled for 10 min. After boiling, serial log dilutions of the sample in sterile distilled H_2O were prepared and then analyzed by PCR.

3. Results

3.1. Primer analysis

Both primers used in this report were 17-mers. They have been checked by the computer program Geneworks (Glynias, 1991). Both primers passed the self complementary test (4 residues) and the repeat test (2 consecutive identical residues). Primer #1 contains 4 Gs and 6 Cs with a G/C content of 59%. Primer #2 contains 5 Gs and 2 Cs with a G/C content of 41%. Melting temperature (T_m) for primer #1 was 54°C, and for primer #2 T_m was 48°C.

3.2. Establishment of the procedure

ILTV infected embryonic CAM (Fig. 1f–i), ILTV infected chicken tracheal tissue (Fig. 1d and e), ILTV infected cells (Fig. 2b–k), and ILTV infected cell culture supernatant (Fig. 3b–d) were used as positive sample sources for the diagnostic PCR reaction. Independent of the source a PCR fragment of 300 bp was identified with infected samples when primers #1 and #2 (see Section 2) were used. The band, which was of expected size, could also be obtained when highly purified ILTV DNA was used as template (Fig. 1k), but could not be obtained when samples not infected with ILTV were used (Fig. 2k, l, and Fig. 3i), indicating that the primers bind specifically to ILTV DNA and not to cellular host DNA.

Though 40 cycles were used in the PCR reaction the result of the analysis could still be obtained within 8 h after receipt of the sample. Sample preparation prior to the PCR reaction was very fast and did not require expensive reagents.
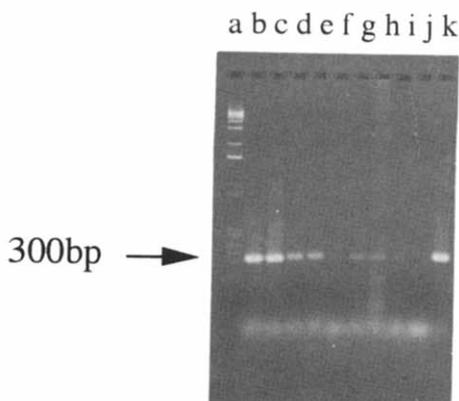
a b c d e f g h i j k

300bp ➡

Fig. 1. Testing of different ILTV isolates by PCR. ILTV-specific primers were used to detect ILTV. Lanes: a, nucleic acid molecular mass standard; b, ILTV vaccine strain; c, ILTV vaccine virus PSG-1; d, trachea from chicken 16614 infected with ILTV (strain NVSL) e, trachea from chicken 16619 infected with ILTV (strain NVSL); f, tracheal ILTV, field isolate A 16670, grown on chorio-allantoic membrane (CAM); g, tracheal ILTV, field isolate A5066, grown on CAM; h, tracheal ILTV, field isolate A3092, grown on CAM; i, tracheal ILTV, field isolate A10242, grown on CAM; j, no template DNA (negative control); k, purified ILTV DNA. All ILTV strains showed the specific 300 bp PCR product independently of the source.

Adjustment of the salt concentration was an essential factor in the performance of the PCR reaction. Since most of the template sources contain salts, a reduced amount of $10 \times$ PCR buffer and $MgCl_2$ was added to the reaction (see Section 2).
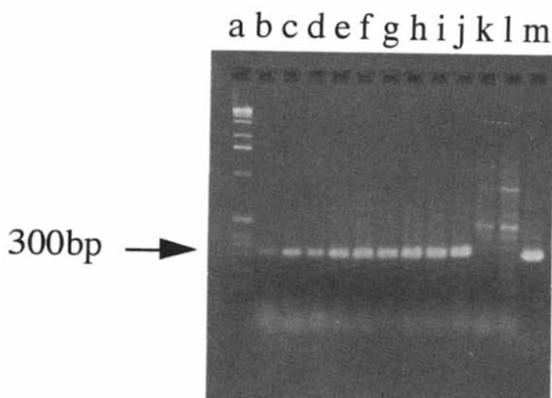
a b c d e f g h i j k l m

300bp ➡

Fig. 2. PCR products for log dilutions of ILTV-infected hepatoma cells. Lanes: 1, nucleic acid molecular mass standard; 2, 1 ILTV infected cell; 3, 5 ILTV infected cells; 4, 10 ILTV infected cells; 5, 100 ILTV infected cells; 6, 500 ILTV infected cells; 7, 1000 ILTV infected cells; 8, 10^4 ILTV infected cells; 9, 5×10^4 ILTV infected cells; 10, 10^5 ILTV infected cells; 11, 10^4 uninfected cells; 12, 10^5 uninfected cells; 13, control with purified ILTV DNA.

