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In Situ Hybridization for the Detection of Infectious Laryngotracheitis Virus in Sections of Trachea from Experimentally Infected Chickens

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Nielsen OL, Handberg KJ, Jørgensen PH: In situ hybridization for the detection of infectious laryngotracheitis virus in sections of trachea from experimentally infected chickens. Acta vet. scand. 1998, 39, 415-421. – An in situ hybridization procedure for the detection of infectious laryngotracheitis virus (ILTV) in experimentally infected chickens is described. Formalin-fixed, paraffin-embedded sections of trachea, taken from chickens on days 3-10 post-inoculation (p.i.) with ILTV were hybridized with a mixture of 2 biotinylated, polymerase chain reaction-generated DNA fragments. The fragments correspond to sequences of the ILTV glycoprotein C and thymidine kinase genes. In situ hybridization was seen in 7 out of 7 chickens examined on day 3 p.i., 2 out of 2 examined on day 4 p.i. and 3 out of 3 examined on day 5 p.i. No hybridization was observed in 3 out of 3 chickens examined on day 10 p.i. ILTV nucleic acid was detected in nuclei of degenerated tracheal epithelial cells and in intranuclear inclusion bodies of syncytia.

Introduction

Infectious laryngotracheitis (ILT) is an acute infection of the upper respiratory tract of chickens caused by a ds-DNA virus designated Gallid herpes virus 1. ILT has a world-wide distribution, and in the severe form of the disease, infection results in haemorrhagic tracheitis and high morbidity and mortality (*Bagust & Guy* 1997).

The first outbreak of ILT in Denmark was diagnosed in backyard chickens in 1993 (Anon. 1993), and ILT has been diagnosed in 10–15 backyard chicken flocks in each of the following years. In 1996 ILT was diagnosed in Sweden for the first time since 1959 in a small backyard chicken flock, and serological screening for ILTV in backyard flocks revealed widespread subclinical ILTV infections (*Engström et al.* 1997).

The causative virus has been identified by various methods including isolation by different cultural methods (*Hughes & Jones* 1988), antigen detecting ELISA (*York & Fahey* 1988), demonstration of typical histological lesions (*Riddell* 1987) and transmission electron microscopy (*Van Kammen & Spradbrow* 1976). Nucleic acid based methods have included polymerase chain reaction (*Shirley et al.* 1990, *Williams et al.* 1994) and filter hybridization (*Keam et al.* 1991, *Key et al.* 1994).

In situ demonstration of ILT virus (ILTV) antigens by immunofluorescence or immunochemistry, using acetone-fixed cell smears or cryostat sections of affected organs, has been described by several authors (*Ide* 1978, *Wilks & Kogan* 1979, *Bagust et al.* 1986, *Guy et al.*

1992, *Abbas & Andreasen, Jr.* 1996). However, information on the use of formalin fixation is very limited, and the aim of the present study was to develop an *in situ* hybridization assay for the detection of ILTV nucleic acid in formalin-fixed, paraffin-embedded tissue samples, an assay which could prove useful in pathogenicity studies.

Materials and methods

Experimental infections

Tissue samples of trachea from 20 specified pathogen-free (SPF) White Leghorn chickens (Lohmann Tierzucht, Cuxhaven, Germany) were included in the study. At 7 weeks of age, 13 chickens were inoculated intra-tracheally with $10^{3.5}$ ELD_{50} (0.4 ml homogenate of foetal membranes in allantoic fluid from inoculated SPF-chicken embryos) of the A96 strain of ILTV (Central Veterinary Laboratory, Weybridge, UK). In addition, 2 chickens were inoculated intra-tracheally with $10^{2.5}$ ELD_{50} (0.4 ml homogenate) of a Danish ILTV field isolate (94-83949). Four chickens, which served as non-infected controls, were mock-infected intra-tracheally with 0.4 ml homogenate from non-inoculated chicken embryos. One additional control chicken was inoculated intra-tracheally with 0.2 ml of the H52 strain of infectious bronchitis (IB) virus ($10^{6.7}$ ELD/ml) at the age of 8 weeks. The chickens were kept under positive pressure in isolators supplied with sterile filtered air.

