A Sensitive Nested-Polymerase Chain Reaction Protocol to Detect Infectious Laryngotracheitis Virus

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ABSTRACT

Background: Infectious laryngotracheitis virus (ILTV) is a member of the family Herpesviridae that has a worldwide distribution, although it is well controlled in areas of intensive production in which periodic outbreaks of the disease occur. ILTV is an important respiratory pathogen of chickens that may cause severe or mild disease in layers and broilers. Severe disease is characterized by respiratory depression, gasping, expectoration of bloody exudate and high mortality. Mild diseased chickens exhibit milder clinical signs and low mortality, and laboratory techniques are mandatory for a final diagnosis. Several techniques have been described for the detection of ILTV, however they have disadvantages that constrain their use in routine diagnosis. Viral multiplication is limited to respiratory tissue, which makes the trachea the ideal site to look for the virus. The purpose of the present study was to develop a sensitive and specific nested Polymerase Chain Reaction (PCR) protocol to detect ILTV DNA directly from tracheal swabs of naturally or experimentally infected chickens.

Materials, Methods & Results: The nested-PCR was carried out with two sets of primers selected from a portion of the ILTV thymidine kinase gene. PCR sensitivity was determined by using five-fold serial dilutions of a commercial laryngotracheitis vaccine. PCR was specific as determined by testing related respiratory viruses (pathogens of chickens), ILTV strain, and field isolates. Nested-PCR was 250 times more sensitive than virus isolation (VI). To further validate the ability of this assay to detect ILTV from tracheal swabs, experimentally infected chickens and ILTV suspect cases were examined by VI, PCR, and histopathology. VI and nested-PCR both detected virus in tracheas during all the experimental period. With one exception, all positive samples by VI were also positive by the nested-PCR. However, nested-PCR detected 5 additional positive samples in the end of the experimental period. Through direct histopathology, typical syncytia and inclusion bodies were found in only two samples. In the clinical cases of respiratory illness, VI detected ILTV positive samples in 4 out of the 8 flocks with respiratory illness and histopathological analyses detected 3 flocks but the nested-PCR detected 5 positive flocks including those positive by VI and histopathology.

Discussion: In the experimental infection, VI and PCR both detected ILTV in the majority of the samples but PCR detected some additional positive samples close to the end of the experimental period. By comparison of the PCR with VI sensitivity, it can be concluded that the protocol here described has a greater advantage over the previously described protocols that afford a direct comparison. Histopathology was the least sensitive test, since viral inclusion bodies and syncytial cells were only observed in two tracheal sections and a possible explanation for this result may be that necrosis and desquamation destroy infected epithelium. In the detection of the virus from clinical cases, the nested-PCR also detected a greater number of positive samples than VI and histopathology. Comparison of the nested-PCR with VI in experimentally infected broilers indicates that the two diagnostic tests are very efficient to detect ILTV in the early time of infection. However, VI tests in late infection may be not as sensitive as the nested-PCR if majority of the ILTV have been inactivated or become latent. Two distinctive sequences were obtained from the positive controls and field isolates. The sequences were specific to ILTV since they had a minimum of 99.5% similarity with previously described strains. The assay described in the present study indicates that the nested-PCR protocol is more sensitive than previously described tests and can replace histopathology and virus isolation with advantage.

Keywords: infectious laryngotracheitis virus (ILTV), avian pathology, PCR, diagnosis, detection.
INTRODUCTION

Infectious laryngotracheitis virus (ILTV) is a member of the family *Herpesviridae* [25] that has a worldwide distribution, although it is well controlled in areas of intensive production in which periodic outbreaks of the disease occur [10,14]. ILTV has a very narrow host range *in vivo*, and only domestic chickens, pheasants, peafowl [14], and turkeys [24] have been described as natural hosts. ILTV is an important respiratory pathogen of chickens that may cause severe or mild disease in layers and broilers. Severe disease is characterized by respiratory depression, gasping, expectoration of bloody exudate and high mortality. Mild diseased chickens exhibit milder clinical signs as depression, conjunctivitis, sneezing, rales, nasal exudate, and low mortality, and laboratory techniques are mandatory for a final diagnosis [10]. Several techniques have been described for the detection of ILTV, including virus isolation (VI) [16], direct fluorescent antibody tests [11,15,19,29], histopathology for the detection of syncytia and inclusion bodies [23], immunoperoxidase procedures (IP) [13], DNA probe hybridization [22], and Polymerase Chain Reaction (PCR) [2,3,9,18,26,30]. Viral multiplication is limited to respiratory tissue [10], which makes the trachea the ideal site to look for the virus. The purpose of the present study was to develop a sensitive and specific nested PCR protocol to detect ILTV DNA directly from tracheal swabs of naturally or experimentally infected chickens.