3.3. Sensitivity

Fig. 2 shows the PCR products for ILTV-infected hepatoma cells (1×10^7 cells/ml, see Section 2) with log dilutions. A specific 300 bp band was observed with samples up to a dilution of 10^5. Because only 10 μl of each sample was used for analysis, it follows that a positive 300 bp PCR band was generated from analysis of 1 ILTV-infected hepatoma cell.

To evaluate the sensitivity of the procedure for detection of ILTV in cell culture supernatants, supernatant from ILTV-infected hepatoma cells was boiled for 10 min. After boiling, serial log dilutions of the sample in sterile distilled H_2O were prepared (see Section 2). PCR analysis of the serial log dilutions revealed a 300 bp band that appeared up to a dilution of 10^2 (Fig. 3). Because cell culture supernatants of ILTV-infected hepatoma cells contained about 10^5 plaque forming units (pfu)/ml, 10 μl of a 10^2 dilution of the cell culture supernatant would contain 10 pfu, which was the lowest concentration of ILTV that was detected.

3.4. Specificity

To test the specificity of the procedure, different stains of ILTV as well as avian viruses different from ILTV were used in the PCR reaction as controls. Development of the assay was performed with the USDA challenge strain NVSL (Fig 1d and e). Beyond that 4 different field isolates (Fig. 1 f–i) and the vaccine strain PSG (Fig. 1c) were tested in the PCR reaction. All ILTV strains tested showed the specific PCR fragment of 300 bp (Fig. 1). This result indicates that the assay was not strain specific and could therefore be used for the identification of different ILTV strains.
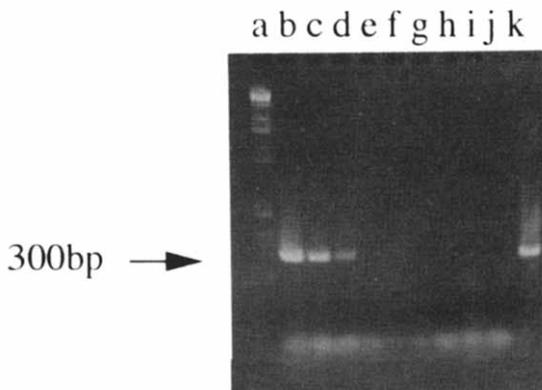

Fig. 3. PCR products for log dilutions of ILTV in cell culture supernatant. Lanes: 1, nucleic acid molecular mass standard; 2, undiluted supernatant of ILTV infected cells with 10^5 pfu/ml; 3, 10^{-1} dilution; 4, 10^{-2}; 5, 10^{-3}; 6, 10^{-4}; 7, 10^{-5}; 8, 10^{-6}; 9, undiluted supernatant of uninfected cells (control supernatant); 10, 10^{-1} dilution of the control supernatant; 11, control with purified ILTV DNA.
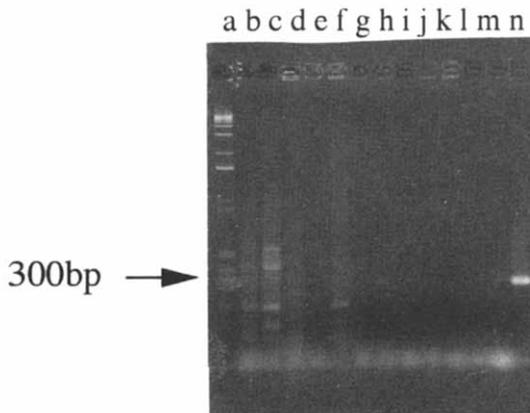
Fig. 4. Differential diagnosis of ILTV from other avian respiratory tract infecting viruses and avian herpesviruses using PCR. Lanes: a, nucleic acid molecular mass standard; b, infectious bronchitis virus (strain Ark 99); c, Newcastle disease virus (strain LaSota); d, gonad tissue from Marek's disease virus (MDV) infected chicken; e, brain tissue of MDV infected chicken (negative control for MDV infection); f, commercial fowlpox vaccine; g, fowlpox virus, tracheal field isolate A8358, grown on CAM; h, fowlpox virus, tracheal field isolate A10625, grown on CAM; i, fowlpox virus, tissue from cutaneous lesion; j, quail bronchitis virus AM14321, yolk fluid; k, Indiana C adenovirus, allantoic fluid; l, Pachecoz herpesvirus, liver tissue of parrot; m, negative PCR control without template DNA; n, purified ILTV DNA (positive control). Only lane n contains a 300 bp PCR product that is specific for ILTV.