From the group of chickens inoculated with the A96 strain of ILTV, tracheal tissue samples were taken from 3 dead chickens and 2 chickens killed on day 3 post-inoculation (p.i.), from 2 dead chickens on day 4 p.i., and from 3 chickens killed on day 5 p.i. Tracheal tissue and serum were taken from 3 chickens killed on day 10 p.i. From the 2 chickens inoculated with the Danish field isolate, tracheal tissue was taken from one dead chicken and one chicken killed on day 3 p.i. The mock-infected chickens were killed and tracheal tissue taken from one chicken on day 3 p.i. and from 3 chickens on day 5 p.i. The IB virus inoculated chicken was killed on day 1 p.i. and tracheal tissue collected.

ILTV-specific probe

The ILTV specific probe was prepared as a mixture of 2 biotinylated DNA fragments, generated by polymerase chain reaction (PCR) on DNA extracted from the allantoic fluid of embryonated eggs inoculated with a Danish ILTV field isolate (95-78930). Extraction was performed according to *Sambrook et al.* (1989). Briefly, 900 μl of allantoic fluid, 100 μl buffer (0.1 M Tris.HCl pH 7.8, 0.05 M EDTA, 5% SDS) and 10 μl of Proteinase K (20 mg/ml) was incubated overnight at 55 °C. One volume (vol.) of buffered phenol was added, and after mixing and centrifugation, the water phase was transferred to new tubes. Subsequently, one vol. of phenol:chloroform:IAA (25:24:1) was added, mixing and centrifugation repeated, the water phase transferred to new tubes, and 1:10 vol. of 3 M sodium acetate pH 5.2 and 7:10 vol. of isopropanol added. The DNA was centrifuged (13,000 rpm for 30 min) and the pellet was washed in 70% ethanol, dried and redissolved in 200 μl TE.

Two sets of primers were used:
gC–1 (TTTCGAGGGCAGCTCGGTGACCC) and
gC–2 (CCATGTACCCAGCGCAGCAGATG);
tk–2 (CAAGTGCCACGCTCTTAAAATTC) and
tk–3 (AGGATGCGGAACATTACGAACCC).
These correspond to the sequences of the genes encoding glycoprotein C (*gC*) (Genbank accession U 06635) and thymidine kinase (*tk*) (Genbank accession L 36139) from ILTV and were used to amplify fragments of 510 bp and 460 bp, respectively. The primers were originally designed for the detection of ILTV by PCR (results not shown), and the *gC* and the *tk* genes

were chosen because their sequences are known for both ILTV and for all 3 serotypes (1,2 and 3) of Marek's Disease virus (MDV). MDV is, like ILTV, an alpha herpes virus, and infections, either by natural route (serotype 1 and 2) or by vaccination (serotype 1, 2 and 3), are common in chickens. Consequently, the gC primers were selected by alignment to fit the ILTV *gC* gene (one sequence published) and to show only non-significant homology with the MDV *gC* genes, and the tk primers were selected to fit all 3 published ILTV *tk* gene sequences and to exclude the MDV *tk* genes and the *tk* gene of the chicken genome. Specificity was further secured by a BLAST search in GenBank.

The PCR amplifications were carried out by mixing the purified DNA with the PCR buffer (x10-stock PCR buffer: 100 mM Tris.HCl pH 9.0, 15 mM $MgCl_2$, 500 mM KCl, 1% Triton X-100, 0.1% gelatin), supplemented with dNTP (2 mM each of dATP, dCTP, dGTP, dTTP), 10 mM DTT, 2 μM of each primer and 2.5 units of superTAQ (H. T. Biotechnology, Cambridge, UK), to a final volume of 50 μl. All tubes, reactants and mixtures were kept on ice until insertion in the thermal cycler. The PCR was performed in an Abacus Thermal Cycler (Hybaid, Teddington, UK) using the following 32 cycles: denaturation 92 °C for 30 s (cycle no. 1 for 300 s); annealing *gC*: 72 °C – 58 °C or tk: 72 °C – 54 °C for 30 s (linear decrease in temperature from cycles nos. 2-25); extension 72 °C for 60 s (cycle no. 32 for 300 s).

The PCR generated fragments were purified in a QIAquick PCR Spin Column (Qiagen) and biotinylated by random priming with the Bio-Prime kit (Gibco BRL) according to the manufacturer's instructions.