MATERIALS AND METHODS

**Viruses**

The ILTV strains were the vaccine Laryngovac¹ and inactivated strain Cover², which were used as positive controls. The field isolates ILTV-BR440, ILTV-1158, ILTV-BR219, ILTV-BR100, ILTV-BR101 were obtained from flocks with respiratory illness collected in the South and Southeast Region of Brazil during the year 2002 [5] when vaccination was not allowed. Tracheal swabs were collected and examined by VI and PCR, tracheal sections were examined by histopathology. Results from these diagnostic assays were compared.

The ILTV strain used for experimental infections was ILTV-BR1158, which caused mild respiratory disease in layers. It was isolated in specific pathogen free (SPF) embryos and identified by the formation of opaque plaques in the chorioallantoic membrane (CAM) in which intranuclear inclusion bodies were present. The virus was grown in 9 day-old embryonated eggs. After 7 days of incubation, the CAMs were harvested, ground with mortar and pestle and resuspended in a solution of phosphate-buffered saline (PBS) with gentamicin and fungizone. The viral suspension was cleared by centrifugation, titrated, and stored as aliquots at -80°C. The tracheas from the originally infected layers and the viral suspension used as inocula in the experimental infection were also tested and resulted negative for the presence of other respiratory pathogens, such as infectious bronchitis virus, Newcastle disease virus, influenza A virus, avian reovirus, *Ornithobacterium rhinotracheale*, *Pasteurella multocida* and *Haemophilus* sp.

**Experimental infection**

Ninety-six broilers from a commercial line were reared in an isolation facility. At 6 weeks of age they were divided into two groups of 48 birds, one of these to be used as a control group and the other to be experimentally infected with ILTV. On day 0, chickens were intratracheally inoculated with 0.2 mL with 0.2 x 10⁴ EID₅₀. On days 2, 4, 6, 8, 10 and 12 post infection (PI), six birds were arbitrarily selected from each group, sacrificed and their tracheas were collected. The medium portion of each trachea was sampled. Then, half of this sample was fixed in formalin for histopathology while the other half was swabbed for PCR and VI were kept at -80°C until use.

**Virus isolation (VI)**

The tracheal swabs were resuspended in PBS, cell debris were removed by centrifugation at 1200 x g for 3 min and supernatants were inoculated into the CAM of 9-day-old embryonated eggs. After 7 days, embryos were killed and the formation of opaque plaques on the CAM was verified. For determination of PCR sensitivity, five-fold serial dilutions of Laryngovac vaccine were also inoculated in embryonated eggs, the procedure was repeated three times.

**Histopathology**

CAMs or a portion of the trachea from broilers experimentally inoculated with ILTV or clinical suspect cases were fixed in neutral buffered formalin and
processed for histopathology. The paraffin embedded tracheas and CAMs were sectioned at 5 μm, stained with haematoxylin and eosin, and examined by light microscopy. Diagnosis of ILTV was based on the observation of typical intranuclear inclusion bodies and syncytia.

**Polymerase Chain Reaction (PCR)**

The tracheal swabs resuspended in PBS were used for DNA extraction by the phenol-chloroform method as previously described [5]. Two sets of nested primers (Table 1) were selected from the published sequence data of the thymidine kinase (TK) gene of ILTV [12].

The internal primers (ILTV/PCR 5', and ILTV/PCR 3' called here as p1 and p2) were as described [2]. A pair of external primers (p3 and p4) was selected in order to increase the sensitivity. The PCR reaction contained 50 ng of template DNA, 20 pmol of each primer, 2.5 mM MgCl2, 10 mM Tris-HCl pH 8.3, 0.2 mM dNTP, and 1 U Taq DNA Polymerase in a final volume of 25 μL. The cycling parameters from the first PCR reaction with primers p3 and p4 included an initial denaturation step of 5 min at 95°C followed by 30 cycles of denaturation at 95°C for 1 min, annealing at 58°C for 1 min, and extension at 72°C for 1.5 min. The final cycle was followed by an extension step at 72°C for 10 min. Two μL from the first reaction were used as template for the second amplification with p1 and p2. Second reaction constitution and cycling parameters were the same used in the first amplification, with exception of the annealing temperate that was 50°C.

PCR products were analyzed by electrophoresis in a 1.2% agarose gel stained with ethidium bromide and the band determined by comparison against a 100-bp DNA ladder.

**Sensitivity and specificity of nested PCR**

PCR sensitivity was determined by PCR and CAM inoculation using five-fold serial dilutions of the ILTV positive control and supernatants of the tracheal pool (15 samples). Five 9-day-old SPF embryonated eggs were inoculated with 0.2 mL of each dilution (5x10⁻¹ to 10⁻⁶). After 7 days of incubation, the CAMs were examined. The EID₅₀ was calculated according to the Sperman-Kärber method [28]. PCR sensitivity was determined by comparison with the egg titration results after adjustments for the dilution and volume.