Control viruses different from ILTV were selected considering the following criteria. (a) Since ILTV is a herpesvirus, two other avian herpesviruses were tested (Marek's disease virus and Pachecoz virus). (b) Viruses which cause symptoms similar to those caused by ILTV in the infected bird and which are therefore difficult to differentiate from ILTV infection were tested (Fowlpox virus, avian adenovirus, infectious bronchitis virus, and Newcastle disease virus). (c) Both DNA (fowlpox virus and avian adenovirus) and RNA viruses (infectious bronchitis virus and Newcastle disease virus) were tested. Fig. 4 shows the banding pattern of the PCR products for various avian viruses. Only purified ILTV DNA (lane n) showed a distinct DNA band with a size of 300 bp. Nonspecific banding patterns were observed in samples obtained from the gonad of a MDV-infected bird (lane d), allantoic fluids containing infectious bronchitis virus (lane b), and Newcastle disease virus (lane c). The PCR products in these reactions differed in size and intensity from the specific PCR product. No banding patterns were observed in control tissues such as trachea from an SPF chicken (data not shown) and brain from a MDV-infected chicken (lane e).

Fig. 4 shows the banding pattern of the PCR products for various avian respiratory DNA viruses (lanes b, c, f–k) as well as that of two other avian herpesviruses (lanes d and l) using the same PCR primers. Only the sample containing ILTV DNA (lane n) had a distinct 300 bp banding pattern. Therefore it is conclusive that the procedure introduced here allows the specific diagnosis for ILTV and is suitable to be used for identification of ILTV and for differential diagnosis of avian infectious respiratory diseases.

4. Discussion

The use of PCR for the diagnosis of viral diseases is not new. The usefulness of the procedure reported here is its simplicity, sensitivity and specificity. The procedure does not require isolation of DNA, no special labelling of samples or primer, and has a sensitivity for detection of one ILTV infected cell or 10 pfu of ILTV. Satisfactory results can be obtained by homogenizing and boiling infected tissue. To distinguish ILTV from other common viral infections of chickens two 17-mer primers homologous to regions of the ILTV genome were chosen. It was concluded that PCR carried out with ILTV-specific primers can be used to differentially detect ILTV.

A specific PCR band of 300 bp was obtained up to a dilution of 10^5 in H_2O of infected hepatoma cells or 10^2 in H_2O for culture supernatant. Because only 10 μl of sample was used in each PCR reaction the results indicate that 1 ILTV-infected cell or 10 pfu of culture supernatant leads to a positive PCR signal. The sensitivity of this diagnostic tool is therefore shown to be extremely high.

The specificity of the PCR procedure was assessed by negative results with infectious bronchitis virus, adenovirus, fowlpox virus, and Newcastle disease virus (negative control). Although multiple nonspecific PCR products would be observed with the Marek's disease virus, only samples containing ILTV showed a PCR product with the expected size of 300 bp. In addition, samples containing ILTV showed only one specific band of high intensity, rather than multiple bands of low intensity. Uninfected tissues would be expected to produce no PCR product.

As a procedure to differentiate ILT from other avian respiratory diseases, it is desirable to show that the reported procedure could apply to all strains of ILTV. We have tested the USDA challenge strain NVSL, the vaccine strain PSG, as well as 4 ILTV strains of field isolates. Our results show that this procedure could apply to all strains tested. Whether it could apply to all ILTV strains available word-wide remains to be elucidated. Due to the finding that the restriction endonuclease digests of nine European wild-type strains of ILTV were similar to that of the American vaccine strains (Leib et al., 1986), it is speculated that-the variation among ILTV strains is minimal and one pair of ILTV PCR primers might be applicable to most, if not all, other strains.

Avian influenza virus was not tested because RNA cannot serve as a template in the PCR, as was shown with Newcastle disease virus. The addition of DNase-free RNase during sample preparation could ensure the exclusion of RNA virus interference.

References

Adair, B.M., Todd, D., McKillop, E.R. and Burns, K. (1985) Comparison of serological tests for the detection of antibodies to infectious laryngotracheitis virus. Avian Pathol. 14, 461–469.