In situ hybridization

The tissue samples were fixed for 24 h in 10% neutral buffered formalin, embedded in paraffin wax, cut in 3-5 μm thick sections, mounted on SuperFrost Plus slides (Gerhard Menzle, Braunschweig, Germany), dewaxed in paraffin, and rehydrated. The sections were pretreated by microwave heating to increase the hybridization efficiency (*Lan et al.* 1996). Briefly, a slide rack containing 20 slides was placed in a Coplin jar, and 0.1 M sodium citrate buffer, pH 6.0, was added up to the 300 ml level mark. A 910 W power output was then applied for 5 min followed by a refill with buffer to the original 300 ml mark. This heating procedure was repeated 3 times followed by 2×5 min washes in tris buffered saline (TBS).

The tissue sections were then post-fixed in 6% neutral buffered paraformaldehyde for 15 min, washed in water for 3×1 min and prehybridized (cover-slip, moist chamber) for 3 h at 56 °C with 0.9 mg/ml sonicated, denatured (5 min at 95 °C) and quenched (5 min at 0 °C) salmon sperm DNA (Sigma cat. no. D 7656, St. Louis, MO, USA) in ×5.5 SSC, 41% formamide and ×4.5 Denhardt's solution.

Hybridization was performed overnight (cover-slip, moist chamber) at 56 °C with a mixture of the 2 biotinylated, ILTV specific DNA fragments, each at a concentration of 2 ng/μl. The hybridization buffer contained 0.9 mg/ml sonicated, denatured and quenched salmon sperm DNA, 9% dextran sulphate, ×4.4 SSC, 33% formamide and ×3.6 Denhardt's solution. After application of the probe, and prior to incubation, the tissue sections were denatured and quenched for 3 min at 95 °C and 0 °C, respectively.

Hybridization was followed by stringency-washing for 4×10 min in ×2 SSC at 20 °C, 2×30 min in ×0.5 SSC with 0.1% Triton X-100 at 50 °C, 2×15 min in ×0.5 SSC at 50 °C, and for 4×10 min in ×2 SSC at 20 °C.

The tissue sections were then washed in TBS with 1% bovine serum albumin (BSA) and 0.1% Triton X-100 at room temperature for 30 min, incubated for 1 h with a 1/200 dilution

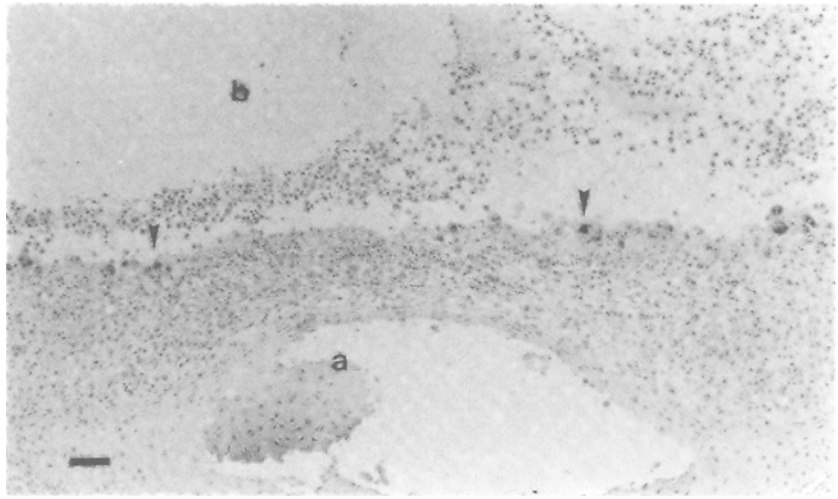

Figure 1 *In situ* hybridization of ILTV in tracheal epithelium from a chicken with experimental ILTV infection. Tissue section reacted with the biotinylated ILTV specific probe. Positive staining locates to the tracheal epithelium (➤). Partial detachment of tracheal cartilage during the staining procedure (a), lumen (b). Counter stained with Meyers haematoxylin. Bar = 50 μm.

(TBS/1% BSA/0.1% Triton X-100) of alkaline phosphatase conjugated streptavidin (DAKO, code D 0396, Glostrup, Denmark), reacted with the substrate New Fuchsin (DAKO, code K 0698, Glostrup, Denmark) for 30 min, counter stained with Meyers haematoxylin, and finally mounted with aqueous mounting medium.

All tissue sections were hybridized with the ILTV specific probe prepared as a mixture of the 2 biotinylated DNA fragments. In addition, duplicates were hybridized with identical concentrations of a biotinylated chicken anaemia virus-specific probe (*Nielsen et al.* 1995) in order to monitor the non-specific background staining.