PCR specificity was verified by using a cow poxvirus strain (CPV) isolated from cattle lesions, a Marek’s disease vaccine and one skin sample with Marek’s disease lesions (MDV), one infectious bronchitis virus (IBV) vaccine, and bovine herpesvirus type 1 (BHV-1) strains Lam, 265, 123 and type 5 strain EVI-88 (BVH-5). The poultry respiratory bacteria *Ornithobacterium rhinotracheale* (ORT) [7], *Pasteurella multocida* (PM), *Haemophilus sp.* (HS), *Mycoplasma gallisepticum*, *Mycoplasma synoviae* and *Escherichia coli*, and tracheal samples from clinically normal birds, SPF chickens and ILTV positive controls were also tested by the nested-PCR.

In order to confirm the identity of the amplification products, the 647-bp internal amplification products from the two positive controls and five field isolates were sequenced. The extension products were purified on Centri-Sep columns. Both strands were sequenced using 30 to 45 ng of PCR DNA templates labeled with 3.2 pmol of the PCR primers and BigDye Terminator v3.1 Cycle Sequencing RR-100. The reaction product was loaded on an automatic sequencer ABI-PRISM 3100 Genetic Analyzer. The Clustal X software [27] was used for sequence analysis, which included the published sequences of ILTV [12, 21].

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence 5'→3'</th>
<th>Position</th>
<th>Amplification product</th>
</tr>
</thead>
<tbody>
<tr>
<td>p1</td>
<td>ACGATGACTCCGACTTTTC</td>
<td>4501-4519</td>
<td>647 bp</td>
</tr>
<tr>
<td>p2</td>
<td>CGTTGGAGGTTGTTGTA</td>
<td>5112-5130</td>
<td></td>
</tr>
<tr>
<td>p3</td>
<td>TGGCAACGGATTCCGGCA</td>
<td>4403-4421</td>
<td>792 bp</td>
</tr>
<tr>
<td>p4</td>
<td>GGCACGTGTTAAGGCTTGGCTCC</td>
<td>5170-5195</td>
<td></td>
</tr>
</tbody>
</table>
RESULTS

PCR

PCR with primers 3 and 4 was 100 times more sensitive than PCR with primers 1 and 2 and 25 times more sensitive than VI. The use of primers 1 and 2 after DNA amplification with primers 3 and 4 (nested-PCR) increased 10 times the sensitivity. The nested-PCR was 250 times more sensitive than VI. With grinded trachea from naturally infected birds, the nested-PCR was 125 times more sensitive than VI. From the same 15 tracheas, the use of PCR with primers 1 and 2 could detect 3 positive samples; however the nested-PCR detected ILTV DNA in all samples.

The nested-PCR protocol was specific, since it amplified an approximately 647-bp product only from ILTV positive controls and field strains (Figure 1) and none of the additional samples from non-infected birds or other bacterial and viral species.

Two distinctive sequences were obtained from the positive controls and field isolates. The Laryngovac, Cover, ILTV-BR100 and ILTV-BR101 strains were identical to a sequence obtained from an isolate from USA [21]. The isolates ILTV-BR440, ILTV-BR1158, and ILTV-BR219 were identical, but were 99.5% homologous to these previously described sequence [21].

Experimental infection

ILTV experimentally infected broilers showed mild respiratory signs 48 hours PI, including depression, and dyspnea. No respiratory signs were observed after day 10 PI. Two of 48 (4.2%) inoculated birds died between days 8 and 10 PI with apparent hemorrhagic tracheal exudates. Neither respiratory signs nor lesions were found in the control chickens.

Table 2 presents the results of the three diagnostic tests performed on ILTV infected birds. VI and nested-PCR both detected virus in tracheas from day 0 through day 12 PI. With one exception, all positive samples by VI were also positive by the nested-PCR. However, nested-PCR detected 5 additional positive samples on days 10 and 12 PI. Through direct histopathology, typical syncytia and inclusion bodies were found in one sample of day 6 and another sample from day 8 PI (Table 2 and Figure 2).

<table>
<thead>
<tr>
<th>Days post-inoculation</th>
<th>VI</th>
<th>Histopathology</th>
<th>Nested-PCR</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0/6</td>
<td>0/6</td>
<td>0/6</td>
</tr>
<tr>
<td>2</td>
<td>3/6</td>
<td>0/6</td>
<td>3/6</td>
</tr>
<tr>
<td>4</td>
<td>4/6</td>
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<tr>
<td>6</td>
<td>5/6</td>
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<td>8</td>
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<tr>
<td>12</td>
<td>2/6</td>
<td>0/6</td>
<td>6/6</td>
</tr>
</tbody>
</table>
Tracheal sections from day 2 PI often-contained normal structures, such as intact cilia and epithelial cells. By day 10 PI, the histological lesions were not characteristic of the disease, since the sections presented necrosis and desquamation of epithelial cells, areas of hyperplasia of the epithelium, subepithelial infiltration of inflammatory cells and presence of bacteria in the lumen of the organs. By day 12 PI, tracheas were histologically normal. The uninfected chicken tracheal samples were negative by the three tests.