Bagust, T.J. (1986) Laryngotracheitis (Gallid-1) herpesvirus infection in the chicken. 4. Latency establishment by wild and vaccine strains of ILT virus. Avian Pathol. 15, 581–595.

Gibbs, C.S. (1933) Massachusetts plan for the eradication and control of infectious laryngotracheitis. J. Am. Vet. Assoc. 83, 214–217.

Glynias, M.J. (1991) Gene Works, Intelligenetics, Inc., Mountain View, California

Griffin, A.M. and Boursnell, M.E. (1990) Analysis of the nucleotide sequence of DNA from the region of the thymidine kinase gene of infectious laryngotracheitis virus; potential evolutionary relationships between the herpesvirus subfamilies. J. Gen. Virol. 71, 841–850.

Guo, P. (1982) Double gel diffusion test for avian infectious laryngotracheitis with hyperimmune serum of rabbit origin. Chinese J. Vet. Med. 8 (7), 18–20.

Guo, P., Scholz, E., Turek, J., Nordgren, R. and Maloney, B. (1993) Assembly of avian infectious laryngotracheitis virus. Am. J. Vet. Res. 54, 2031–2029.

Guo, P., Scholz, E., Maloney, B. and Welniak, E. (1994) Construction of recombinant avian infectious laryngotracheitis virus (ILTV) expressing the β-galactosidase gene and DNA sequencing of the insertion region. Virology (in press).

Hanson, L.E. (1984) Diseases of Poultry, 8th edn. (Hofstad, M.S., Ed.), Iowa State University Press, Ames, IA, pp. 444–451.

Hitchner, S.B.J., Fabricant, J. and Bagust, T.J. (1977) A fluorescent-antibody study of the pathogenesis of infectious laryngotracheitis. Avian Dis. 21, 185–194.

Hitchner, S.B., Shea, C.A. and White, P.G. (1958) Studies on a serum neutralization test for the diagnosis of laryngotracheitis in chickens. Avian Dis. 2, 258–269.

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

Hughes, C.S., Williams, R.A., Gaskell, R.M., Jordan, F.T.W., Bradbury, J.M., Bennett, M. and Jones, R.C. (1991) Latency and reactivation of infectious laryngotracheitis vaccine virus. Arch. Virol. 121, 213–218.

Keeler, C.L., Kingsley, D.H. and Adams Burton, C.R. (1991) Identification of the thymidine kinase gene of infectious laryngotracheitis virus. Avian Dis. 35, 920–929.

Leib, D.A., Bradbury, J.M., Gaskell, R.M., Hughes, C.S. and Jones, R.C. (1986) Restriction endonuclease patterns of some european and american isolates of avian infectious laryngotracheitis virus. Avian Dis. 30, 835–837.

Roizman, B. (1982) The Herpesviruses, Vol. 1 (Roizman, B., Ed.), Plenum Press, New York, pp. 1–23.

Scholz, E., Welniak, E., Nyholm, T. and Guo, P. (1993a) An avian hepatoma cell line for the cultivation of infectious laryngotracheitis virus and the expression of foreign genes with a mammalian promoter. J. Virol. Methods 43, 273–286.

Scholz, E., Zhang, C.L. and Guo, P. (1993b) Transactivation of the early SV40 promoter by avian infectious laryngotracheitis virus in avian hepatoma cells. J. Virol. Methods 45, 291.

van der Heide, L., Chute, L. and O'Meara, D.C. (1967) A comparative investigation of the course of an experimental infection with infectious laryngotracheitis in chickens. Avian Dis. 11, 149–153.

Watrach, A.M., Vatter, A.E., Hanson, L.E., Watrach, M.A. and Rhoades, H.E. (1959) Electon microscopic studies of the virus of avian infectious laryngotracheitis. Am. J. Vet. Res. 20, 537–544.

Williams, R.A., Bennett, M., Bradbury, J.M., Gaskell, R.M., Jones, R.C. and Jordan, F.T.W. (1992) Demonstration of sites of latency of infectious laryngotracheitis virus using the polymerase chain reaction. J. Gen. Virol. 73, 2415–2420.

Winterfield, R.W., Fadly, A.M. and Gallina, A.M. (1973) Adenovirus infection and disease. 1. Some characteristics of an isolate from chickens in Indiana. Avian Dis. 17, 334–342.