Serological assay

Sera from the 3 chickens killed on day 10 p.i. were analysed for the presence of anti-ILTV antibodies by means of a commercial ELISA kit (Kirkegaard & Perry Laboratories, Maryland, USA).

Results

Tracheal sections from the 12 chickens inoculated with ILTV (either the A96 strain or the Danish field isolate) examined on days 3-5 p.i. all showed histological lesions characteristic of ILT (*Riddell* 1987), i.e. acute tracheitis and formation of syncytia of tracheal epithelial cells containing intranuclear inclusion bodies. The syncytia were either located in the epithelium or were lying in the tracheal lumen. In addition, various degrees of degeneration, desquamation and necrosis of the tracheal epithelium, and exudation of neutrophils and bleeding, were observed.

Positive in situ hybridization, identified as precipitated New Fuchsin dye, was detected in the tracheal epithelium (Fig. 1), and was present in 7 out of 7 chickens examined on day 3 p.i. (5 inoculated with the A96 strain and 2 inoculated with the Danish field isolate), 2 out of 2 examined on day 4 p.i. (A96 strain) and 3 out of 3 examined on day 5 p.i. (A96 strain). Staining was

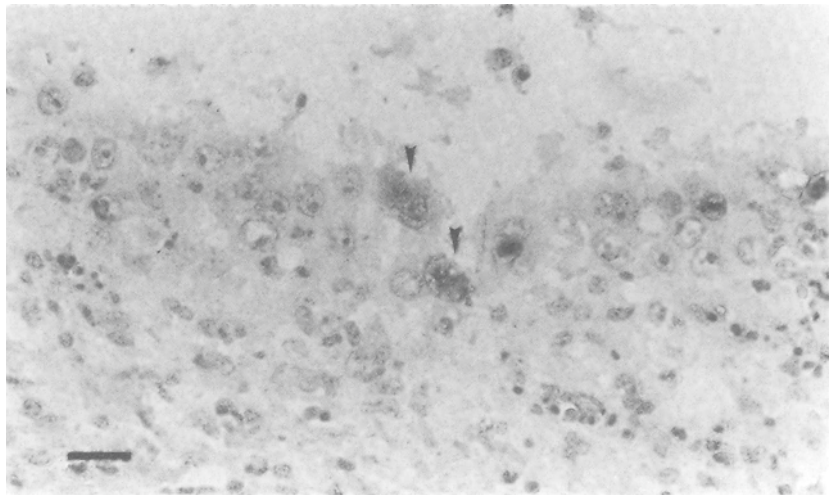

Figure 2. In situ hybridization of ILTV in tracheal epithelium from a chicken with experimental ILTV infection. Tissue section reacted with the biotinylated ILTV specific probe Weak positive staining locates to nuclei of swollen degenerated epithelial cells (➤). Bar = 20 μm

seen in the nuclei of swollen, degenerated epithelial cells (Fig. 2) and intense staining co-localized with intranuclear inclusion bodies within syncytia (Fig. 3).

The 3 ILTV-inoculated chickens killed on day 10 p.i. (A96 strain) all showed slight epithelial hyperplasia of the tracheal epithelium and a moderate, lichenoid infiltrate with lymphocytes. Positive staining was not seen. All 3 chickens had serum antibodies against ILTV.

No histological lesions were seen in tracheal sections from any of the 4 mock-infected chickens, whereas the chicken inoculated with infectious bronchitis virus showed acute purulent tracheitis. Positive staining was not seen in any of the 5 control chickens. Staining was not seen when the ILTV-specific probe was replaced with the chicken anaemia virus-specific probe.

Discussion

In situ detection of ILTV antigen by immunofluorescence or immunochemistry in cell smears or cryostat sections from experimentally infected chickens, using acetone fixation, has been described extensively (*Ide* 1978, *Wilks & Kogan* 1979, *Bagust et al.* 1986, *Guy et al.* 1992, *Abbas & Andreasen, Jr.* 1996). In these studies, ILTV antigen was detected 1-14 days p.i., predominantly 2-8 days p.i. To our knowledge, formalin fixation has previously been described only by *Bagust et al.* (1986), who detected antigen by immunofluorescence using standard procedures, and by immunofluorescence in formalin fixed duplicates. However, the consequences of formalin fixation were not specified.