### Clinical cases

VI detected ILTV positive samples in 4 out of the 8 flocks with respiratory illness. Histopathological analyses detected 3 flocks as ILTV positive. The nested-PCR detected 5 positive flocks including those positive by VI and histopathology (Table 3).

#### Table 3. Results of ILTV detection in tracheas of suspect cases by virus isolation (VI), histopathology, and nested-PCR (number of positive samples/total number of samples per flock).

<table>
<thead>
<tr>
<th>Flock</th>
<th>440</th>
<th>1158</th>
<th>219</th>
<th>100</th>
<th>101</th>
<th>59</th>
<th>71</th>
<th>72</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nested-PCR</td>
<td>10/15</td>
<td>15/15</td>
<td>12/15</td>
<td>9/16</td>
<td>1/1</td>
<td>0/9</td>
<td>0/6</td>
<td>0/6</td>
</tr>
<tr>
<td>VI</td>
<td>3/5</td>
<td>4/5</td>
<td>1/5</td>
<td>0/5</td>
<td>1/1</td>
<td>0/9</td>
<td>0/6</td>
<td>0/6</td>
</tr>
<tr>
<td>Histopathology</td>
<td>1/5</td>
<td>1/4</td>
<td>0/1</td>
<td>0/5</td>
<td>1/1</td>
<td>0/1</td>
<td>0/1</td>
<td>0/1</td>
</tr>
</tbody>
</table>

### DISCUSSION

The nested-PCR protocol was developed to detect ILTV DNA from tracheal swabs of poultry. Since there is considerable similarity between the TK genes from different ILTV strains [12, 21], it was decided to select diagnostic primers from this region. PCR based on ILTV TK gene primers offered a specific means of detecting various ILTV strains and field isolates but did not amplify DNA from other viruses, bacteria and poultry tracheal swabs from clinically normal birds. All sequenced amplification products were specific to ILTV since they had a minimum of 99.5% similarity with previously sequenced strains [12, 21].

In the sensitivity test, the nested-PCR was 100 times more sensitive than a previously described PCR protocol [2] and 25 times more sensitive than VI. The nested-PCR established to detect ILTV gE [8] was 4.5 times more sensitive than VI. A previously described real time PCR was 3.7 times more sensitive than VI [6]. By comparison of the PCR with VI sensitivity, it can be concluded that the protocol here described has a greater advantage over the previously described protocols that afford a direct comparison [2, 6, 8]. In the detection of the virus from clinical cases, the nested-PCR also detected a greater number of positive samples than VI and histopathology. Comparison of the nested-PCR with VI in experimentally infected broilers indicates that the two diagnostic tests are very efficient to detect ILTV in the early time of infection. However, VI tests in late infection may not be as sensitive as the nested-PCR if majority of the ILTV have been inactivated or become latent [4, 17]. Some authors [3] used conjunctival swabs and their results showed on day 5 and 6 PI more PCR positives (19%) than VI (3%). Although no sample was positive by VI on day 8 PI, 11% of the samples were PCR positive. In the present study, VI and PCR both detected ILTV in 100% of the samples on day 8 PI but PCR detected 100% more positive samples on days 10 and 12 PI. Histopathology was the least sensitive test, since viral inclusion bodies and syncytial cells were only observed in two tracheal sections on day 6 and 8 PI. These results agree with the ones from other experimental infections that indicated that ILTV is not detected or detected inconsistently [1, 14]. One possible explanation for this result may be that necrosis and desquamation destroy infected epithelium [14].

The assay described in the present study indicates that the nested-PCR protocol described is more sensitive than previously described protocols and specific to detect ILTV DNA from tracheal swabs and can replace histopathology and virus isolation with advantage.
Sources and Manufacturers
1 Solvay Animal Health, Charles City, IL, USA.
2 Spafas, Storrs, CT, USA.
3 Amersham Biosciences Corp, Piscataway, NJ, USA.
4 Rhodia Mérieux Veterinária Ltda, Brazil.
5 Intervet International B.V., Boxmeer, Holland.
6 Instituto de Pesquisas Veterinárias Desidério Finamour, FEPAGRO, RS, Brazil.
7 Princeton Separations, Adelphia, NJ, USA.
8 Applied Biosystems, Foster, CA, USA.

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References