The present *in situ* hybridization procedure could detect ILTV nucleic acid in formalin-fixed, paraffin-embedded sections of trachea from 12 out of 12 experimentally infected chickens examined on days 3-5 p.i., while no viral nucleic acid could be detected in 3 chickens on day 10 p.i. The presence of serum antibodies against ILTV in these 3 chickens con-

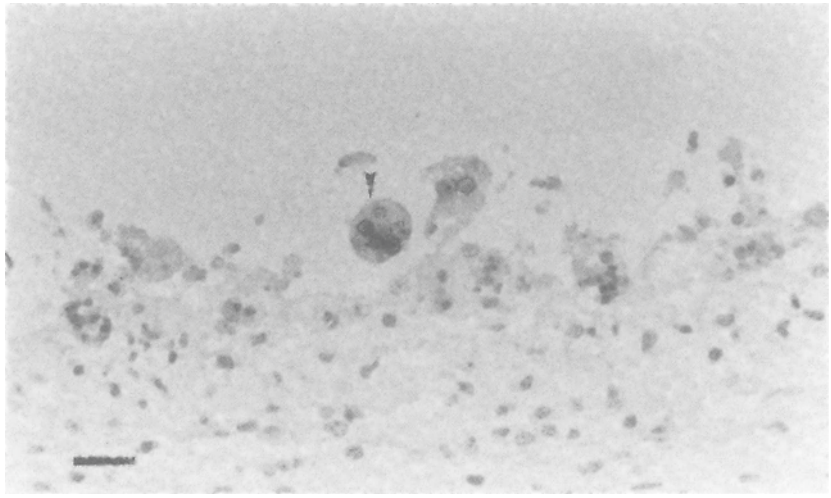

Figure 3. In situ hybridization of ILTV in tracheal epithelium from a chicken with experimental ILTV infection Tissue section reacted with the biotinylated ILTV specific probe Intense positive staining co-localizes with intranuclear inclusion bodies within a syncytium lying in the tracheal lumen (➤). Bar = 20 μm

firmed the successful inoculation of the chickens. Thus, the sensitivity of the in situ hybridization assay seems comparable with the sensitivity of the immunofluorescence and immunochemical techniques. Furthermore, detection of ILTV nucleic acid was seen with both the A96 strain and the Danish field isolate of ILTV. Thus, the results fulfill the intention of the present study, i.e. to develop an *in situ* hybridization assay for the detection of ILTV. The simple demonstration of ILT-typical histological lesions in haematoxylin and eosin stained sections of trachea (*Riddell* 1987) presents, however, no difficulties and is still preferable for the rapid diagnosis of ILT in field material. Until now, ILT in Sweden (*Engström et al.* 1997) and in Denmark has been limited to backyard chickens. ILT in the commercial poultry production in other parts of the world was believed to be controlled by vaccination, but ILT has reappeared in broilers, both in European countries and in North America, in recent years (*Alexander & Gough* 1997). The ability of ILTV to establish latent infections in the trigeminal ganglion (*Bagust et al.* 1986) may be responsible for this. Therefore studies of ILT pathogenesis are of current interest, and the hybridization method presented here, which combines detection of ILTV nucleic acid *in situ* with the preservation of histological details, is a useful tool for such studies.

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Sammendrag

In situ hybridisering af infektiøs laryngotracheitis virus i snit af luftrøret fra eksperimentelt inficerede kyllinger

Udvikling af en metode til in situ hybridisering af infektiøs laryngotracheitis virus (ILTV) i eksperimentelt inficerede kyllinger beskrives. Formalin-fikserede, paraffin-indstøbte snit af luftrøret, udtaget fra kyllinger 3-10 dage efter inokulation med ILTV, blev hybridiseret med en blanding af 2 biotinylerede, "polymerase chain reaction" (PCR) baserede DNA fragmenter. De 2 fragmenter repræsenterer generne for henholdsvis viralt glykoprotein C og tymidin kinase In situ hybridisering kunne iagttages hos 7 ud af 7 kyllinger undersøgt 3 dage efter inokulation (p i.), hos 2 ud af 2 kyllinger undersøgt 4 dage p i. og hos 3 ud af 3 kyllinger undersøgt 5 dage p i 3 ud af 3 kyllinger undersøgt 10 dage p.i. var uden hybridiserings-signal ILTV nukleinsyrer sås udelukkende i degenererede luftrørs-epithelcelle-kerner og i intranucleære inklusionslegemer i syncytier.

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